

**Characterisation of the Multifunctional Protein,
CREAP**

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Kristy Lea Shipman

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ABBREVIATIONS

8-Br-cAMP	8-bromoadenosine cAMP
ACTH	adrenocorticotrophin releasing hormone
ATCC	American tissue culture collection
ATF	activating transcription factor
BrUTP	5-bromo-UTP
BSA	bovine serum albumin
bZIP	basic leucine zipper
cAMP	cyclic adenosine monophosphate
CBC	cap binding complex
cDNA	complementary DNA
CDXA/CDXRE	caudal type homeobox response element
CH	cysteine-histidine
CMV	cytomegalovirus
Co-IP	co-immunoprecipitation
CRE	cAMP response element
CREAP	CRE associated protein
CREB	CRE binding protein
CREM	CRE modulating protein
CRH	corticotrophin releasing hormone
CRH-BP	CRH binding protein
CROP	cisplatin resistance over-expressed protein
CsCl	cesium chloride
CTD	carboxyl terminal domain
Dex	dexamethasone
DHEAS	dehydroepiandrosterone
DMEM	Dulbecco's modified eagle's medium
DNA	dideoxy-ribonucleic acid
E.coli	eshericia coli
ECL	enhanced chemiluminescence
EcRE	ecdysone response element

EMSA	electrophoretic mobility shift assay
ERE	estrogen response element
EST	expressed sequence tag
FBS	fetal bovine serum
GFP	green fluorescent protein
GR	glucocorticoid receptor
GST	glutathione-s transferase
HBS	Hepes buffered saline
HLH	helix loop helix
hnRNPs	heteronuclear ribonuclear protein
HPA	hypothalamic pituitary adrenal axis
HRE	hybrid steroid response element
HRP	horseradish peroxidase
HTH	helix turn helix
IGC	interchromatin granule clusters
IL	interleukin
IP	immunoprecipitation
IPTG	isopropyl-beta-D-thiogalactopyranoside
IR	immunoreactivity
KAc	potassium acetate
KRE	lysine/arginine/glutamine
LB	lauria broth
Luc	luciferase
lv	long-version
MAPK	mitogen activated protein kinase
Mg ₂ Cl	magnesium chloride
mRNA	messenger RNA
MTE	multiple tissue expression array
Mut	mutant
NaCl	sodium chloride
NBCS	new born calf serum
NLS	nuclear localisation signal
NTD	amino terminal domain

NuPAGE	neutral polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol ester
Pol	polymerase
PR	progesterone receptor
RLU	relative luciferase units
RNA	ribonucleic acid
RRM	RNA recognition motif
rRNA	ribosomal RNA
RS domain	Arginine-serine domain
SC35	splicing component 35kDa
Scr	scrambled
SF2/ASF	splicing factor 2/alternative splicing factor
siRNA	small-interfering RNA
SNP	single nucleotide polymorphism
snRNA	small nuclear RNA
snRNP	small nuclear ribonuclear protein
SR Protein	Serine-arginine protein
sv	short-version
TBS	tris buffered saline
TBST	tris buffered saline with Tween-20
TE	tris-EDTA
TF	transcription factor
tRNA	transferase RNA
Trx	thioredoxin
UV	ultraviolet

PUBLICATIONS

“Identification of a Family of DNA-Binding Proteins with Homology to RNA Splicing Factors”. **Kristy L. Shipman**, Phillip J Robinson, Bruce R King, Roger Smith, Richard C Nicholson. Biochemistry and Cell Biology, Volume 84, Number 1, 1 February 2006, pp. 9-19(11).

CONFERENCE ABSTRACTS

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“Identification of a Novel Transcription Factor Family that Binds to the cAMP Regulatory Element.” 85th Annual Meeting of the Endocrine Society, 2003, Philadelphia, USA

“The CREAPs: A Family of CRE Binding Proteins.” 46th Annual Scientific Meeting of the Endocrine Society of Australia, 2003, Melbourne, Australia

“Analysis of Transcription Factors regulating Placental CRH through the CRE.” 45th Annual Scientific Meeting of the Endocrine Society of Australia, 2002, Adelaide, Australia.

Abstract

Pre-term birth is still the leading cause of perinatal mortality and morbidity. CRH is a hormone that is involved in the timing of labour, therefore investigation of its regulation is of importance in understanding human parturition. The CRE is a central regulatory element on the CRH promoter and in investigating proteins that bind to this element a novel protein was discovered. CREAP or cAMP Regulatory Element Associated Protein, was initially discovered by its ability to bind to the CRE. Its sequence encodes a unique set of modular domains including two zinc fingers, two leucine zippers, two coiled-coils and an RS-rich domain. These domains point to functions in both DNA binding/transcription and RNA splicing, with the leucine zippers being characteristic of bZIP transcription family and the RS domain characteristic of the SR Protein family of splicing factors, to represent a new protein family.

In this thesis, molecular reagents were produced for the study of CREAP together with a polyclonal antibody. This antibody was used in western blotting to detect a 58 kDa full-length CREAP protein and a shorter 25-30 kDa truncated splice variant. CREAP was localised to the nucleus and to intranuclear splicing speckles, with co-localisation and co-immunoprecipitation with the splicing factor SC35, strongly suggesting a role in splicing. To test the transcriptional activity of CREAP, specifically if it regulates CRH expression, luciferase reporter studies were conducted. However, CREAP showed negligible effect on CRH or CRE promoter activities suggesting that it is not involved in CRH regulation. CREAP did however react with a large number of transcription factors in an *in vitro* assay, mostly from the bZIP and zinc finger families. siRNA mediated knockout of CREAP was conducted and the effect on genome-wide expression analysed using a microarray. CREAP knockdown caused an over-representation of genes from the protein transport, metabolism, signal transduction and transcription factor processes. Overall, CREAP appears to be a multifunctional protein that is ubiquitously expressed, and is involved in both splicing and transcriptional processes.

Chapter 1. Literature Review

1.1 Parturition and Preterm Birth

1.1.1 Background

In most cases pregnancy results in the birth of a full term, healthy baby, with no complications. However the problem of pre-term, birth, that is birth before 37 weeks of gestation, accounts for 75% of perinatal mortality and over 50% of long-term morbidity (McCormick 1985). Despite years of research, the mechanisms that govern the timing of birth and the pathologies that lead to premature delivery are still not completely known. There are several theories on the timing of parturition and it seems most likely that the process is multifactorial. One theory that has been investigated in our laboratory involves the hormone Corticotrophin Releasing Hormone or CRH. CRH is produced by the placenta and maternal serum levels rise exponentially during gestation, suggesting a “placental clock” is involved in the timing of birth. This lab has studied the regulation of CRH gene expression in both the placenta and hypothalamus in the hope of elucidating its role in pregnancy. A novel regulatory factor was discovered during these studies and the initial focus of this thesis was investigating this protein and characterising its role in CRH regulation. During the course of this work the results indicated that this protein has functions beyond CRH regulation and that it may be member of a novel multifunctional protein family with roles in both transcription and splicing.

1.1.2 Parturition Theories

1.1.2.1 CRH, Cortisol and Estrogen

The exponential rise in CRH concentration in the weeks before normal parturition is associated with a surge in fetal glucocorticoid and the production of estriol (Fencl et al. 1980; McGregor et al. 1995). One of the leading theories on parturition involves a positive feed forward loop between CRH and the endogenous glucocorticoid, cortisol. In contrast to the hypothalamus, glucocorticoids stimulate placental CRH, instead of inhibiting it. It is proposed that placental CRH enters the fetal circulation through the umbilical vein, then stimulates the fetal pituitary-adrenal axis to produce cortisol and cortisol sulfate, which are capable of further stimulating placental CRH production, creating a positive feed forward loop (Majzoub et al. 1999b).

The primate placenta lacks the enzymes 17-hydroxylase/17-20 lyase. Without these enzymes the placenta cannot directly synthesise estradiol from progesterone (the only steroid that the placenta can produce de novo) (Majzoub et al. 1999b). Therefore, the fetal adrenal serves as the major source of the estradiol precursor, dehydroepiandrosterone sulfate (DHEAS) during pregnancy. Consequently, placental CRH production may have evolved to stimulate the fetal adrenal system to produce DHEAS, to satisfy the high demand for estrogens (Karalis et al. 1996). Smith et al. have found that CRH has direct effects on human fetal adrenal cortical cells to stimulate the production of glucocorticoids (Smith et al. 1998). CRH increased DHEAS production by cultured human fetal adrenal cortical cells in a dose-dependent fashion and was as effective at stimulating DHEAS as ACTH. CRH appears to preferentially stimulate DHEAS directly from the adrenal, as it is only 30% as effective as ACTH in

directly stimulating cortisol (Smith et al. 1998). The positive feed forward loop between placental CRH and the fetal adrenal provides cortisol for maturation of the fetal lung and other organs and DHEAS for estrogen production to potentially stimulate parturition through prostaglandin synthesis, oxytocin receptor expression and gap junction formation in the myometrium (Majzoub et al. 1999b). The dual stimulation of cortisol and DHEAS by placental CRH is beneficial for postnatal survival because it synchronises the glucocorticoid effects on fetal organ maturation with the possible DHEAS/estrogen effects on parturition (Karalis et al. 1996). These concepts are displayed figuratively in Figure 1.1.

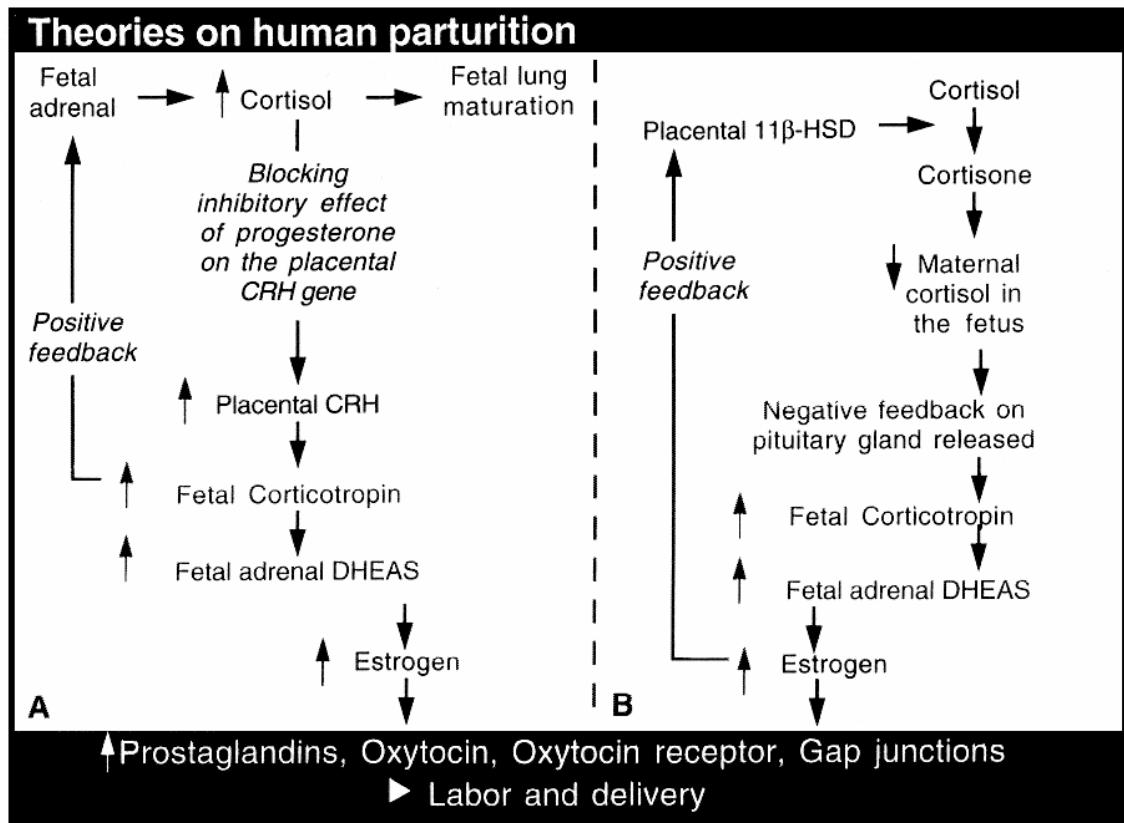


Figure 1.1. Two Theories on Human Parturition.

A. Placental CRH is secreted into the fetal circulation and stimulates fetal corticotropin from the pituitary. This stimulates the fetal adrenal to produce cortisol, which further stimulates placental CRH production creating a positive feed forward loop and causes fetal lung maturation. Fetal DHEAS is also stimulated to provide estrogen for parturition. B. During the second half of gestation, the increase in estrogen stimulates placental 11 β -hydroxysteroid dehydrogenase. This converts maternal cortisol to inactive cortisone, thus lowering maternal cortisol levels in the fetus. This decreases the negative glucocorticoid feedback on the fetal pituitary resulting in increased secretion of cortisol and DHEAS, promoting organ maturation and parturition (adapted from (Majzoub et al. 1999a).

1.1.2.2 The Placental Clock

Studies have found that there is a relationship between CRH concentrations and the length of gestation and the length of labour (Smith 1999a). McLean *et al.* found that CRH levels in the second trimester predicted the length of gestation. High levels of CRH in the second trimester were linked to pre-term delivery while low concentrations were associated with post-term delivery (McLean *et al.* 1995). They suggest that a ‘placental clock’ may be active to control the timing of parturition and that this clock is functioning from as early as 16-20 weeks gestation (McLean *et al.* 1995). Wadhwa *et al.* also found that maternal CRH levels in the early third trimester predict the length of gestation (Wadhwa *et al.* 1998). CRH will be discussed in more detail in Section 1.5.

1.1.2.3 CRH Binding Protein

By 21 weeks CRH is detectable in placental tissues (Riley *et al.* 1991) and around 16-20 weeks it is detectable in maternal plasma with plasma CRH levels increasing exponentially throughout pregnancy and peaking during labour (McLean *et al.* 1995). However, much of this CRH is inactivated by a circulating CRH-binding protein (CRH-BP) (Behan *et al.* 1989). The concentrations of CRH-BP are stable in non-pregnant women and for the first 25 weeks of pregnancy. The plasma concentration decreases slightly between 25 and 30 weeks, and then rapidly decreases at 4-6 weeks before labour at term (Fadalti *et al.* 2000) (see Figure 1.2). The decrease in binding protein leads to a rise in free and possibly active CRH. The placental clock hypothesis proposes that the rate of increase in maternal plasma CRH through pregnancy influences the timing of labour by determining when saturation of CRH-BP will occur and thus when free CRH becomes available to act as a parturition trigger (McLean and Smith

2001). The CRH binding protein is thought to exist to prevent placental CRH inappropriately stimulating the anterior pituitary (Linton et al. 1990).

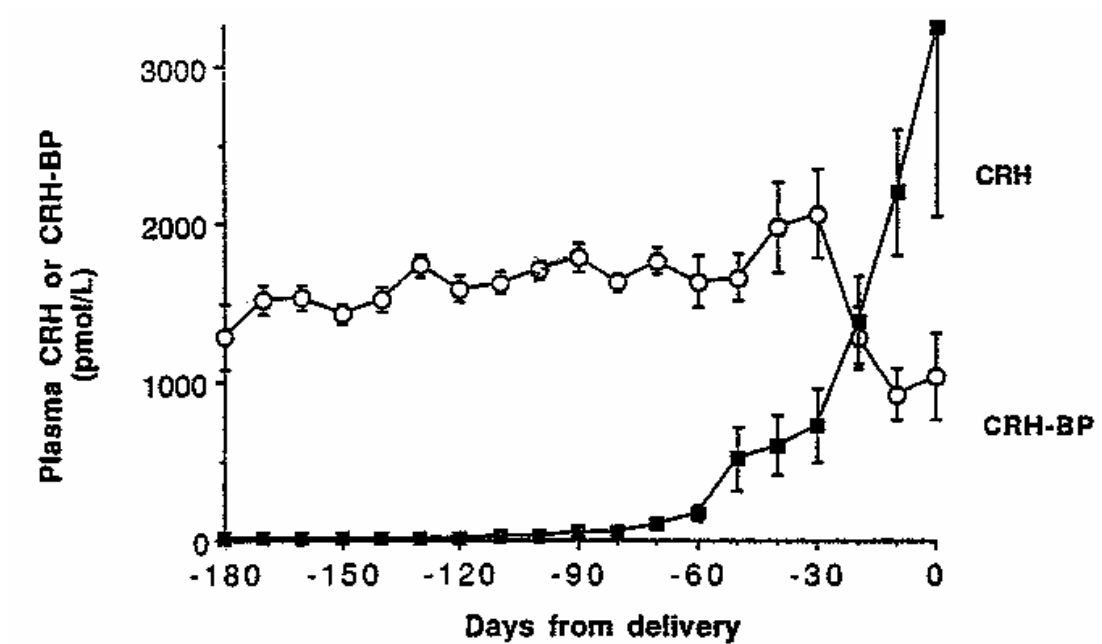


Figure 1.2. Plasma CRH and CRH-BP Levels.

Comparison of the molar concentrations of CRH (■) and CRH-BP (O) in maternal plasma during the final 180 days gestation of spontaneous term delivery pregnancies (McLean et al. 1995).

1.1.2.4 Progesterone

In most mammals, labour is preceded by progesterone withdrawal and estrogen activation. However this is not the case in primates and humans, where progesterone

levels increase linearly over gestation. Mesiano et al. hypothesized that functional progesterone withdrawal occurs by increased expression of the type A progesterone receptor (PR-A), which suppresses progesterone responsiveness mediated by progesterone receptor B (PR-B), and that functional estrogen activation occurs by increased myometrial expression of ER α and/or ER β . They found that the ratio of PR-A to PR-B was important in mediating the functional withdrawal of progesterone (Mesiano et al. 2002). It has also been found that prostaglandins acting via the PKC pathway facilitate functional progesterone withdrawal by increasing the myometrial PR-A/PR-B expression ratio (Madsen et al. 2004). It has also been demonstrated that progesterone regulates CRH gene transcription via a CRE in the CRH promoter and that PR-A and PR-B exhibit different actions in the regulation of CRH gene expression (Ni et al. 2004b). Overexpression of progesterone receptor A (PR-A) or glucocorticoid receptor in primary placental cells resulted in a decrease in CRH promoter activity following progesterone treatment, whereas an increase in promoter activity was observed with overexpressed PR-B (Ni et al. 2004b).

Related to the CRH-cortisol theory is the cortisol blockade of progesterone model. There is evidence that progesterone has an inhibitory effect on placental CRH production. When progesterone is added to placental trophoblast cultures there is a decrease in CRH mRNA (Karalis et al. 1996) and peptide (Jones et al. 1989) levels. It is thought that high levels of progesterone early in gestation could compete with cortisol for the glucocorticoid receptors thus inhibiting cortisol and its stimulatory effect on CRH (Karalis et al. 1996). The production of placental CRH is thought to be sufficient to stimulate some cortisol production over pregnancy (Majzoub et al. 1999b). Inhibition

of CRH would only be reversed after the rise in fetal cortisol secretion in late gestation is great enough to overcome progesterone at the receptor level (Majzoub et al. 1999b). The blockade of cortisol receptors by progesterone would be overcome and cortisol would be able to further stimulate CRH to create a positive feed forward loop and high levels of CRH and cortisol (Majzoub et al. 1999b).

1.1.2.5 11 β -Hydroxysteroid Dehydrogenase

Another leading theory on parturition involves the enzyme 11 β -hydroxysteroid dehydrogenase, which converts cortisol to inactive cortisone. This theory proposes that maternal cortisol suppresses the fetal hypothalamic pituitary axis (HPA) during the first half of gestation (Pepe and Albrecht 1995) (Figure 1.1). The rise in estrogens during the second half of gestation is believed to stimulate placental 11 β -hydroxysteroid dehydrogenase, thus converting maternal cortisol to inactive cortisone (Majzoub et al. 1999b). In this way, less maternal cortisol would cross the placenta and the negative glucocorticoid feedback on the fetal HPA would be released. The fetal HPA could then increase secretion of ACTH, cortisol and DHEAS leading to fetal organ maturation and stimulation of parturition (Pepe and Albrecht 1995; Schwartz 1997). However, this model has been studied mostly in the baboon and its relevance to human parturition is unclear.

In cases of congenital adrenal hyperplasia, where there is an enzymatic block in cortisol synthesis, maternal cortisol is unable to suppress the fetal adrenal at 8-10 weeks gestation. The fetal pituitary tries to compensate for the cortisol deficiency by increasing the synthesis of ACTH resulting in over stimulation of the adrenal cortex and

virilization of female fetuses (Pang 1997). Treatment with exogenous glucocorticoid decreases fetal steroid production and virilization, demonstrating that maternal cortisol alone is insufficient to suppress the fetal adrenal early in gestation (Pang 1997). Also, estrogen levels (derived almost exclusively from the fetal adrenal) increase progressively over gestation (Tulchinsky et al. 1972) suggesting that the fetal adrenal is progressively activated during the first half of gestation. However, 11 β -hydroxysteroid dehydrogenase usually only inactivates about 60% of maternal cortisol (Benediktsson et al. 1997), even at term therefore a considerable amount of cortisol is capable of reaching the fetus very late in gestation. Thus, it appears that this theory is not as well supported in humans as the CRH-cortisol positive feed forward model.

1.1.2.6 Prostaglandins

There is a potential interaction between CRH and prostaglandin (PG) production in the amniotic compartment that may promote parturition through cervical maturation and uterine contractility. CRH induces the synthesis of PGE₂ and PGF₂ α at physiological concentrations in vitro (Jones et al. 1989) and both PGE₂ and PGF₂ α are potent promoters of cervical maturation and uterine contractility. These prostaglandins are capable of further stimulating CRH secretion from the placenta and fetal membranes (Petraglia et al. 1989) creating a positive feedback loop.

1.1.2.7 Inflammation

Increasing evidence suggests that inflammatory pathways are involved in the process of parturition. Thomson and colleagues, using histological staining, found that there is a

massive influx of inflammatory cells, in the form of neutrophils and macrophages, into both the lower and upper segment of the myometrium during labour (Thomson et al. 1999). Leukocytic invasion of the myometrium and cervix is accompanied with up-regulation of the pro-inflammatory cytokines, IL-1 β , IL-6 and IL-8 (Osman et al. 2003). IL-1 β has been shown to induce basal and store operated calcium entry into myometrial smooth muscle cells (Tribe et al. 2003), to stimulate phosphodiesterase activity (Oger et al. 2002) and to induce PGF2 α and its receptor (Rauk and Chiao 2000), all of which increase the myometrial contractile ability. There is no increase in inflammatory cells in the fetal membranes at labour (Osman et al. 2006) but the membranes have been shown to have an acute inflammatory gene signature (Haddad et al. 2006). The source of this inflammatory activity is unclear. Many of the inflammatory events associated with labour are also found in cases of preterm labour due to infection. A causal pathway model for the activation of the myometrium found that an inflammatory aetiology was most likely for labour (Bisits et al. 2005).

1.1.3 Myometrial Contractility and CRH

Functional CRH receptors can be found in the myometrium of the uterus, suggesting a role for CRH in myometrial contractility. There are multiple receptor subtypes and pregnancy results in differential expression (Grammatopoulos et al. 1998; Rodriguez-Linares et al. 1998; Linton et al. 2001). The different receptor subtypes are able to exert different actions on the myometrium of pregnant versus non-pregnant women (Grammatopoulos et al. 1994). The receptors are largely linked to the adenylate cyclase second messenger system during the pregnant state (Grammatopoulos et al. 1994) and the production of cyclic adenosine monophosphate (cAMP) in the myometrium is

associated with relaxation. CRH inhibits the production of prostaglandin E2 (a contractile agent) in myometrial cells, aiding in maintaining quiescence (Grammatopoulos and Hillhouse 1999b). However, as term approaches this effect is decreased due to reduced coupling of the G α s regulatory protein of the CRH-receptor with the catalytic component of adenylate cyclase (Grammatopoulos et al. 1996). It is thought that oxytocin exerts a direct inhibitory effect on adenylate cyclase in the myometrium and also switches the CRH receptors to a low affinity state thus uncoupling it from adenylate cyclase (Europe-Finner et al. 1994). The reduction in cAMP increases myometrial excitability. Therefore, it seems likely that oxytocin-sensitive CRH receptors are responsible for maintaining myometrial relaxation through cAMP and thus inhibition of these receptors by oxytocin in later pregnancy enables CRH to have a different, even reverse effect, in the control of uterine contractility and thus labour (Grammatopoulos and Hillhouse 1999a).

CRH potentiates the effects of uterotonics such as prostaglandin F2 alpha and oxytocin (Quartero and Fry 1989; Benedetto et al. 1994). CRH alone has no effect on the contractility of myometrial strips in vitro, but the presence of CRH (or preincubation with CRH) causes a greatly enhanced contractile response to oxytocin and PGE2 (Quartero and Fry 1989; Benedetto et al. 1994). However the work on CRH receptors in the myometrium needs further work to verify exactly what is regulating them, especially the role of oxytocin, rather than just predicting possible mechanisms.

1.1.4 Preterm Birth

Preterm birth is usually defined as birth before 37 completed weeks gestation (Smith 1999b). In the USA, the preterm delivery rate is 12-13%, in Europe and other developed countries the reported rates are 5-9% (Slattery and Morrison 2002; Hamilton et al. 2006; Martin et al. 2006), while in Australia it is around 5-7% (Tracy et al. 2007). Two recent studies on low risk primiparous women in Denmark and Australia found an increase in the rate of spontaneous preterm singleton delivery over a ten year period, despite advances in obstetric treatments (Langhoff-Roos et al. 2006; Tracy et al. 2007). A similar trend has occurred in the USA with rate increasing from 9.5% in 1981 to 12.7% in 2005 (Hamilton et al. 2006). There is an unexplained difference in preterm rates between different ethnic groups with black African-American women having a preterm rate of 16-18%, compared with 5-9% in their Caucasian counterparts, while Hispanic and East Asian women typically have low rates (Goldenberg et al. 2008). Low socioeconomic and educational status, low and high maternal age and single marital status are also risk factors for preterm birth (Goldenberg et al. 2008).

Approximately 50% of preterm births are idiopathic, that is they have no known cause, while intrauterine infection accounts for 25-40% (Goldenberg et al. 2000). Although affecting only around 10% of pregnancies, preterm birth is associated with a 75% neonatal death rate and over 50% of long term morbidity (Challis et al. 2001). Preterm babies have an increased risk of neurologic, metabolic and respiratory disorders. Since preterm birth is the leading cause of perinatal morbidity and mortality among neonates (Wadhwa et al. 1998), a predictor of preterm birth such as maternal CRH levels would be very useful: for example, knowing what causes preterm birth and how CRH may be

involved in the process. Such understanding could lead to prevention of preterm delivery rather than just predicting those cases that are destined to deliver early. Therefore the challenge of this thesis is to elucidate the molecular and biochemical processes that may be involved in placental CRH regulation, thus providing the knowledge to implement preventative treatments.

1.2 Transcription

1.2.1 From DNA to Protein

A complete understanding of gene expression and regulation requires knowledge of the whole process from DNA to protein. The genetic information, or gene, is encoded by DNA through the sequence of the nucleotides in a strand. Genes contain the instructions for producing protein molecules. The genetic information is transmitted by transcribing the DNA gene into messenger RNA (mRNA) which is then translated into protein to carry out a function. RNA is transcribed from DNA by enzymes called RNA polymerases. The mRNA moves to the cytoplasm and directs the synthesis of a protein through the process of translation. This whole process is tightly regulated so that the cell will function correctly, and synthesise the appropriate protein at the relevant time.

1.2.2 The General Process

Transcription is the process that converts the genetic information contained in our DNA into protein coding mRNA. Transcription consists of three main processes: initiation, elongation and termination. Primary transcripts of eukaryotic mRNAs undergo extensive modifications such as 5' capping, splicing and 3'poly-A strand addition.

These RNA processing steps are highly coupled to the transcription steps (Sperling 2007).

1.2.2.1 RNA Polymerases

Three RNA polymerases are involved in transcribing genes in the nucleus. They share some common subunits and structural features but transcribe different genes. RNA polymerase I transcribes most of the ribosomal RNAs (28S, 18S and 5.8S), RNA polymerase II transcribes mRNAs as well as some small nuclear RNAs (snRNAs) and RNA polymerase III synthesises 5S rRNA, transfer RNAs (tRNAs) and some other snRNAs (Cramer et al. 2001).

1.2.2.1.1 RNA Polymerase II

RNA polymerase II (Pol II) promotes the synthesis of pre-mRNA. It consists of twelve subunits, with the largest subunit containing a carboxyl terminal domain (CTD) that is phosphorylated during initiation. The CTD of Pol II has 52 seven-amino-acid repeats in mammals, with three serines per heptad repeat, two of which are subject to phosphorylation (Dahmus 1996). Complete phosphorylation by CTD kinases causes a huge change in the ionic nature of the domain, with the potential to acquire 104 negatively charged phosphate groups. It is likely that CTD phosphorylation switches transcription from initiation to elongation through release of a substantial part of the Pol II initiation complex (O'Brien et al. 1994).

1.2.2.2 Initiation

Initiation begins with proteins known as transcriptional activators binding to specific DNA sequences to help attract Pol II to the transcription start site. This step is necessary for Pol II and the general transcription factors to overcome the difficulty of binding to DNA that is packaged in chromatin (Alberts et al. 2002). A protein complex known as the mediator is also needed to allow proper communication between the transcriptional activator and RNA Pol II.

The majority of RNA Pol II-dependent promoters are characterised by a TATA box. This is a highly conserved sequence found approximately 25-35 base pairs upstream of the start site and is a site rich in A and T, hence the name TATA box. In vitro studies have shown that even a single mutation in the TATA box dramatically decreases transcription by RNA Pol II (Lodish et al. 2000). The TATA box positions RNA Pol II correctly for transcription initiation.

Transcription initiation requires a set of general multi-protein transcription factor complexes (TFIID, TFIIA, TFIIB, TFIIF, TFIIE, TFIIH) (Sperling 2007). TFIID contains the TATA box binding protein (TBP) and TFIIH the helicase to separate DNA strands along with the kinase to phosphorylate RNA Pol II. The transcription process starts with the binding of TFIID/TBP to the TATA box of the promoter, which distorts the DNA to bring the sequences on either side of the binding site together, to allow subsequent protein assembly to form the transcription initiation complex. Once the TBP has bound the TATA box, TFIIB binds followed by TFIIF and RNA Pol II. TFIIE then binds creating a docking site for TFIIH which completes the assembly of the

general transcription factors. In the presence of ATP, the helicase activity of TFIIF unwinds the DNA duplex at the start site allowing Pol II to begin transcribing the template strand. TFIIF phosphorylates the CTD region of Pol II at multiple sites to switch the transcription process from initiation to elongation (Lodish et al. 2000).

1.2.2.3 Elongation

Once transcription has been initiated, RNA Pol II is associated with a series of elongation factors which help stop the polymerase from dissociating from the DNA before it reaches the end of the gene. To contend with chromatin structure, Pol II is also associated with chromatin remodelling complexes. Whether these complexes move with the polymerase along the template or just rescue a stalled polymerase is unclear (Alberts et al. 2002).

Phosphorylation of the RNA Pol II tail and its dissociation from the initiation complex allows the association of not just elongation factors but also pre-mRNA processing factors. Some of these processing proteins are able to jump from the Pol II tail onto the newly transcribed mRNA to begin processing it as it emerges from the polymerase. Thus the process of transcription and pre-mRNA processing are tightly coupled.

1.2.2.4 Capping

Cap addition is the first RNA-processing event to occur soon after transcription initiation has started. The 7-methyl guanine cap is added to all Pol II transcripts when the RNA is about 25 bases long, soon after the 5' end emerges from the exit channel of

the polymerase (Shuman 1997). The cap is added by three enzymes: RNA triphosphatase, guanylyltransferase, and 7-methyltransferase (Shuman 1995). The guanylyltransferase and methyltransferase bind to the phosphorylated Pol II CTD so they can act on the pre-mRNA immediately after transcription initiation and is another example of the coordination between transcription and pre-mRNA processing (McCracken et al. 1997). The 5' cap distinguishes mRNA from other types of RNA. The cap is bound by a CBC (cap-binding complex) which helps the pre-mRNA to be properly processed and exported to the cytoplasm.

1.2.2.5 Termination and 3' End Processing

The pre-mRNA is capped almost immediately after transcription begins and splicing occurs as the RNA Pol II moves along the template, mediated largely by proteins transferred from the CTD tail of Pol II. Similarly, two important 3' end processing proteins, CstF (cleavage stimulation factor) and CPSF (cleavage and polyadenylation specificity factor) travel with the CTD of RNA Pol II (Alberts et al. 2002). As the end of a gene is reached cleavage and polyadenylation signals are transcribed into the RNA and recognised and bound by CPSF and CstF and additional proteins recruited. The mRNA is cleaved at the end of the gene and the enzyme poly-A polymerase adds consecutively, approximately 200 adenine (A) nucleotides to the 3' end of the mRNA to form a poly-A tail. Poly-A binding proteins assemble on the tail and act to determine the length of the tail and remain bound as the mRNA is exported from the nucleus. RNA Pol II may continue to transcribe for several hundred nucleotides past the DNA template 3' cleavage site but soon dissociates from the DNA and transcription terminates. The excess RNA is degraded. It is thought that the transfer of the 3' end

processing proteins from Pol II to the mRNA causes a conformational change on the polymerases that results in dissociation from the DNA template, or that the lack of a cap on the excess RNA signals the polymerase to terminate transcription (Alberts et al. 2002).

1.3 Transcriptional Regulation

1.3.1 General

Regulation of transcription initiation is the most widespread form of gene control in eukaryotes. Transcription is controlled by transcription factor proteins binding at cis-acting regulatory sequences on the DNA, which are often located quite far from the promoter they regulate (Lodish et al. 2000). Therefore, a single promoter may be regulated by multiple transcription factors binding to alternative control elements allowing complete control of gene expression.

1.3.2 The Promoter

As well as the TATA box (Section 1.2.2.2), most genes have additional non-coding DNA sequence upstream of the transcription site that are generically described as the promoter. This region of DNA contains regulatory sequences which control transcription and gene expression and where RNA Pol II assembles. There are a combination of DNA sequences on class II promoters including basal promoter elements, proximal elements and distal enhancer elements. The best characterised basal elements are the TATA box itself and the pyrimidine-rich initiator element located at the start site (Patikoglou and Burley 1997). Promoter proximal elements can occur

from 50 to 200 base pairs upstream on the start site and transcription factors can bind to these sites to regulate transcription. Distal enhancer elements can be located far from the start site in either direction and make up another group of DNA targets for factors that modulate RNA Pol II activity (Patikoglou and Burley 1997). The transcription factors that bind all of these elements are often transducing both extracellular and intracellular stimuli and other cellular signals to control gene expression.

1.3.3 Transcription Factors

1.3.3.1 Classification of Transcription Factors

Transcription factors are the mediators of gene regulation, often responding to extra and intra-cellular signalling pathways to control gene expression by binding to the promoter. They contain a variety of motifs that interact with specific DNA sequences (Figure 1.3). They are often classed according to the type of DNA-binding domain that they contain, and many have characteristic consensus protein sequences (Lodish et al. 2000). They can bind to the gene promoter and other regulatory sequences to affect gene expression.

1.3.3.2 Helix-Turn-Helix Proteins

The helix-turn-helix (HTH) motif was the first DNA-binding motif to be recognised. This motif has been found in hundreds of proteins from both eukaryotes and prokaryotes. This conserved motif consists of an α -helix, a turn and second α -helix (Steitz et al. 1982; Ohlendorf et al. 1983). The HTH motif cannot-fold or function by itself so is usually part of a larger DNA binding domain (Pabo and Sauer 1992). Outside of the HTH motif, the protein sequence and structure varies greatly between

different proteins giving a great versatility to the number of DNA sequences that can be recognised. Helix-turn-helix proteins often bind DNA as symmetrical dimers providing two similar symmetric “half-sites”, greatly increasing binding affinity. HTH proteins may also contain additional domains that are important for regulating activity.

1.3.3.3 Homeodomain Proteins

The homeodomain is a DNA-binding motif that is present in a large family of eukaryotic transcription factors. The homeodomain contains a helix-turn-helix (HTH) motif (Kissinger et al. 1990; Wolberger et al. 1991). The overall structure of the homeodomain consists of an extended N-terminal arm and three α -helices, with helices 2 and 3 forming the HTH motif. Residues in helix 3 contact the DNA in the major groove while residues in the N-terminal arm fit into the minor groove (Pabo and Sauer 1992). Protein-protein interactions may also have a role in modulating homeodomain-DNA interactions such as through heterodimerisation

1.3.3.4 Zinc Finger Proteins

A variety of proteins have regions that-fold around a central zinc ion. They form a finger-like bundle held together by a zinc ion. The common zinc finger motif has an α helix and two β -strands with antiparallel orientation. This motif is often found in proteins that bind RNA or DNA. There are several different types of zinc fingers but most consist of different arrangements of cysteine and histidine residues, such as C2H2 and C3H, to hold the zinc ion (Klug and Schwabe 1995).

The C2H2 zinc finger binds one zinc ion through two cysteine and two histidine side chains. The two cysteines are near the turn of a β sheet region, while the two histidines are in an α -helix (Pabo and Sauer 1992). This produces a compact domain which can insert its α -helix into the major groove of DNA (Lodish et al. 2000). The C2H2 zinc finger is one of the most common DNA binding motifs found in transcription factors and often occur as tandem repeats.

C3H zinc fingers are characterised by three cysteine and one histidine residue that coordinate the zinc ion. C3H zinc fingers have been found in proteins that tend to bind AU-rich RNA sequences (Hall 2005). The RNA is recognised by a combination of hydrogen bond and stacking interactions with the bases. Most C3H zinc fingers contain more than one zinc motif but it has been shown that RNA binding is modular and a single C3H zinc finger can bind weakly but specifically to AU-rich RNA (Michel et al. 2003).

1.3.3.5 Steroid Receptors

The steroid receptor family are an important class of regulatory proteins that include receptors for steroid hormones, retinoids, vitamin D and thyroid hormone. They contain separate domains for hormone binding, DNA binding and transcriptional activation (Evans 1988; Beato 1989). The DNA binding domain is characterised by a four cysteine double loop-zinc-helix motif (Freedman and Luisi 1993). Steroid receptors can function as homo- and/or heterodimers, recognising both inverted and directly repeated half sites (Patikoglou and Burley 1997).

1.3.3.6 Helix-Loop-Helix Proteins

The helix-loop-helix (HLH) proteins have some similarities to the leucine zipper family. They have a basic region that contacts the DNA and a dimerisation region (Voronova and Baltimore 1990). The dimerisation region forms an α -helix, a loop and a second α -helix (Murre et al. 1989). HLH proteins also form heterodimers to modulate their activity.

1.3.3.7 Leucine Zipper/bZIP Proteins

The leucine zipper motif is another motif present in a large class of transcription factors, the bZIP (basic zipper) proteins. They consist of a basic region and an α -helix with the hydrophobic amino acid, leucine at every seventh position over a region of 30-40 residues to allow helix formation. They form dimers with the helices forming a coiled-coil, while the basic regions grip the major groove of the DNA much like a pair of scissors. The dimers can be homodimers or heterodimers, greatly increasing the regulatory potential. The basic region is usually rich in arginine and lysines, but also contains other conserved residues. The basic region is primarily responsible for specific sequence binding as swapping zipper domains between proteins does not affect DNA binding (Agre et al. 1989)1989). Many bZIP proteins bind to cAMP regulatory elements (CREs) in DNA promoters.

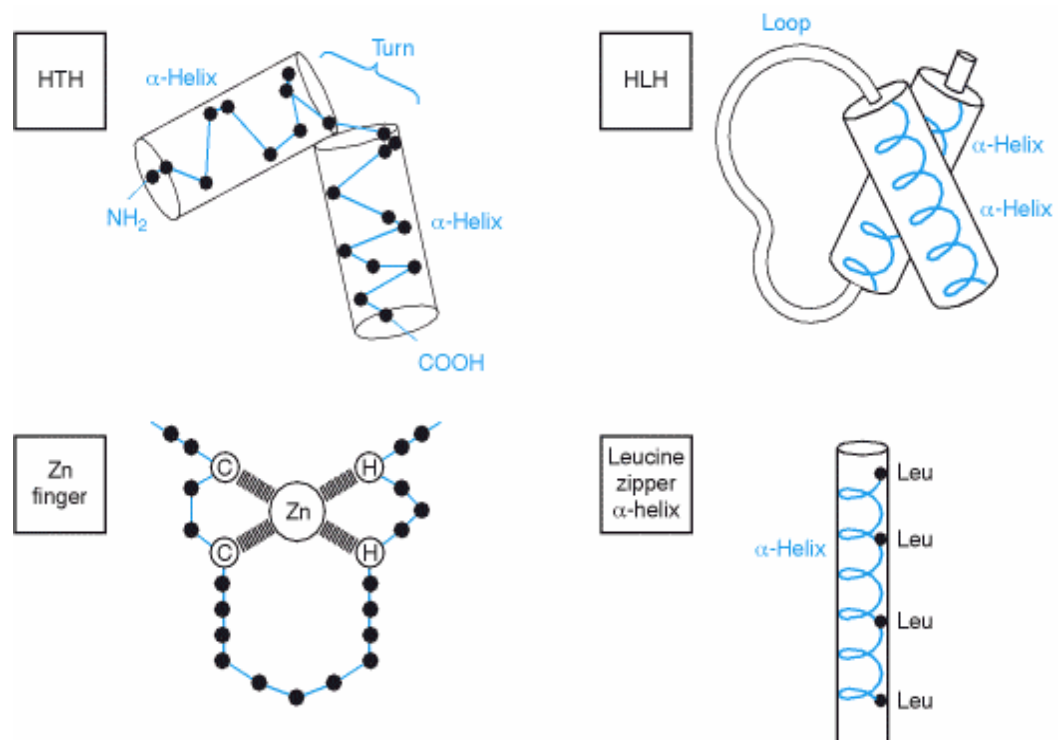


Figure 1.3. Structural motifs commonly found in transcription factors and DNA-binding proteins.

Abbreviations: HTH, helix-turn-helix; HLH, helix-loop-helix. Two amphipathic α -helices can align with their hydrophobic faces in opposition to form a coiled-coil structure. Adapted from Human Molecular Genetics 2, T. Strachan and A. Read. New York and London: Garland Science ;c1999.

1.4 Splicing

1.4.1 Introns and Exons

Most genes in higher animals and plants contain non-coding DNA called introns, dispersed throughout the coding, or exon, DNA. Both introns and exons are transcribed

into pre-mRNA, and in many cases the introns are considerably longer than the exons (Lodish et al. 2000). The non-coding introns need to be removed before the transcript can be translated into a functional protein. Splicing is the process that removes the introns to create a coding mRNA. Most mammalian genes are highly intronic, with over 90% of the gene made up of non-coding introns (Proudfoot 2000). Each splicing event removes one intron through two sequential reactions known as transesterifications. The two exons are joined while the intron is removed as a lariat (Alberts et al. 2002).

Introns range in size from 10 nucleotides to over 100,000 nucleotides. They are bounded by conserved sequences that define their start and finish, the 5' and 3' splice sites respectively. Intron sequence removal involves three sites on the RNA, the 5' splice site, 3' splice site, and the branch point which forms the base of the excised lariat. These sites generally have a consensus sequence that is similar from intron to intron (Alberts et al. 2002).

1.4.2 snRNAs and snRNPs

Splicing is carried out by both RNA and protein molecules. There are five RNA molecules involved, known as small nuclear RNAs (snRNAs). They are less than 200 nucleotides long and designated U1, U2, U4, U5 and U6. Each is complexed with at least seven protein subunits to form a small nuclear ribonuclear protein (snRNPs). These snRNPs form the core of the spliceosome that performs pre-mRNA splicing. U1 snRNP is the first snRNP to assemble with the pre-mRNA. Purified human U1 snRNP contains only three specific proteins, U1-A, U1-C and U1-70k (Luhmann et al. 1990).

1.4.3 The Spliceosome

The spliceosome assembles on the pre-mRNA from multiple components, and subunits enter and leave as the splicing reaction proceeds. The spliceosome contains snRNAs and more than 300 other proteins including the snRNPs and RNA helicases (Nilsen 2003). There is evidence to suggest that the spliceosome is largely pre-assembled (Nilsen 2002). As of 1999, around 100 splicing factors had been identified, but with improved spliceosomal purification and mass spectrometry techniques that number has already doubled (Burge et al. 1999; Jurica and Moore 2003).

Recognition of the splice sites is accomplished largely by base pairing between snRNAs and the pre-mRNA. During splicing the spliceosome undergoes several RNA-RNA interactions where one set of RNA interactions is broken and replaced with another, such as U6 replacing U1 at the 5' splice site. It is thought that these rearrangements occur several times and is a way of checking and rechecking the pre-mRNA sequence to ensure the accuracy of splicing (Alberts et al. 2002).

1.4.3.1 The Splicing Process

Assembly of the spliceosome onto pre-mRNA occurs in an ordered fashion. In metazoans, the pre-mRNA is committed to the splicing pathway by the formation of the E complex. Assembly of the E complex involves recognition of the 5' splice site by U1 snRNA and association of non-snRNP splicing factors such as SR proteins and the U2 auxiliary factor (U2AF) which binds to the 3' splice-site (Hastings and Krainer 2001).

U2 snRNA base pairs with the branch point during formation of the A complex and the ensuing association of the U4/U6-U5 tri-snRNP with the pre-mRNA results in formation of the B complex. Finally, remodelling of the interactions forms complex C and creates the catalytically active spliceosome (Hastings and Krainer 2001).

The catalytic site of the spliceosome is mostly formed by RNA molecules rather than proteins. U2 and U6 form the structure that juxtaposes the 5' splice site and the branch point and performs the first transesterification/cleavage reaction. The 5' and 3' splice sites are brought together in a similar way, involving U5, for the second transesterification/cleavage reaction (Alberts et al. 2002). Once the splicing reaction is complete, the snRNPs remain bound to the lariat and the spliced product is released (see Figure 1.4).

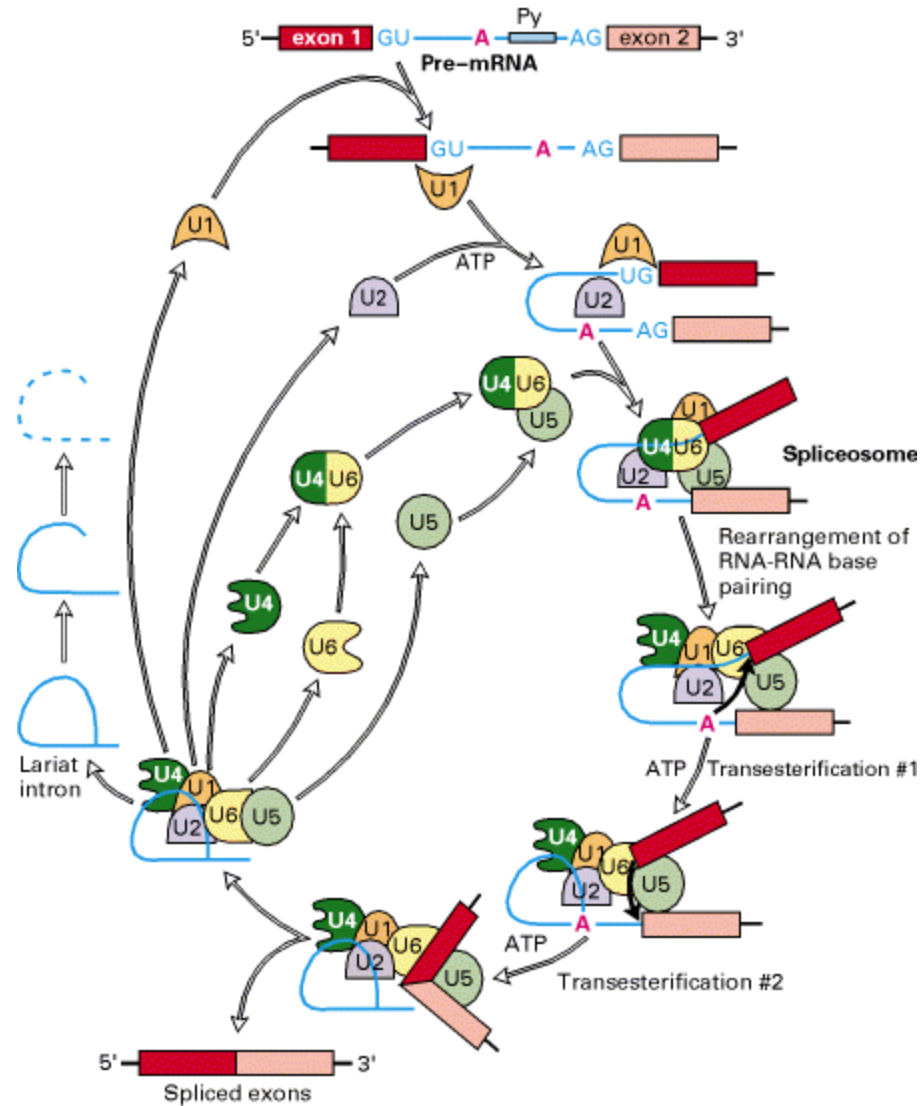


Figure 1.4. The Spliceosomal Splicing Cycle.

The splicing snRNPs (U1, U2, U4, U5, and U6) associate with the pre-mRNA and with each other in an ordered sequence to form the spliceosome. This large ribonucleoprotein complex then catalyses the two transesterification reactions that result in splicing of the exons (light and dark red) and excision of the intron (blue) as a lariat structure. Adapted from M. J. Moore et al., 1993, in R. Gesteland and J. Atkins, eds., *The RNA World*, Cold Spring Harbour Press, pp. 303-357.].

1.4.4 Alternative Splicing

Alternative splicing is the most important mechanism used to generate a large number of mRNA (and hence, protein isoforms) from a limited number of genes. During splicing, the small exons within the non-coding introns, are recognised alternatively so that an exon can either be incorporated into the mRNA or is excised as part of an intron (Stamm et al. 2005). In this way, mRNA with different sets of exons can be generated from the same gene to give rise to different proteins. This was initially believed to be only a minor processing path, but DNA microarray experiments have indicated that 74% of all human genes are subject to alternative splicing (Johnson et al. 2003a; Johnson et al. 2003b). This high percentage is also supported by EST (expressed sequence tag) database analyses which indicate that 35-60% of all human gene products are alternatively spliced (Mironov et al. 1999; Brett et al. 2000; Kan et al. 2001). As splice sites tend to be degenerate, additional sequences present in the exon or adjacent introns aid their recognition and binding by regulatory proteins such as SR proteins and hnRNPs (Stamm et al. 2005). Alternative splicing events which change the sequence of a protein thus provide an additional level of regulating protein function, changes that can alter the binding properties, the intracellular localisation, protein stability, post-translational modifications and enzymatic activity.

1.4.5 SR Proteins

1.4.5.1 The SR Protein Family

The SR proteins are a highly conserved family of structurally and functionally related non-snRNP splicing factors. They have a modular domain structure consisting of one

or two RNA recognition motifs (RRMs) and a C-terminal domain rich in arginine and serine called an RS domain (Fu 1995). The RRM domains determine RNA binding specificity while the RS domain mediates protein-protein interactions and directs localisation to nuclear splicing speckles (Caceres et al. 1997). The prototypical SR protein with two RRM domains is SF2/ASF while the typical single RRM SR protein is SC35. Both SF2/ASF and SC35 interact with U1 70K during spliceosome formation (Wu and Maniatis 1993).

The SR family of proteins function in early spliceosome formation and in multiple steps of the splicing reaction (Staknis and Reed 1994). They recruit U1 snRNP to the 5' splice site (Eperon et al. 1993; Jamison et al. 1995) and bridge the 5' and 3' splice sites via RS domain interactions with U1 and U2 snRNP-associated proteins (Wu and Maniatis 1993). They also facilitate the recruitment of the U4/U6-U5 tri-snRNP complex (Roscigno and Garcia-Blanco 1995).

SR proteins appear to be involved in the recognition of specific 5' splice sites and alternative splicing, generally promoting proximal splice sites (Fu 1995). They either selectively recruit U1 snRNP to a specific 5' site or they promote U1 snRNP binding to all potential sites and the most proximal site is selected by an additional mechanism that has not been characterised (Eperon et al. 1993).

1.4.5.2 SR-like Proteins

There are other RS domain containing proteins that lack recognisable N-terminal RRM domains yet still appear to function in splicing. These proteins are called SR-like proteins or SR protein related polypeptides (SRrps) (Fu 1995). The U1 snRNP component, U1 70K is

the only snRNP known to contain an RS domain (Theissen et al. 1986; Spritz et al. 1987). The small subunit of U2AF, known as U2AF35 in human, contains an RS domain but lacks an RNA binding motif, and has been found to be involved in specific protein-protein interactions with SR proteins and to be associated with the E complex during splicing (Wu and Maniatis 1993; Staknis and Reed 1994).

1.4.6 Nuclear Speckles

Much of the pre-mRNA splicing machinery, including snRNPs, spliceosome subunits and other non-snRNP splicing factors such as SR proteins, is localised to intranuclear speckles, also known as 'SC35 domains' (Wansink et al. 1993) or splicing factor compartments (Phair and Misteli 2000). The speckles correspond to interchromatin granule clusters (IGC's) and to a lesser extent, perichromatin fibrils, that are seen by electron microscopy (Lamond and Spector 2003). These fibrils also contain splicing proteins and occur on the periphery of IGC's, as shown in Figure 1.5, in addition to being present in the nucleoplasm (Fakan 1994).

The majority of pre-mRNA splicing factors are enriched in speckles and not at the sites of active transcription (Zimber et al. 2004), being on average 5-10-fold more enriched in speckles than in the nucleoplasm (Wei et al. 1999). Nuclear speckles are thought to function in the storage and assembly of spliceosomal factors (Lamond and Spector 2003) while perichromatin fibrils are considered to be the sites of transcription and pre-mRNA processing (Fakan 1994; Cmarko et al. 1999). *In situ* hybridisation and BrUTP labelling studies have localised active genes and Pol II transcripts mostly at the periphery of (most likely in perichromatin fibrils), and to a lesser extent within speckles,

allowing coordinated transcription and splicing (Wei et al. 1999; Lamond and Spector 2003). Although most transcription takes place at the periphery of nuclear speckles, there are also proteins that are involved in transcription that associate with splicing speckles (Larsson et al. 1995; Mortillaro et al. 1996; Zeng et al. 1997).

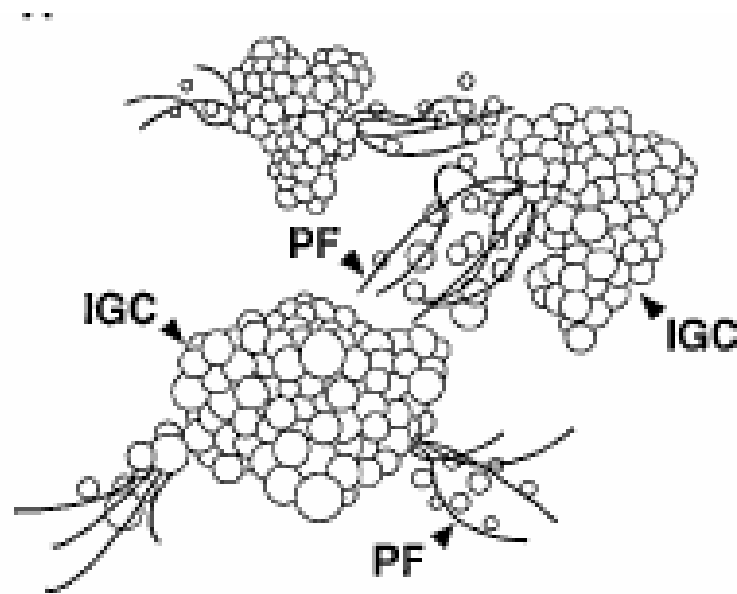


Figure 1.5. The Organization of Splicing Factors from a Transcriptionally Active Nucleus.

In a transcriptionally active nucleus, splicing factors localize to interchromatin granule clusters (IGC) and perichromatin fibrils (PF). The interchromatin granule clusters are irregular in shape and often connected, probably representing perichromatin fibrils, are observed between interchromatin granule clusters. The irregular shape of the interchromatin granule clusters probably represents a freeze-frame of dynamic movements within the cluster that are occurring in living cells. Adapted from (Spector 1996).

1.5 Corticotropin Releasing Hormone

1.5.1 General

Corticotropin-releasing hormone (CRH) is a 41 amino acid neuropeptide that is produced in the hypothalamus and is involved in the stress response (Vale et al. 1981). Hypothalamic CRH secretion is stimulated by many forms of physical or psychological duress and is a vital coordinator of the neuroendocrine response to stress (McLean and Smith 2001). CRH was first isolated from the sheep hypothalamus by Vale *et al.* in 1981 (Vale et al. 1981). CRH stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which in turn, stimulates glucocorticoid production in the adrenal cortex (Lightman and Young 1989; Orth 1992). CRH is also found in extra-hypothalamic sites of the central nervous system (Thompson et al. 1987; Seasholtz et al. 1988; Dorin et al. 1993) and peripheral tissues including the placenta (Frim et al. 1988; Usui et al. 1988; Zoumakis et al. 1997).

Placental CRH is identical in structure, immunoreactivity and bioactivity to hypothalamic CRH (King et al. 2001b). It is present in the syncytiotrophoblasts and intermediate trophoblasts of the placenta and also in the fetal membranes and musculature of the umbilical vessels (Riley et al. 1991; Petraglia et al. 1992). The production of placental CRH only occurs in humans and higher primates (Robinson et al. 1989; Smith et al. 1999) suggesting that there are different mechanisms involved in regulating human pregnancy than there are in other mammals. CRH is hypothesised to have a significant role in parturition, therefore, investigation of its regulation is of importance. The CRH promoter contains DNA regulatory elements that are involved in regulating its expression. Of the DNA regulatory elements involved in regulating CRH

expression at the promoter level, one of the most important is the cAMP regulatory element (CRE).

1.5.1.1 CRH Stimulants

Various endogenous biochemical substances stimulate CRH release from the placenta and hypothalamus. These agents include IL-1, angiotensin II, oxytocin, arginine vasopressin, norepinephrine, epinephrine and acetylcholine (Petraglia et al. 1989). They act through the cAMP dependent protein kinase pathway to turn on specific genes via the CRE (Deutsch et al. 1988). Exogenous agents such as forskolin and 8-Br-cAMP also increase the expression of CRH in the placenta and hypothalamus via cAMP-mediated protein kinase A (PKA) activation (Suda et al. 1985; Turkelson 1988; Itoi et al. 1996). Prostaglandin E₂ (PGE₂) and prostaglandin F₂ α (PGF₂ α) also increase placental CRH in a concentration response manner (Dibbs et al. 1997).

1.5.2 Placental CRH

Large amounts of placental CRH are secreted into the maternal circulation during the third trimester of human pregnancy. CRH secretion increases as much as 100 times during the last 6 to 8 weeks of gestation (Majzoub and Karalis 1999) and peaks during labour to values of 1000-10,000 pg/ml, a concentration similar to that found in the hypothalamic-pituitary portal blood during stress (Goland et al. 1986). This suggests that CRH has an important role in parturition. Studies have found that there is a relationship between CRH concentrations and the length of gestation (Smith 1999a). McLean *et al.* found, through a prospective longitudinal cohort study of 485 women, that CRH levels in the second trimester predicted the length of gestation. High levels of

CRH were linked to pre-term delivery while low concentrations were associated with post-term delivery. They found, through linear regression analysis, that CRH levels increased as a constant exponential function throughout gestation and that the exponential curves for women who would have pre-term, on time or post-term deliveries were parallel when plotted on log linear scales (McLean et al. 1995) (Figure 1.6). They suggest that a 'placental clock' may be active to control the timing of parturition and that this clock is functioning from as early as 16-20 weeks gestation (McLean et al. 1995). Wadhwa *et al.* also found that maternal CRH levels in the early third trimester predict the length of gestation (Wadhwa et al. 1998). In a study of 63 pregnant women they found that women who delivered preterm had significantly higher CRH levels than those who delivered at term, although sample numbers were quite low in this study and serial sampling was not conducted (Wadhwa et al. 1998). Similar results were found in a prospective observational study in a Chinese population, though many of the results showed a trend rather than statistically significant data (Leung et al. 2000). An interesting finding was that the strength of the association between CRH levels and preterm delivery is greatest in very premature deliveries.

These findings point to CRH being a clinically useful predictor of pre-term delivery (Smith 1999a). Further, the predictive effect of CRH levels is increased by combining with measurement of alpha-fetoprotein and clinical risk factor score (McLean et al. 1999). However, a previous study by Berkowitz *et al.* found no significant increase in maternal CRH levels in preterm versus term labour when measured from 20 weeks gestation (Berkowitz et al. 1996). One point of difference is that this earlier study used a different assay with a higher threshold for detection of CRH than the subsequent work

by McLean *et al.*, and there were population and the blood sample preparation differences. Nevertheless, the values for CRH binding protein levels were similar between the two studies. Therefore, until the completion of more definitive studies the predictive power of plasma CRH levels remains controversial.

Some of the controversy on the role of the levels of the circulating CRH may reflect the effects of CRH on the length labour. Thus, Stalla *et al.* discovered that women with high CRH concentrations at the time of labour tend to have shorter labours whereas women with low CRH had longer labours (Stalla 1998). Mclean *et al.* also found an inverse relationship between CRH concentrations and the duration of labour (McLean et al. 1994).

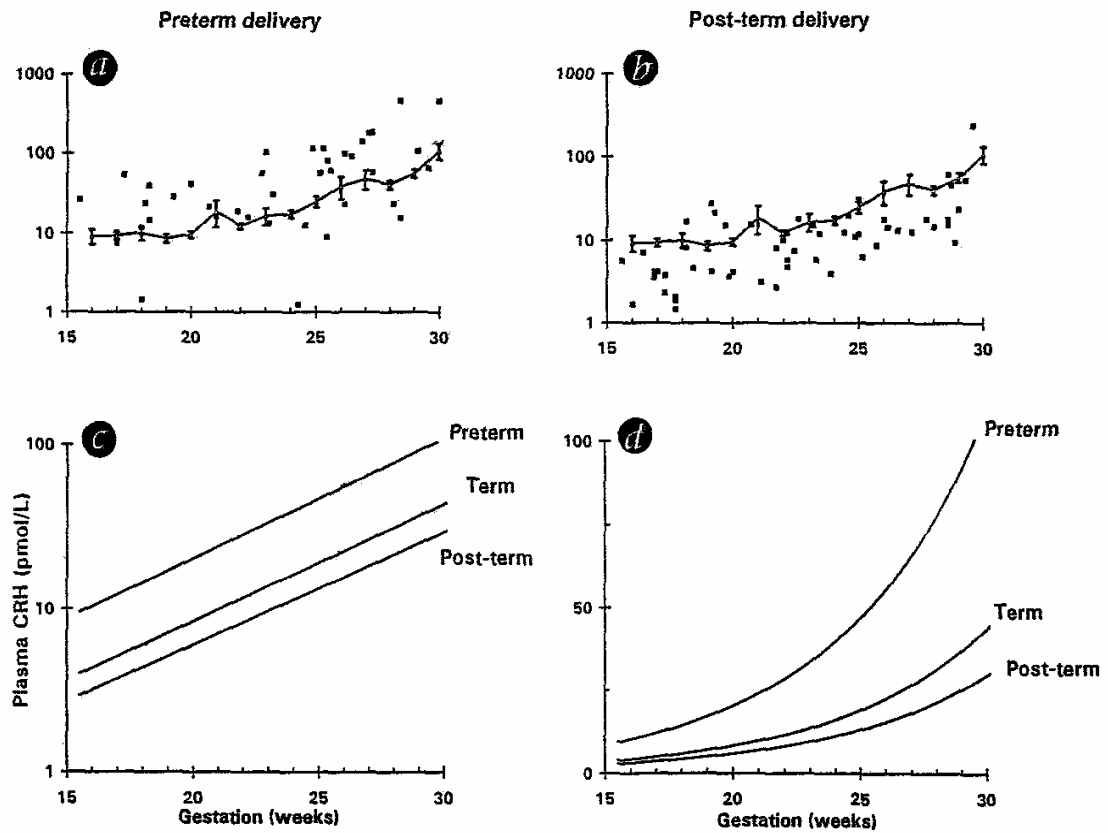


Figure 1.6. Maternal plasma CRH levels throughout mid-gestation in women whose pregnancies ended in spontaneous preterm labour, term labour or post-term delivery.

a, Mean plasma CRH in term deliveries compared with individual samples from women delivering preterm, or *b*, post-term. *c*, Log-linear regression curves fitted to plasma CRH results for the three groups. *d*, Non-logarithmic representation of the regression curves demonstrating the exponential increase in CRH (adapted from (McLean et al. 1995)).

1.5.3 Localisation of CRH Immunoreactivity

In the human brain, immunoreactive CRH has been found in the posterior pituitary, thalamus, hypothalamus, cerebral cortex, cerebellum, pons, medulla oblongata, spinal cord and cerebrospinal fluid and in peripheral tissues it has been localised to adrenal medulla, ovary, testis, heart, lung, liver, stomach, duodenum and pancreas (Suda et al. 1984).

Riley *et al.* used immunohistochemical techniques to determine the localisation and distribution of CRH immunoreactivity (CRH-IR) in human placenta, fetal membranes, decidua and umbilical cord from tissues throughout gestation. CRH-IR was not detectable in tissues from 6-8 weeks gestation (Riley et al. 1991). This is consistent with the low maternal serum CRH levels during the first trimester. Positive CRH staining appears in the placenta between 8 and 20 weeks gestation. In the placenta, positive CRH staining was found in the syncytiotrophoblast and intermediate trophoblast, but not in cytotrophoblast cells. The trophoblast that had replaced the endothelium of maternal blood vessels in the decidua also had positive CRH staining. CRH-IR was present in the epithelium and some cells of the subepithelial layer of the amnion, while the trophoblast layer and some cells of the reticular and cellular layers of the chorion also showed CRH-IR. The decidua shows positive CRH staining in invasive trophoblast and stromal cells, whilst the amnion epithelium of the umbilical cord and the musculature of the umbilical vessels are also CRH positive.

1.5.4 The CRH Gene

One of the most interesting puzzles in considering the role of CRH in human parturition is the fact that placental CRH is expressed in humans and higher primates but in no other species examined, including rat, guinea pig and lemur (Robinson et al. 1989; Smith et al. 1999). Further, the regulation of human CRH expression differs between the placenta and the hypothalamus (Robinson et al. 1988; Jones et al. 1989; King et al. 2001c; King and Nicholson 2007). Despite this, in all species, CRH is encoded by a single gene, localised to chromosome 8 in humans (Arbiser et al. 1988).

Differences in the CRH promoter might provide one simple explanation for these intra-species variations, however, examination of the CRH promoter in humans, sheep, mice and rats reveals that it is highly conserved across the different species with considerable homology over the first 336bp of the promoter in humans, sheep, mice and rats (King et al. 2001b) (Figure 1.7). Further, there is complete conservation of several consensus regulatory elements including a cAMP regulatory element (CRE), a $\frac{1}{2}$ estrogen regulatory element (1/2ERE), an ecdysone regulatory element (EcRE), 2 TATA boxes (TBP), and a homeobox protein-binding site (CDXA or CDXRE) (King et al. 2001b). Therefore it seems probable that specific proteins within the nucleus determine whether a species expresses placental CRH.

In seeking to identify species-specific protein binding to regions of the CRH promoter Scatena and Adler compared human and rat choriocarcinoma cell lines transfected with CRH-luciferase constructs and found that a region between –532 and –400bp inhibited rat cells while the –400 to –340bp region increased promoter activity in the rat but not

human cells (Scatena and Adler 1996). Thus CRE had a major role in human cells but appeared inactive in rat cells. This demonstrated that differences in trans-acting factors, not cis-acting factors, were responsible for determining the expression of CRH in placental cells. Therefore, it seems that different transcription factors are present in different species.

Human	TATGGCCTTTCATAGTAAG <u>AGGTCA</u> ATATGTTTTC-ACACTTGGGAAATCTCATTCAAGAATTTT
Sheep	TTTGGCCTTTCCTAGTAAG <u>AGGCCA</u> GTATGTCTTC-ACACTTGGGAAATCTCATTCAAGAATTTT
Mouse	TCTGGCCTATCATAGTAAG <u>AGGTCA</u> GTCTGTTTTCACACTTGGATAGTCTCATTCAAAAATTTT
Rat	---GGCCTATCATAGTAAG <u>AGGTCA</u> GTATGTTTTCACACTTGGATAATCTCATTCAAGAATTTT
	<u>HRE/½ ERE</u>
Human	<u>TGTCAATGGACA</u> AGTCATAAGAAGCCCTTCCATTTTAGGGCTCGTT <u>TGACGTCA</u> CCAA-GAGGCGA
Sheep	<u>TGTCAATGGACA</u> AGTCATAAGAAGCCCTTCCATTTTAGGGCTCTTT <u>TGACGTCA</u> TCAAGGAGGCGA
Mouse	<u>TGTCAATGGACA</u> AGTCATAAGAAACCCTTCCATTTTCGGGCTCGTT <u>TGACGTCA</u> CCAAGGAGGCGA
Rat	<u>TGTCAATGGACA</u> AGTCATAAGAAGCCCTTCCATTTTAGGGCTCGTT <u>TGACGTCA</u> CCAAGGAGGCGA
	<u>EcRE</u> <u>CRE</u>
Human	TAAATATCTGTTGAT <u>TATAAT</u> TGGATGTGAGATTCAAGTGTGAGATAGCAAAA-TTCTGCCCCCTCG
Sheep	TAAATATCTGTTGAT <u>TATAAT</u> TGGATGTGAGATTCAAGTGTGAGATAGCAAAAATCTGCCCCCTCG
Mouse	TAAATATCTGTTGAT <u>TATAAT</u> TGGATGTGAGATTCAAGTGTGAAATAGCAGAA-CTCTGTCCCTCG
Rat	TAAATATCTGTTGAT <u>TATAAT</u> TGGATGTGAGATTCAAGTGTGAAATAGCAGAA-CCCTGTCCCTCG
	<u>TBP (2)</u>
Human	TTCTTTGGCAG <u>GGCCCTATGATTATGCAGG</u> AGCAGAGGCAGCACGCAATCGAGCTGTCAAGAGA
Sheep	TTCCCGGGCAGG--CCTATG <u>ATTTATGC</u> AGGAGCAGAGGCAGC-GGCAATCCAGCTGTCAAGAGA
Mouse	CTCCTTGGCAGGGCCCTATT <u>ATTTATGC</u> AGGAGCAGAGGCAGCACGCAATCGAGCTGTCAAGAGA
Rat	CTCCTTGGCAGGGCCCTATT <u>ATTTATGC</u> AGGAGCAGAGGCAGCACGCAATCGAGCTGTCAAGAGA
	<u>Human-placenta specific</u>
	<u>CDXRE</u>
Human	GCGTCAGC-TATTAGGCAAATGCTGCGTGCTTTTGAAGAGGGTCGACACT <u>TATAAA</u> ATCCCACTC
Sheep	GCGTCAG-TTATTAGGCAAATGCTGCGTGCTTCTGAAGAGGGTCGACACT <u>TATAAA</u> ATCCCCTTC
Mouse	GCGTCAGCTTATTAGGCAAATGCTGCGTGCTTCTGAAGAGGGTCGACAT <u>TATAAA</u> ATCTCACTC
Rat	GCGTCAGCTTATTAGGCAAATGCTGCGTGCTTCTGAAGAGGGTCGACGT <u>TATAAA</u> ATCTCACTC
	<u>TBP</u>
Human	CAGGCTCTGGAGTGG
Sheep	CAGGCTCTGGTGTGG
Mouse	CAGGCTCTGGTGTGG

Figure 1.7. Comparison of the DNA sequence of the promoter regions of CRH genes from human, sheep, mouse and rat.

This shows the region from -1 to -337 of the human CRH gene with regulatory elements underlined and bold (adapted from (King et al. 2001b)).

1.5.5 CRH Promoter Regulatory Elements

The CRH promoter contains several regulatory elements that are responsive to different factors. These elements and how they relate to CRH expression are discussed in more detail below. A model of how these elements may be interacting on the CRH promoter in the placenta is shown in Figure 1.8.

1.5.5.1 The CRE of the CRH Promoter

The CRE appears to have an important role in the regulation of human CRH gene expression. Spengler *et al* identified the consensus CRE using progressive 5' end deletions of the human CRH (hCRH) gene promoter (Spengler *et al.* 1992). The CRE is located between –221 and –228bp in the CRH promoter (Spengler *et al.* 1992). It consists of a palindromic DNA sequence, TGACGTCA (Yamamoto *et al.* 1988; Shaywitz and Greenberg 1999). Cheng *et al.* explored the effect of cAMP on the CRH promoter in primary human placental cell cultures (Cheng *et al.* 2000a). They demonstrated that cAMP stimulates CRH gene expression through the CRE in the proximal promoter region, in a dose-dependent manner. Through deletion analysis they also found that cAMP responsiveness was lost, and basal promoter activity decreased by 30%, when the region from –342 to –212 bps was deleted, thus removing the CRE (Cheng *et al.* 2000a). This demonstrates the necessity of the CRE for CRH expression in placental cells. It has also been demonstrated that progesterone regulates CRH gene transcription via the CRE in the CRH promoter (Ni *et al.* 2004b) (Section 1.1.2.4). The CRE is also involved in estrogen and glucocorticoid regulation of CRH (see below).

1.5.5.2 *The CDXA/CDXRE*

The CRH promoter contains a caudal type homeobox response element (CDXA or CDXRE) at -125 to -118 base pairs. The role of this element in CRH regulation was discovered more by chance than design. Scatena and Adler identified a proximal cAMP responsive region within the CRH promoter from -200 to -99bp that does not contain any characterised classic CRE (Scatena and Adler 1998). They also isolated a 58 kDa placental, human specific factor that binds within this region from -128 to -109 bps but this factor does not bind to a CRE and does not contain classical CRE-binding proteins such as CREB or ATF-2. King *et al.* also found a cAMP responsive site between -213 and -99bps in a mouse corticotroph cell line (AtT-20 cells) (King et al. 2001a). Using deletion studies of the CRH promoter, they found that deletion to -213bp (hence removing the consensus CRE) reduced cAMP stimulation of promoter activity to 3.47-fold above baseline. However, the construct containing only the first 99bp of the promoter showed no significant induction of activity over baseline. This indicated that there was a cAMP responsive region between -213 and -99bps. Analysis of this region revealed a CDXA/CDXRE. Mutation of this site resulted in significantly reduced cAMP induced promoter activity compared to the wild type promoter (King et al. 2001a). Therefore, cAMP stimulates the CRH promoter through two separate response elements, a consensus CRE and a consensus CDXA. They also suggest that these two regions may act synergistically. This indicates that the CDXA is responsive to cAMP in both the placenta and mouse pituitary cells.

1.5.5.3 *The EcRE*

The CRH promoter contains a consensus ecdysone regulatory element (EcRE) at –273 to –263 bps (King et al. 2001b). Ecdysone is an insect steroid hormone that has no known role in mammalian gene regulation and there are no ecdysone receptors present in mammals. However, when this region is mutated and transfected into primary placental cell cultures, there is an increase in promoter activity above that of the wild type (Cheng et al. 2000b). This suggests that this region may act as an inhibitor in placental cells. The region –278 to –246 bps (that includes the EcRE) was identified by Malkoski *et al.* as a negative glucocorticoid response element (nGRE) in AtT-20 cells (Malkoski and Dorin 1999) and this region corresponds to one of the GR binding sites identified by Guardiola-Diaz *et al.* (Guardiola-Diaz et al. 1996). However, Cheng *et al.*'s mutations in the EcRE that up-regulated CRH promoter activity in placental cells had no effect in AtT-20 cells (King and Nicholson 2007). The role of the EcRE in placental CRH regulation needs further elucidation, but a role as an inhibitor seems logical, as gene regulation should include both stimulation and repression for optimum control.

1.5.5.4 *Estrogen Response Element*

The CRH promoter contains five estrogen regulatory element half sites (1/2 ERE) (Vamvakopoulos and Chrousos 1993). However, Dibbs *et al.* transfected CRH-luciferase reporter genes into human placental choriocarcinoma cell lines and found no effect from administration of estrogen (Dibbs et al. 1997). They also observed no binding of estrogen receptor to the 1/2 EREs. The CRH promoter is almost certainly exposed to estrogen, given that the placenta converts fetal DHEA to estrogen, but the

role that estrogen has, if any, on CRH expression is unknown. However, in primary placental cells it has been shown that estrogen has an inhibitory effect on CRH mRNA and protein levels and the effect is mediated by ER α (Ni et al. 2002). It was also found that the inhibitory effect of estrogen on CRH in the placenta required the presence of a consensus CRE (Ni et al. 2004a).

1.5.5.5 Glucocorticoids

The CRH promoter does not contain a consensus glucocorticoid responsive element (GRE) (Vamvakopoulos and Chrousos 1993), however, Guardiola-Diaz *et al.* identified three regions of the CRH promoter that can bind glucocorticoid receptors (GRs) in mouse AtT-20 cells; -313 to -301, -270 to -258 and -202 to -175bps (Guardiola-Diaz et al. 1996). In contrast to the glucocorticoid-mediated inhibition of CRH gene expression in the hypothalamus, the expression of placental CRH mRNA and protein is stimulated by glucocorticoids. Cheng *et al.* found that glucocorticoids up regulate the transcription of CRH in human primary placental cells but they also established that the synthetic glucocorticoid, dexamethasone, stimulates placental hCRH gene transcription through the CRE and not the regions capable of binding the GR sites (Cheng et al. 2000b). Using 5' deletions of the hCRH promoter, they found that dexamethasone responsiveness was lost when the region between -342 and -213bp was removed. This region contains the CRE, a hybrid steroid HRE (Truss et al. 1991) and the EcRE (Fisk and Thummel 1998). Mutational analysis found that mutation of the CRE caused a 30% decrease in basal promoter activity and abolished the dexamethasone responsiveness. Mutation of the HRE had no effect while EcRE mutation resulted in an increase in basal activity and maintenance of dexamethasone induction. They also found that fragments

of the hCRH promoter containing the CRE confer glucocorticoid responsiveness to a heterologous promoter (Cheng et al. 2000b). These results indicate that a functional CRE is necessary and adequate for glucocorticoid stimulation of CRH gene expression in the placenta. However, studies have demonstrated that the glucocorticoids and their receptor (GR) do not bind directly to the CRE (Guardiola-Diaz et al. 1996). Using super-shift electrophoretic mobility shift assay (EMSA), King *et al.* have shown that the placental nuclear proteins that bind to the CRE cannot be super-shifted using anti-GR antibodies, indicating that GR is not binding directly to the CRE (King et al. 2001a). It is probable that glucocorticoid effects in placental cells occur through protein-protein interactions at the CRE, which do not require ongoing protein synthesis (Cheng et al. 2000b; King et al. 2001b). King *et al.* additionally found that the region from –213 to –99bps (containing the CDXA), that is also stimulated by cAMP, can be stimulated by glucocorticoids (King et al. 2001a). This suggests that it is not just protein-protein interactions at the CRE, but interactions with the cAMP pathway that are mediating glucocorticoid stimulation of CRH promoter activity.

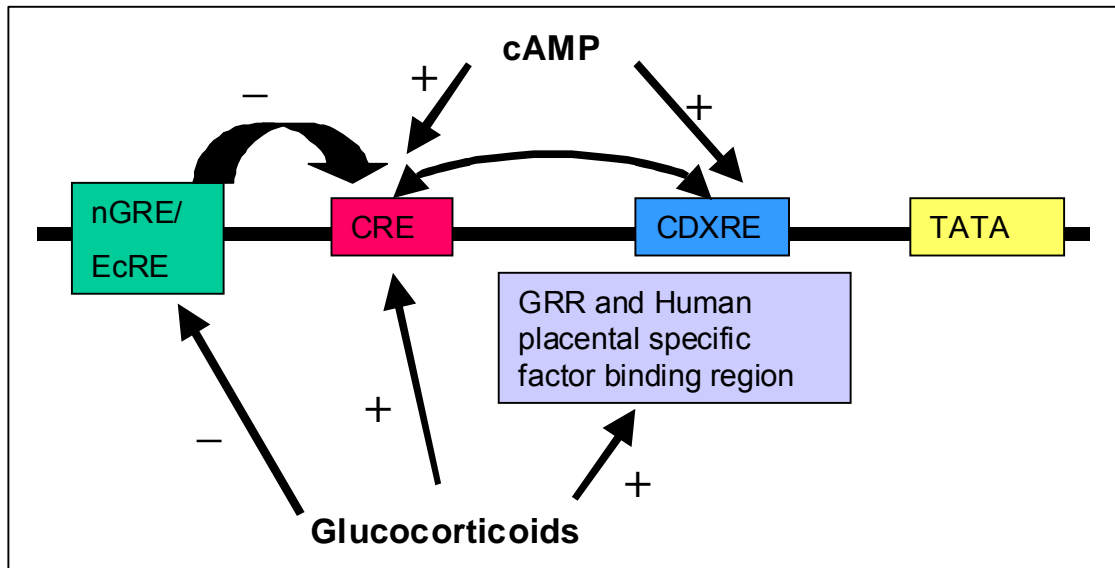


Figure 1.8. CRH Promoter Functional Elements.

A schematic model of the CRH promoter with potential regulatory elements mediating the effects of cAMP and glucocorticoids. nGRE/EcRE represents the negative glucocorticoid regulatory element, which also contains the EcRE. CRE is the cAMP responsive element, CDXA is the caudal type homeobox element, GRR represents the region from -213 to -99bps that is stimulated by glucocorticoids and also is the site of human placental specific factor binding and TATA is the TATA box. The - and + signs indicate inhibitory and stimulatory effects, respectively, while the double headed arrow indicates synergistic stimulatory interactions (adapted from (King et al. 2001a).

1.5.6 The CRE and the cAMP Pathway

The CRE plays a pivotal role in the regulation of CRH expression in both the placenta and the hypothalamus. The CRE not only mediates the actions of the cAMP pathway, but also mediates effects of glucocorticoids, estrogens and progesterone on CRH regulation. Therefore an understanding of the cAMP pathway is imperative.

The activation of adenylate cyclase enzyme by extra- or intracellular signals leads to the production of the second messenger, cAMP (cyclic adenosine monophosphate) by adenylate cyclase. This in turn activates protein kinase A (PKA) which enters the nucleus and phosphorylates the cAMP regulatory element binding protein (CREB). The phosphorylated CREB then binds to the CRE and activates transcription (see Figure 1.9).

1.5.6.1 The CRE

The cAMP response element (CRE) consists of an eight base pair palindrome, TGACGTCA, and is usually located within 100 base pairs of the TATA box (Montminy 1997). The palindromic CRE can be separated into two CGTCA motifs which can be on the same or opposite strands to function cooperatively (Fink et al. 1988). The ATF (activating transcription factor) sequence is almost identical to the CRE with a consensus of TGACGT(C/A)(G/A) (Lin and Green 1988), and there is an overlap of binding proteins between the two sites.

1.5.6.2 Protein Kinase A

PKA, also known as cAMP dependent protein kinase, consists of two regulatory and two catalytic subunits. The R subunit exists in two forms, RI and RII, while just one form of the C subunit is present in eukaryotic cells. Both R subunits (RI and RII) and the single C subunit exist as multiple isoforms ($C\alpha$, $C\beta$, $C\gamma$, $RI\alpha$, $RI\beta$, $RII\alpha$, $RII\beta$). In unstimulated cells there is almost no PKA in the nucleus (Adams et al. 1991), however

upon cAMP induction, the regulatory units are bound by cAMP and dissociate from the catalytic subunit allowing it to passively translocate to the nucleus and phosphorylate target proteins, such as CREB (Adams et al. 1993; Harootunian et al. 1993).

PKA is required for cAMP-stimulated transcription. Cells deficient in PKA activity are unable to stimulate a CRE reporter gene (Montminy et al. 1986), overexpression of the PKA inhibitor, PKI, specifically abolishes cAMP dependent transcription (Grove et al. 1987), and microinjection of purified PKA catalytic subunit induces a CRE reporter (Riabowol et al. 1988). PKA mediates the effect of cAMP to regulate promoters containing CRE's.

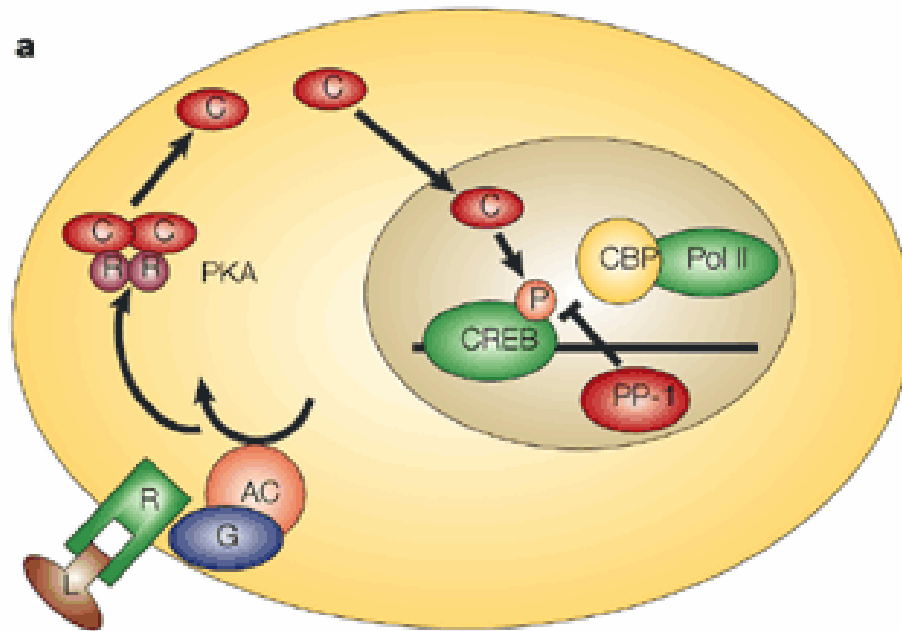


Figure 1.9. The PKA Pathway.

Induction of adenylyl cyclase (AC) by ligand (L)-bound receptor (R) proceeds through activation of the heterotrimeric G protein (G). Increases in the levels of cellular cAMP promote dissociation of the protein kinase A (PKA) heterotetramer, which consists of paired regulatory (R) and catalytic (C) subunits. Liberated C subunits migrate into the nuclear compartment by passive diffusion and phosphorylate the cyclic AMP response element (CRE)-binding protein (CREB) at a single phospho-acceptor site, Ser133. Ser133 phosphorylation promotes transcription by recruitment of the co-activator CREB-binding protein (CBP). CBP, in turn, mediates transcriptional activation through its association with RNA polymerase II (Pol II) complexes and through intrinsic histone acetyltransferase activity. Target gene activation is terminated by the serine/threonine phosphatase PP-1-mediated dephosphorylation of CREB. (Mayr and Montminy 2001).

1.5.6.3 Adenylate Cyclase

Adenylate cyclase is the enzyme that synthesises cAMP from ATP through catalysis. There are nine isoforms (types I-IX) and each has its own unique regulation. Adenylate cyclase has low basal activity but is stimulated upon binding of the G-protein α subunit ($G_{s\alpha}$), through activation of G-protein coupled receptors (Tesmer and Sprang 1998). In addition to regulation by $G_{s\alpha}$, mammalian adenylate cyclase is also regulated by other G-proteins, forskolin, calcium and phosphorylation (Tang and Hurley 1998).

1.5.7 CRE-Binding Proteins

1.5.7.1 Most CRE-Binding Proteins are Members of the bZIP Family

The only common feature of bZIP proteins is an α -helical basic region and the leucine zipper domain. The bZIP proteins can be subdivided into families such as the Fos, Jun, C/EBP, CREB/ATF and NF-E2 families (Hurst 1995). The CRE and other related sequences have dyad symmetry, consisting of two 'half-sites'. Therefore the dimerisation ability of bZIP proteins makes them ideal to provide to basic DNA binding regions to contact both sites.

Classical CRE-binding proteins include CREB, ATF2, ATF3, ATF4, and ATF6 (Hurst 1995). However, other bZIP proteins can bind the CRE under certain conditions. AP1 (activating protein 1) consists of homo- and heterodimers of Jun, Fos and ATF. Jun-Jun and Jun-Fos dimers preferentially bind the AP-1 site (TGACTCA) while Jun-ATF and ATF-ATF dimers prefer the related CRE site (TGACGTCA) (Hai and Curran 1991).

C/EBP has its own binding site but also recognises the CRE (Bakker and Parker 1991; Kageyama et al. 1991).

1.5.7.2 *CREB*

One of the best characterised transcription factors that bind to the CRE is the cAMP response element binding protein (CREB) (Shaywitz and Greenberg 1999). CREB was discovered during studies of cAMP regulation of the somatostatin gene. Montminy and Bilezikjian used a DNase I footprinting assay on nuclear extracts from PC12 cells to detect DNA binding proteins that interact with the CRE of the somatostatin promoter (Montminy and Bilezikjian 1987). To purify the protein binding to the CRE, a sequence specific DNA affinity column was constructed and PC12 extracts passed over the column. The resulting protein was determined to be 43 kDa and named CREB for CRE-binding Protein (Montminy and Bilezikjian 1987).

CREB binds to its target sequence as a dimer through a conserved structural motif, the leucine zipper, formed by a heptad repeat of leucine residues (Yamamoto et al. 1988),(Yun et al. 1990). DNA binding is facilitated by a basic lysine- and arginine-rich domain located at the N-terminal (Dwarki et al. 1990). The leucine zipper and basic domain place CREB in the bZIP family of transcription factors, which includes c-Fos, c-Jun, c-Myc, and C/EBP , ATF and yeast Gcn4 (Struhl 1989; Vinson et al. 1989). Using super-shift EMSA, King *et al.* have found that CREB and c-Jun are part of the placental cell nuclear protein complex that binds to the CRE from the CRH promoter (King et al. 2001a).

PKA phosphorylates CREB at serine 133 (Ser133) and this phosphorylation site is required for signal-induced transcription *in vivo* (Montminy and Bilezikjian 1987; Gonzalez and Montminy 1989; Gonzalez et al. 1989). CREB is activated by phosphorylation and phosphorylated CREB then binds to the CRE (Wolfl et al. 1999). CREB is also capable of being phosphorylated by CaM kinases I, II and IV, the Ras-MAPK pathway, PKC, glucagon synthase kinase III and casein kinase II but the role of these enzymes in the placenta is unclear (Tsagarakis et al. 1991; Xing et al. 1996; King et al. 2001b).

1.6 cAMP Regulatory Element Associated Protein- CREAP

1.6.1 Discovery

Because the CRE appears to have a major role in both cAMP and glucocorticoid stimulated CRH promoter activity, our laboratory used the yeast one-hybrid system with the CRE as the bait in screening a placental cDNA library to identify possible regulatory proteins (Shipman et al. 2006). This resulted in the isolation of a cDNA encoding a protein with leucine-zipper like repeats (Figure 1.10). This sequence was not homologous with any known transcription factor but the presence of the leucine-zipper-like repeats suggested that it has DNA, specifically CRE, binding properties, similar to the bZIP transcription factor family. This was substantiated with Electrophoretic Mobility Shift Assays (EMSA) when it was shown that protein from yeast transformed with CREAP specifically binds to the CRE and competition with unlabelled CRE oligonucleotides compete away binding (see Figure 1.11). The complex formed on the CRE was not super shifted with antibodies to a variety of known

transcription factors. This novel, putative transcription factor was named CREAP for cAMP regulatory element associated protein.

1	MISAAQLLD	ELMGRDRNLA	PDEKRSNVRW	DHESVCKYYL	CGFCPAELFTN
51	TRSDLGPCE	KIHDENLRKQ	YEKSSRFMKV	<u>GYERDFLRYL</u>	<u>QSLLAEVERRI</u>
101	<u>RRGHARLAL</u>	SQNQSSGAA	GPTGKNEEK	<u>I QVLTDKIDVL</u>	<u>LQOIEEL</u> GSEG
151	KVEEAQG	MM KLVEQLKEER	ELLRSTTSTI	ESFAAQEKQM	EVCEVCGAFLI
201	VGDAQSRVD	DHLMGKQHMG	YAKIKATVEE	LKEKLRKRTE	EPDRDERLKKE
251	KQEREEREK	EREREREERE	RKRRREEEER	EKERARDRER	RKRSRSRSRHS
301	SRTSDRRCS	RSRDHKRSRS	RERRRSRSD	RRRSRSHDRS	ERK HRSRDR
351	RRSKSRDRK	SYK HKSRD	REQDRKSKEK	EKRGSDDKKS	SVKSGSREKQS
401	EDTNTEske	SDTKNEVNGT	SEDIKSEGDT	QSN	

Figure 1.10. Amino Acid Sequence of the Coding Region of CREAP.

The amino acid sequence of CREAP is shown with the leucine zipper-like regions in bold and double underlined.

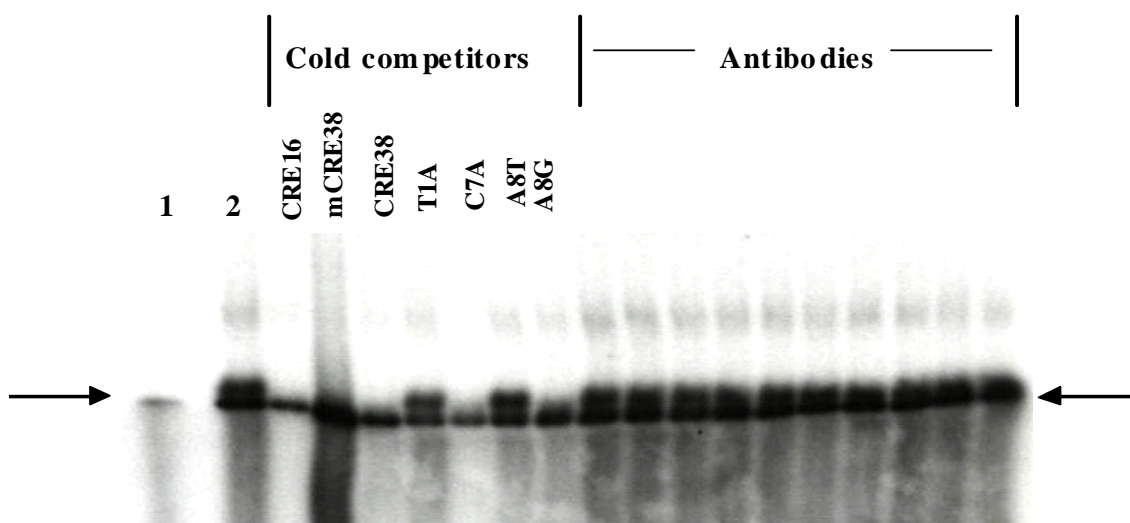


Figure 1.11. EMSA of CREAP Proteins Using ³²P Labelled CRE16 as the Probe.

Protein from yeast transformed with the CREAP clone form a specific complex (arrow) on the CRE (but not with protein from control yeast, lane 1). Binding characteristics were determined by unlabelled oligonucleotide binding competition. CRE16= cold CRE16, CRE38= cold 38bp CRE, mutCRE= mutant CRE38, T1A, C7A, A8T and A8G= mutant CRE16 with mutation of specific nucleotides eg. C7A is the 7th C changed to A. Supershift is not observed with antibodies to a variety of transcription factors. (Shipman et al. 2006).

1.6.1.1 CROP

Although CREAP appeared to be a novel transcriptional protein, a database sequence search revealed that CREAP is identical to CROP, a protein discovered by Nishii and colleagues (Nishii et al. 2000). CROP stands for Cisplatin Resistance-Associated Overexpressed Protein, and was isolated from the cisplatin resistant cell lines, ACHN/CDDP and NC65/CDDP. These authors identified CROP as a novel putative SR/splicing protein, whereas CREAP has been characterised as a putative transcription factor and they emphasised several domains that are consistent with such a function (Figure 1.12). CREAP/CROP is now listed in the gene databases under both names and in the protein database under accession number O95232, along with a protein only identified as Okadaic acid-inducible phosphoprotein OA48-18 (Chin et al. 2000).

1.6.1.2 hLuc7A

In their initial paper describing CROP, the Japanese group noted that it had homology to a yeast protein, termed Luc7p, shown to be involved in 5' splice site recognition and essential for vegetative growth (Nishii et al. 2000). Subsequently, towards the completion of this thesis, Puig and colleagues used EST-based sequence tags to clone the human homologue, which they termed hLuc7A, that is identical in sequence to CREAP and CROP (Puig et al. 2007). Consistent with a role in splicing, hLuc7A co-precipitated with U1 snRNA from human cell extracts. If HeLa cells nuclear extracts are depleted of Luc7A, the splicing of several reporters is reduced two to three-fold (Puig et al. 2007). Overexpression of hLuc7A in a HeLa cell splice-site assay switched

5' splice-site utilisation towards the more distal splice site (Puig et al. 2007). These results show that hLuc7A does function as a splicing factor.

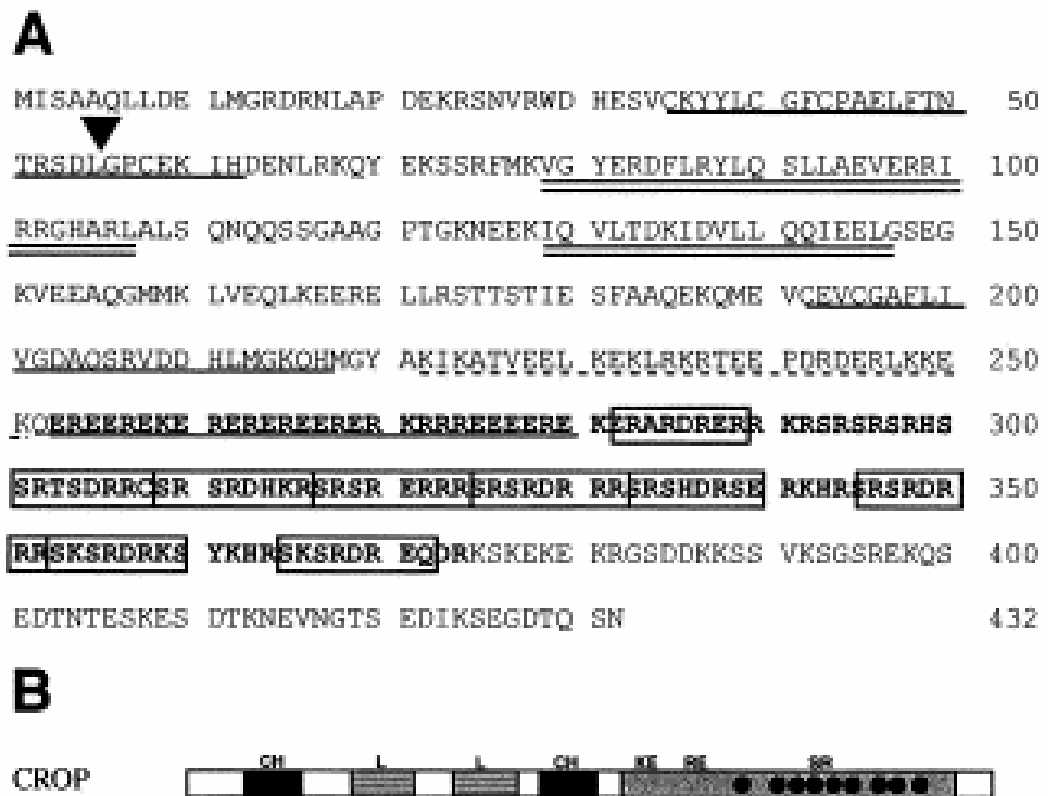


Figure 1.12. CROP Nucleotide Sequence and Domain Structure.

Predicted amino acid sequence of CROP (A), and schematic domain organization of CROP (B). A: The cysteine/histidine motifs are underlined once. The leucine zipper-like repeats are underlined twice. The lysine/glutamate-rich region is underlined with a dashed line. The arginine/glutamate-rich region is in bold and underlined letters. The arginine/serine-rich region is in bold letters. 8 Amino acid imperfect repetitive motifs in serine/arginine-rich domain are in boxes. B: CH, cysteine/histidine motifs; L, leucine zipper-like repeats; KE, lysine/glutamate-rich region; KRE, lysine/arginine/glutamate-rich region; RD, arginine/serine-rich region; RS, arginine/serine-rich region; •, 8 amino acid imperfect repetitive motif. ((Nishii et al. 2000)

1.6.2 Multifunctional Proteins

Thus, from our own data, it appears that CREAP may be involved in the transcriptional regulation of some genes, whereas other data on CROP (and yeast Luc7p) suggest a role in pre-mRNA splicing. Such multifunctionality is an emerging feature of certain nucleic acid binding proteins. There are at least eight proteins that were initially categorised as DNA binding transcription factors, but have been shown to have apparent RNA-binding activities and functions and it is likely that there are more (Cassiday and Maher 2002). This includes TFIIIA, WT-1, TRA-1, Bicoid, p53, STAT1, TLS/FUS and σ^{70} . Many of these proteins contain C2H2 zinc fingers. TFIIIA contains nine C2H2 zinc fingers and in vitro analysis found that the first three zinc fingers contribute most of the DNA affinity while fingers 4-6 are responsible for RNA binding (Clemens et al. 1993). C2H2 zinc fingers are also involved in both DNA and RNA binding of Wilms' tumour protein (WT1) and the *C.elegans* protein TRA-1 contains multiple zinc fingers (Cassiday and Maher 2002).

1.6.3 Tissue Distribution

1.6.3.1 Distribution of CREAP

Clues to the primary function of a protein can be gleaned from its tissue distribution. To determine the distribution of CREAP, a Multiple Tissue Expression Array was used by our laboratory. The Array consisted of RNA from a variety of tissue and cell line sources including most human tissues, cell lines and fetal tissues. The CREAP probe hybridised to the majority of human adult, fetal and cell line RNAs, present on the membrane with particularly high mRNA expression in the cerebellum, pituitary,

putamen, thymus and several fetal tissues, implicating a possible role in development. RT-PCR has also been conducted on HeLa, JEG-3 and placental cells using CREAP specific primers (unpublished data). Therefore it seems that CREAP is expressed in the majority of human tissues (Figure 1.13).

1.6.3.2 CROP Tissue Expression

In their examination of the distribution of CROP, Nishii and colleagues used Northern Blot analysis. CROP mRNA of 3.5kb was detected in almost all the tissues tested (heart, brain, placenta,, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte) (Nishii et al. 2000) (Figure 1.14). These findings are broadly in agreement with our results using the tissue arrays. The slightly different results obtained with their localisation compared to the CREAP MTE Array (eg. CROP was strongly expressed in heart and ovary, and CREAP expression was low in these tissues) are likely explained by the use of different methods (northern blotting versus MTE Array) and/or different hybridisation probes. We used the first 800bp of the CREAP sequence as a probe while the probe used for the CROP results is not indicated. Interestingly, there also appears to be a shorter mRNA present in some tissues, including placenta, which is absent in others, however this finding was not discussed by the authors.

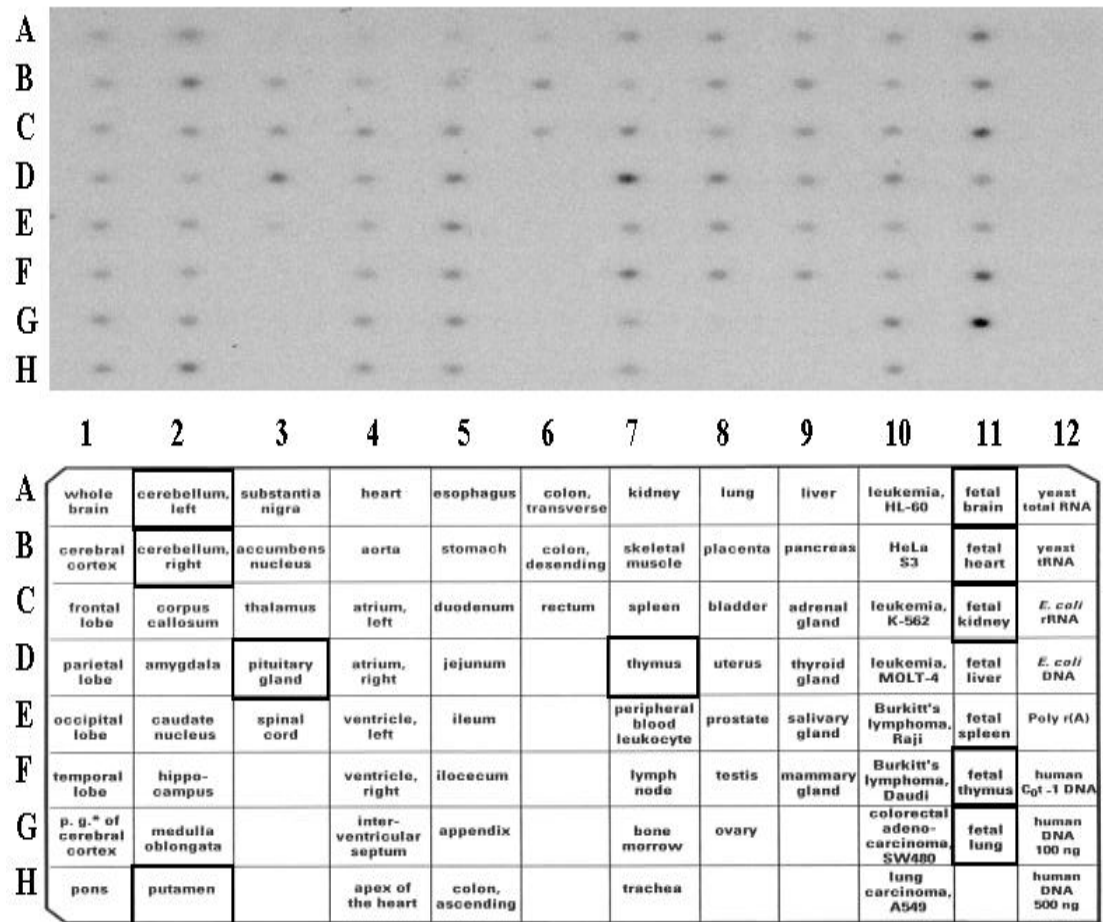


Figure 1.13. Multiple Tissue Expression Array Blot Results.

The top panel shows the autoradiogram of the MTE Array probed with CREAP. The intensity of the RNA dots relates to the level of expression of CREAP in that particular tissue or cell. The bottom panel shows the identity and position of the RNA samples on the membrane. Highly expressed tissues are boxed with a thick line. Column 12 contains negative control RNA to which CREAP should not hybridise. (Shipman et al. 2006).

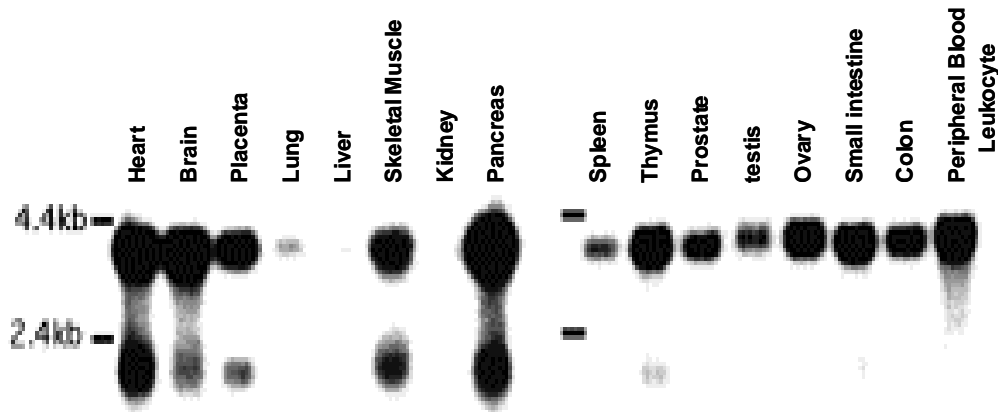


Figure 1.14. Expression of CROP mRNA in Human Tissues.

Human multiple tissue Northern blot I, II (Clontech), containing approximately 2 µg of poly(A) RNA per lane from the indicated human tissues, were analysed by Northern blot hybridization with a CROP-specific probe. (Nishii et al. 2000)

1.6.3.3 Subcellular Localisation of CROP

A green fluorescent protein (GFP)-CROP fusion protein was used to determine the subcellular localisation of CROP. The GFP-CROP fusion protein was shown to localise to intranuclear speckles, and co-localised with the SR protein, SC35 in the splicing speckles (Umehara et al. 2003). They also showed that both the serine-arginine (RS) and arginine-glutamate (RE) rich regions are necessary for localisation to speckles.

Treatment with the chemotherapeutic agent, cisplatin, changed the speckled distribution of CROP so that the nuclear speckles appear less defined. Interestingly, the distribution of the SR protein, SC35, was also changed by cisplatin treatment (Umehara et al. 2003).

It is possible that part of the resistance to cisplatin may be mediated by a redistribution of splicing factors.

1.6.4 Structural Domains of CREAP/CROP

The CREAP peptide sequence has a unique set of domains including two leucine zipper-like regions, two zinc fingers, two coiled-coils and an arginine/serine (RS)-rich region. The leucine zipper-like regions are very similar to other bZIP transcription factors such as CREB, CREM, ATF-1, Jun and Fos (Shipman et al. 2006). The C-terminal of CREAP is very hydrophilic and has regions rich in lysine and glutamate (KE), arginine and glutamate (RE) and arginine and serine (RS).

Nishii et al, analysed the serine/arginine-rich region of CROP and found that it contained a series of eight amino acid imperfect repetitive motifs with the consensus sequence Ser-Arg-Ser-Arg-Asp/Glu-Arg-Arg-Arg (Nishii et al. 2000). This motif has also been found in the RNA helicase, HEL117 (Sukegawa and Blobel 1995), and the 70K component of U1 snRNP (Theissen et al. 1986). There are also regions with homology to a RNA binding protein from *Aribidopsis thaliana*. The RS regions of SR proteins have been shown to mediate protein-protein interactions, to function as nuclear localisation signal and to contribute to spliceosome assembly.

1.6.5 Possible Binding Partners

King *et al.* have shown that CREB and c-Jun are part of the placental nuclear protein complex that binds to the CRE with super shift EMSA (King et al. 2002). However the

size of the unshifted complex suggests that there are other unidentified proteins in this complex. It is possible that CREAP may be part of this complex as it is present in the placenta, has demonstrated CRE binding properties and contains leucine zipper like repeats, similar to the transcription factors CREB and c-Jun.

The arginine/serine rich region of CREAP is similar to the RS region of the SR protein family involved in splicing. This RS region is known to be involved in protein-protein interactions so represents another possible interaction domain. Umehara and colleagues investigated this possibility by performing a yeast-two hybrid assay to determine binding partners for CROP. They tested for interactions with the SR protein, SF2/ASF and CROP itself and found evidence for both (Umehara et al. 2003). It was also found that the RE and RS domains are necessary for CROP-CROP protein interactions.

1.6.6 Protein Modifications

Many SR proteins have been reported to be phosphorylated at multiple serines in the RS domain (Roth et al. 1991; Colwill et al. 1996). CROP was tested for phosphorylation by the SR protein kinases mSRPK1, mSRPK2 and Clk1 in vitro. Both CROP and SF2/ASF were phosphorylated by all three recombinant kinases (Umehara et al. 2003).

1.6.7 Yeast Luc7p and Human Luc7A

1.6.7.1 Yeast and Human Homologues of CROP and CREAP

The novel yeast U1 snRNP, yLuc7p, is essential for vegetative growth and is involved in pre-mRNA splicing (Fortes et al. 1999a). The human homologue of yLuc7p is

hLuc7A, which is identical to CROP and CREAP. yLuc7p was identified by a mutation that causes lethality in a yeast strain lacking the nuclear cap-binding complex (CBC) (Fortes et al. 1999b). The LUC (Lethal Unless CBC is produced) genes encode several components of the commitment complex and U1 snRNP, with some of the proteins being conserved between yeast and vertebrates (Fortes et al. 1999a). All metazoan LUC7 family members contain C-terminal extensions with multiple RS and RE repeats, placing them in the SR protein family, the yeast Luc7p terminates shortly after the second zinc finger. There are two other human Luc7 related proteins, designated, hLuc7B1 and hLuc7B2 that contain two zinc fingers and RS and RE domains (Puig et al. 2007). Whereas there are a large number of SR proteins involved in splicing in vertebrates, yeast has no or very few SR proteins. The presence of the SR region in hLuc7A/CREAP could change the function of the human protein compared to yLuc7p's role in yeast.

1.6.7.2 Luc7p is a Component of Yeast U1 snRNP

Luc7p co-precipitates with the yeast U1 snRNP proteins Snu71p and Nam8p and also with U snRNAs, indicating that Luc7p is a stable component of yeast U1 snRNP (Fortes et al. 1999a). This result was also confirmed in a biochemical study of U1 snRNP composition (Rigaut et al. 1999). A minor amount of the snRNP proteins, yCBC80 and Npl3p, also co-precipitated with Luc7p (Fortes et al. 1999a). In contrast to the situation in yeast, the vertebrate Luc7 proteins do not appear to be stable components of U1 snRNP (Luhrmann et al. 1990). Luc7p is involved in splicing and can affect U1 snRNP composition. However, mutation of Luc7p caused only a minor affect on the splicing of

some reporter introns *in vitro*, suggesting that Luc7p- may only be required for the splicing of specific introns (Fortes et al. 1999a).

1.7 Hypothesis and Aims

CREAP has a unique set of domains that implicate it as a multifunctional protein that may be involved in both transcription/DNA binding and splicing. Studies by other researchers on CREAP identical proteins strongly suggest a role in splicing, however work in this lab has identified CREAP as a CRE-binding protein, similar to the bZIP transcription factor family, which allows the hypothesis that CREAP may be regulating CRH expression and thus has a role in pregnancy. Therefore the aims of this investigation are:

1. To further characterise CREAP, especially its expression and localisation.
2. To determine if CREAP has a role in CRH regulation by assessing its function as a transcriptional regulator *in vitro*.
3. To investigate if CREAP interacts with additional transcription factors or proteins, especially members of the bZIP family.
4. To investigate the effect of CREAP knockdown on genome wide expression.

Chapter 2. Materials and Methods

2.1 General Reagents

Specific chemicals and reagents are listed in Table 2.1 or described in the text. All other general chemicals were of molecular biology grade.

Reagent	Supplier
8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP)	Sigma or Fluka
Ampicillin	Sigma
Bovine Thrombin	Sigma
CIAP	Promega
Complete Protease Inhibitor Cocktail	Roche
DAPI	Invitrogen
Dexamethasone	Sigma
DMEM	Sigma
DNase I	Sigma
Dpn I	Promega
Dulbecco's PBS	GIBCO
EcoRI	Promega
Ethidium Bromide	Sigma
Gentamicin	Sigma
Glutathione Sepharose 4B	Amersham Pharmacia Biotech
Hanks Balanced Salt Solution	GIBCO
HindIII	Boehringer Mannheim
K ₃ Fe(CN) ₆	Sigma
K ₄ Fe(CN) ₆ ·3H ₂ O	Sigma
Lauria Broth	Sigma
LB Agar	GIBCO
Lysozyme	Pierce
NotI	Promega
Penicillin	GIBCO
Percoll	Amersham Pharmacia Biotech
Protein A Agarose	Santa Cruz Biotech
Reduced Glutathione	Amersham Pharmacia Biotech
Sac I	Promega
Slowfade Gold Mounting Media	Invitrogen
Streptomycin	Sigma
T4 DNA ligase	Promega
Taq DNA polymerase	MBI Fermentas
Trypsin	Worthington
X-gal	Sigma

Table 2.1. List of Reagents and their Supplier.

2.2 Competent Bacteria and Transformation

2.2.1 Strains

For general cloning purposes the bacterial strain XLI Blue (Stratagene) and DH5 α (Invitrogen) were used. XL10 Gold Ultracompetent cells (Stratagene) were used for more difficult cloning. BL21 Competent cells (Stratagene) were used for GST fusion protein expression. All strains were grown in Luria Broth (LB) or on LB agar plates (Sigma) with the appropriate antibiotic (Sigma).

2.2.2 Preparation of Chemically Competent Cells

50ml of a 1:100 dilution of bacterial culture was grown till the OD₅₅₀ reached 0.4-0.6. The cells were then chilled on ice and pelleted by centrifugation at 2000g. The pellet was resuspended in 15 ml Freezer Buffer (100 mM KCl, 50 mM CaCl₂, 10% glycerol, 10 mM KAc, pH 6.2). The cells were then pelleted again and resuspended in 4ml Freezer Buffer before aliquoting into pre-chilled tubes and freezing at -70°C.

2.2.3 Transformation

Competent cells were thawed on ice then mixed with ng-ug amounts plasmid DNA. Cells were incubated on ice for 20 min then heat shocked at 42°C for 45s before fast cooling on ice for 2 min. 500 μ l of warm LB was added and the cells allowed to recover at 37°C for 30-60 min. 100-250 μ l of the transformation was then spread on appropriate LB agar plates and incubated at 37°C overnight.

2.3 Plasmid DNA Preparation

2.3.1 Minipreps

Miniprep plasmid DNA purifications were carried out using the GenElute Mini Kit, Sigma, according to the manufacturers recommended protocol. Briefly, 1-5ml of overnight bacterial culture was pelleted at 13,000rpm for 1min in a microfuge. The pellet was resuspended in Resuspension Buffer with RNase, then lysed with an alkaline lysis buffer. The lysate was neutralised with a sodium-acetate containing buffer and the cell debris pelleted by centrifugation. The DNA containing supernatant is passed over a silica column where the plasmid DNA is bound. Contaminants are washed away and the plasmid DNA eluted in water or TE buffer.

2.3.2 Maxipreps

The PerfectPrep Maxi Kit (Eppendorf) was used according to the manufacturer's protocol to purify larger amounts of plasmid DNA for use in transfection. The Perfectprep Plasmid Maxi protocol is based on coupling two basic processes; alkaline lysis followed by binding of plasmid DNA to a matrix. 300ml of overnight bacterial culture was pelleted and the bacteria resuspended in Solution 1. The cells were lysed by addition of alkaline lysis Solution 2. The sample was then neutralized with Solution 3 and the cell debris pelleted by centrifugation at 15,000g for 15min at 4°C. The DNA containing supernatant was mixed with matrix and the plasmid DNA bound to the DNA Binding Matrix under high salt conditions. Under such conditions the DNA is rapidly driven from the lysate onto the matrix, whereas small RNA molecules remain in solution. The DNA-bound matrix particles were pelleted by centrifugation, and washed

on a Spin Column with an alcohol buffer containing low salt. The DNA remains bound to the matrix while weakly-bound substances and salts are washed off. Plasmid DNA is eluted from the matrix with Elution Buffer, concentrated by ethanol precipitation, and finally resuspended in Elution Buffer or Molecular Biology Grade Water.

2.3.3 *CsCl* gradients

Large Scale (litres of culture) plasmid purifications were conducted using the cesium chloride (CsCl) gradient equilibrium centrifugation method. Overnight bacterial cultures were pelleted and resuspended in GTE (50 mM glucose, 25 mM Tris.Cl pH 8, 10 mM EDTA) plus lysozyme. Alkaline lysis was conducted with 0.2N NaOH and 1% SDS. The solution was neutralised with potassium acetate (3M potassium, 5M acetate) and the cell debris pelleted with centrifugation at 4000rpm for 15 min at 4°C. The supernatant was filtered through gauze and the DNA precipitated using isopropanol.

The DNA pellet was resuspended in TE (25 mM Tris, 10 mM EDTA, pH 8) and 1gm CsCl added for every ml solution. Ethidium bromide (EthBr) solution was added to a final concentration of 740 µg/ml. The solution was centrifuged at 45,000rpm in the NVT 65 rotor in a XL-90 Beckman Ultracentrifuge at room temperature for 16 hours. The plasmid/EthBr band was removed from the gradient by puncturing the tube with a 19g needle and syringe. The EthBr was removed from the solution using butanol extractions and the plasmid DNA dialysed against TE to remove the CsCl.

2.4 Plasmid DNA Manipulations

2.4.1 General Cloning Techniques

2.4.1.1 Restriction Enzyme Digestion

Plasmid DNA was digested with restriction enzymes according to the manufacturer's recommended protocol. Briefly, the plasmid DNA was diluted in the appropriate 1x restriction enzyme buffer and 10-20U enzyme added and the digest incubated at 37°C for at least 2 hours.

2.4.1.2 Dephosphorylation of Digested Vectors

Restriction enzyme vector digest reactions were immediately treated with CIAP (Calf Intestinal Alkaline Phosphatase) (Promega) to dephosphorylate the ends and prevent recircularisation of the vector. The digest reaction was made up to 50 µl in 1x CIAP buffer and 0.01U/pmol ends of CIAP. The reaction was incubated at 37°C for 30 min, another aliquot of CIAP added before a further 30min incubation.

2.4.1.3 Ligation

T4 DNA ligase (Promega) was used to ligate insert and vectors. Typical ligations use 10-200ng of vector and insert to vector ratios of 1:1, 1:3 and 3:1. The ligation consisted of the cut vector, the insert DNA, 1x ligase buffer and 1U T4 ligase. The ligation was incubated for a minimum of 1 hour at room temperature or overnight at 4°C.

2.4.1.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to assess plasmid size, check restriction sites and purify inserts. Generally 1% agarose gels with 0.5 µg/ml ethidium bromide in TBE buffer (90 mM Tris, 90M Boric acid, 2 mM EDTA, pH 8) were run at 80-100V to separate the DNA. The gels were viewed on an UV transilluminator and photographed. DNA bands requiring purification were cut from the gel using a scalpel blade and extracted using the Agarose Gel DNA Extraction Kit (Roche Applied Science).

2.4.2 CRH and CRE Promoter Plasmids

The human CRH genomic clone, CRH1001+, was the generous gift of Joseph Mazjoub, Harvard University Medical School, Boston, MA, USA. CRH and CRE vectors were previously cloned by Dr You Hong Cheng and Dr Bruce King, in the Mothers and Babies Research Centre, and the following is a brief description of their cloning. The CRH 5'-flanking DNA sequence was previously sub cloned into the promoterless Photinus (firefly) luciferase reporter vector pGL3-basic (Promega, Madison, WI) (Figure 2.1) (Cheng et al. 2000a). Serial 5' deletions of a 5500bp CRH promoter region have been previously described (Cheng et al. 2000a) to create CRH-663 and CRH-99. To create the CRE-Globin construct a 38bp fragment of the CRH promoter containing the CRE (5'CCTTCCATTTTAG GGCTCGTTGACGTCACCAAGAGGCG) was inserted upstream of a minimal mouse β-globin promoter (Nicholson et al., 1990) in pGL3-basic. QuikChange Site Directed Mutagenesis Kit (Stratagene) was used to create a CRE in the CRH-99 construct (CRE-CRH-99) as previously described (King et al. 2002).

To create a plasmid with the β -globin promoter driving the luciferase reporter the rabbit β -globin promoter sequence (-109 to +10 bp) was removed from pGLOB-CAT by *Bam*HI and *Bgl*III double digestion, and ligated into the *Bgl*III site of the pGL₃-Basic vector to make the GLOB-pGL₃ vector. To construct the CRE-globin promoter plasmid, the *Bgl*III site of a luciferase construct containing the hCRH promoter region from -340 to -215 bp (which had been constructed by linking a hCRH PCR fragment into the *Bgl*III and *Mlu*I sites of the pGL₃-promoter vector) was converted to a *Xho*I site using a linker oligonucleotide. *Mlu*I and *Xho*I double digestion removed the hCRH promoter sequences from this plasmid, and the fragment was ligated into *Mlu*I- and *Xho*I-digested pGLOB-GL₃ to create pCRE-GLOB-GL₃. pCMV-CREB was purchased from Clontech in the CREB Dominant Negative Vector Set.

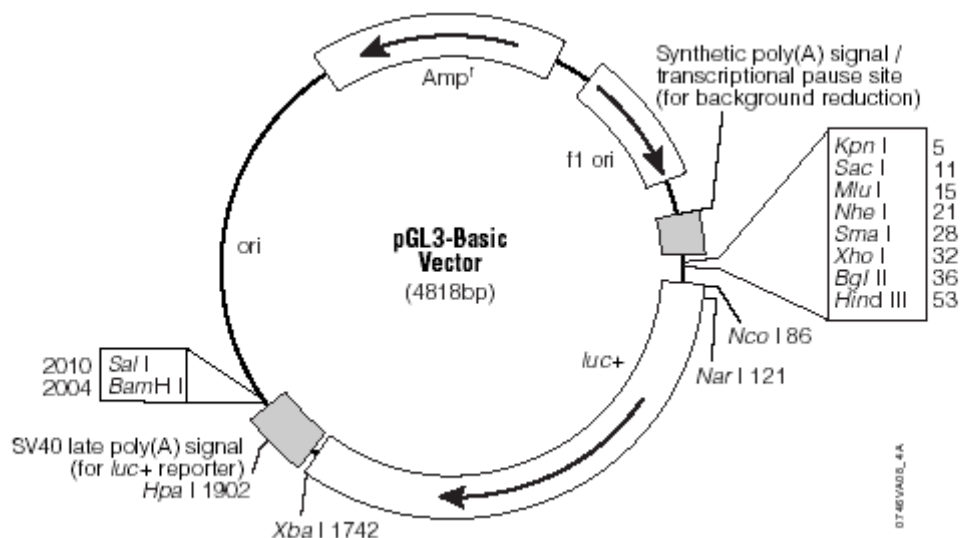


Figure 2.1. pGL3-Basic Vector Circle Map.

Additional description: *luc+*, cDNA encoding the modified firefly luciferase; *Amp^r*, gene conferring ampicillin resistance in *E. coli*; *f1 ori*, origin of replication derived from filamentous phage; *ori*, origin of replication in *E. coli*. Arrows within *luc+* and the *Amp^r* gene indicate the direction of transcription; the arrow in the *f1 ori* indicates the direction of ssDNA strand synthesis.

2.4.3 CREAP Expression Vectors

Dr Richard Nicholson, Mothers and Babies Research Centre, previously cloned the CREAP cDNAs from a placental cDNA library. Briefly, cDNAs encoding proteins capable of binding to the CRE were cloned using the MATCHMAKER one-hybrid system (Clontech) following the manufacturer's recommended protocols. An oligonucleotide pair containing the sequence 5'-CGCGTGACGTCATGACGTCATGACGTCA was inserted into the *Mlu* I site of the pHISi vectors and transformed into the YM4271 yeast strain. This new CRE yeast strain was then transformed with a human placental MATCHMAKER cDNA library

(Clontech), following the manufacturer's recommended protocols, and selected for growth in the absence of histidine. The cDNA obtained from the yeast one-hybrid screen was then used as a probe to screen a λ -TriplEX2 human placental cDNA library (Clontech), following the manufacturers' recommended protocol. Several near identical and overlapping clones were obtained and used to determine the full-length DNA sequence of CREAP-1. DNA sequencing was performed at the Australian Genome Research Facility (University of Queensland, Brisbane, Australia).

Primer	Purpose	Sequence 5' to 3'
5EcoR1hCREAP	Site Directed Mutagenesis	GGCGCAGTTGTTGGATGAATTCATGGGCCG
3EcoR1hCREAP	Site Directed Mutagenesis	CCCGGCCCATGAATTCATCCAACAACAAC C
CREAPforEco	PCR full length clone, plus insert 5' EcoR1 site for cloning	CGAATTCGTGTTTTCGTTGGCGGGTG
CREAPrevXba	PCR full length clone	CTCTAGAGGCACACTAATGGGGGCA

Table 2.2. PCR and Mutagenesis Primer Sequences.

2.4.3.1 Polymerase Chain Reaction to Create Full- Length CREAP Coding Region

To create a full-length expression plasmid, CREAPSV and CREAPLV were cut at the HindIII site and ligated, and used as the template to PCR a ~1600bp full length CREAP product. The CREAPSV/LV ligation was used as the template in a PCR reaction to amplify the ~1600bp coding region using sequence specific primers. The reaction consisted of 1.5 mM MgCl₂, 0.2 mM dNTPs, 1X Taq Buffer (MBI Fermentas), 0.6 μM CREAPforEco and CREAPrevXba primers, 1 μl ligation and 1 μl Taq Polymerase (MBI Fermentas). The cycling conditions were as follows

94°C 2min
94°C 30sec
67°C 30sec
72°C 30sec
72°C 2 min

} 30 cycles

2.4.3.2 CREAPlv Vector Cloning

The full length CREAP PCR product was ligated into the pGEM-T Easy vector system (Promega, Madison, WI) which utilises the A-overhangs created by Taq polymerase to clone into the complementary T-overhangs of pGEM-T Easy (Figure 2.2). The full length CREAP sequence was then cut out of pGEM-T Easy with EcoR1 and sub-cloned into the pCI-Neo Mammalian Expression Vector (Promega) (Figure 2.3) to create the full length CREAPlv expression plasmid.

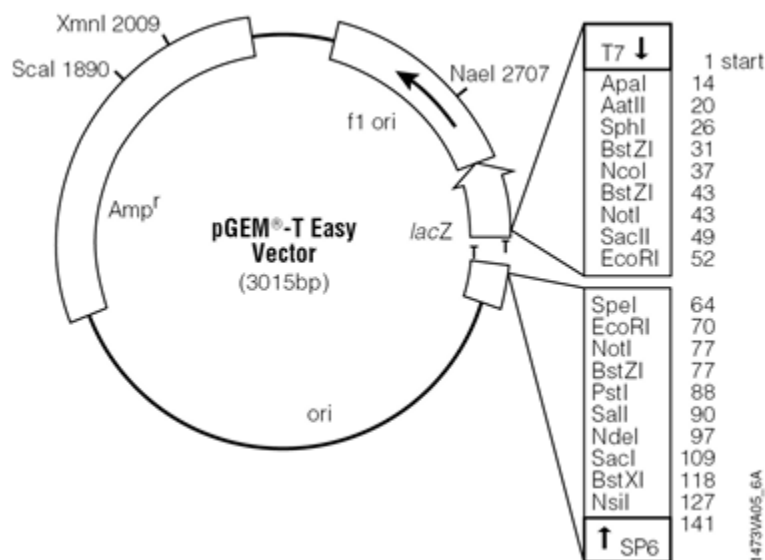


Figure 2.2. pGem T-Easy Vector Map.

Cloning vector for enhanced cloning of PCR products. Amp^r, gene conferring ampicillin resistance in *E. coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in *E. coli*. Arrows within lac⁺ and the Amp^r gene indicate the direction of transcription; the arrow in the f1 ori indicates the direction of ssDNA strand synthesis.

2.4.3.3 CREAPsv Vector Cloning

An 800bp, truncated version of CREAPSV was also sub-cloned into pCI-Neo Mammalian Expression Vector. The insert was cut from the CREAPSV cDNA plasmid through EcoRI and NotI sites, and the ends dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIAP) (Promega) treatment. The insert was ligated into the EcoRI/NotI sites of the pCI-Neo vector to create CREAPsv expression plasmid.

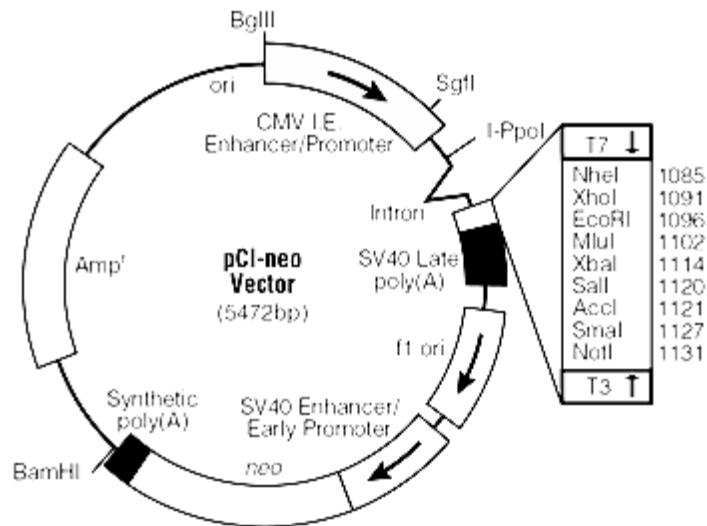


Figure 2.3. pCI-Neo Vector Map

Mammalian expression vector with CMV promoter. Amp^r, gene conferring ampicillin resistance in *E. coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in *E. coli*.

2.4.3.4 Site-Directed Mutagenesis

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to create an in-frame EcoRI site in pGEM-T Easy CREAP to allow for full length fusion proteins to be made. This Kit employs a PCR-based method using PfuTurbo® DNA polymerase™ and a temperature cycler. Primers containing the desired mutation, in this case the insertion of an EcoRI site at position 133-138 (GAATTC), each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuTurbo* DNA polymerase. The primers used were 5EcoR1hCREAP and 3EcoR1hCREAP. Incorporation of the primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5'-Gm⁶ATC-3') is specific for methylated and

hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. The nicked vector DNA containing the desired mutations is then transformed into XL1-Blue supercompetent cells.

The reaction conditions consisted of 5 μ l of 10x Pfu buffer, 15ng CREAPSV plasmid, 150ng of each primer, 1 μ l of 10 mM dNTPs, 1 μ l Pfu Turbo and 40 μ l water. The reaction was temperature cycled as described below. The reaction was digested with 1 μ l DpnI for 1-1.5h at 37°C. 1 μ l of the reaction was then used to transform XL1 Blue competent cells.

QuikChange Cycling Conditions

95°C 30s

95°C 30s	}	12 Cycles
55°C 1min		
75°C 9min		

2.4.4 GST Fusion Protein Vector

To create an in-frame GST-fusion protein with CREAP, an EcoR1 site was created in CREAP-SV at position 133-138 (GAATTC) using the QuikChange Site Directed Mutagenesis Kit (Stratagene) as described above. The mutated vector was then digested with EcoR1 and HindIII (Hind III site at nt645-650) and ligated into the GST gene fusion vector, pGEX-AHK (a modified version of pGEX2TK, Amersham Pharmacia Biotech), a generous gift of Dr Patsie Polly, University of New South Wales, Sydney, NSW. The in-frame EcoRI site allows the GST and CREAP proteins to be correctly expressed.

2.5 Recombinant Protein Expression and Purification

BL-21 strain bacterial cultures containing the pGex-CREAP fusion plasmid as well as a thioredoxin plasmid were grown to OD₆₀₀ 0.6-0.9. Protein expression was then induced with 250µM IPTG for 4 h at 33°C. Cell lysis was performed with 200 µg/ml lysozyme. The cells were sonicated and incubated at 4°C with 1% Triton X-100 for 1 h with rotation. The lysate was centrifuged and the supernatant collected. The bacterial supernatant was passed over a column containing Glutathione Sepharose 4B beads (Amersham Pharmacia). The beads were thoroughly washed with PBS. CREAP protein was cleaved away from the GST tag by bovine thrombin (Sigma) digestion for 16 h at room temperature. The protein was then collected and quantified by BioRad Protein Assay (BioRad) before being frozen at -80°C.

2.6 BioRad Protein Assay

The BioRad Protein Assay is based on the Bradford method (Bradford 1976). Protein quantification was carried out according to the manufacturer's protocol. It relies on a colour change in Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. A standard curve of bovine serum albumin (BSA) (0-10 µg/ml) was prepared along with the unknown protein samples (20 µl). 1ml of diluted BioRad Dye Reagent (diluted 1:5 in water). The samples were incubated for at least 5min before the optical density was measured at 595nm wave length in a spectrophotometer. Linear standard curves were then plotted in Excel and the concentration of the protein samples determined.

2.7 Polyclonal Antibody Production

Polyclonal antibody production in rabbits was conducted with the approval of the Newcastle Animal Care and Ethics Committee (ACEC 7531203). Pre-immunisation rabbit serum was collected 1 month before immunisation was commenced. 40 µg of purified recombinant CREAP protein was combined with complete Freund's adjuvant (Sigma) for the first immunisation, and incomplete Freund's adjuvant for all subsequent immunisations, and vortexed for 30 min to create a thick emulsion. 100 µl of the antigen was then injected subcutaneously in four places on the dorsal surface of each rabbit using a 26 gauge needle. Two weeks later 10 ml of blood was collected from the ear vein and the blood allowed to clot. The antibody-containing serum was aliquoted and frozen at -20°C. This schedule was repeated monthly for 6 months.

2.8 Cell Culture

All cell cultures were incubated at 37°C in 5% CO₂.

2.8.1 Primary Placental Cell Culture

Human term placentas were obtained from normal pregnant women after spontaneous vaginal delivery or elective caesarean section. Collection of placentas was performed with the approval of the Hunter Area Health Service (Newcastle, Australia) and the University of Newcastle Human Ethics Committees, with informed consent at John Hunter Hospital. Cytotrophoblasts were obtained according to Kliman's method (Kliman et al. 1986). Placentas were collected within 40 min of delivery and the

chorionic villi tissue was dissected from the maternal side of the placenta using forceps and surgical scissors. The procedure was carried out in Class II Biohazard Hoods using sterile technique. The placental tissue was thoroughly washed with saline to remove excess blood. The dissected placental cotyledons were minced finely with scissors in DMEM plus antibiotics. Cells were dispersed by digestion in 0.125% trypsin and 0.04% DNase I in DMEM at 37°C, with agitation for 30 min. The tissue was allowed to settle and the supernatant 45 ml transferred to sterile 50ml tubes, and the digestion repeated two more times, with collection of the supernatants.

The supernatants in 50ml tubes were underlayered with 5ml of foetal bovine serum (FBS) (GIBCO) to neutralise the trypsin and centrifuged at 2,300rpm for 15 min at room temperature in a Beckman GPR centrifuge with swing-bucket rotor (GH 3.7). The resulting cell pellets were resuspended in 20 ml of DMEM and pooled. Cell fractions were filtered through 90µm pore size nylon gauze into 50ml tubes. The filtered cells were pelleted and resuspended in a total of 10 ml DMEM.

To obtain a highly purified fraction of cytotrophoblasts, cells were fractionated on a 5-70% Percoll (GE Life Sciences). The 90% stock of Percoll was diluted with 1x Hank's Balanced Salt Solution (Invitrogen) to create 5% increments in Percoll concentration (see Table 2.3). The gradients were formed in 50ml tubes using 15cm stainless steel canula connected to tubing and a syringe to allow gradient formation from the bottom of the tube. 3 ml of each Percoll percentage starting with 5% was carefully underlaid to create the gradient.

Density (%)	90% Percoll (ml)	1X Hank's (ml)
5	2	34
10	4	32
15	6	30
20	8	28
25	10	26
30	12	24
35	14	22
40	16	20
45	18	18
50	20	16
55	22	14
60	24	12
65	26	10
70	28	8

Table 2.3. . Percoll Density Gradient Dilution Table.

To acquire the necessary percentage of Percoll, 90% stock Percoll was diluted in 1x Hanks Balanced Salt Solution (without Phenol red).

The resuspended cell pellet was gently layered on top of the gradient and the tubes centrifuged at 2,500rpm for 20 min. The cell fractions were collected from the bottom using a cannula connected to a 25 ml syringe. The first 10 ml containing red blood cells is discarded and the next 15 ml containing the purified cytotrophoblasts collected. The cytotrophoblasts are washed in DMEM and spun down several times to remove Percoll. The final cell pellet was resuspended in 10 ml culture media and the cell number determined using trypan blue staining and a hemocytometer. Appropriate cell numbers

are seeded into culture plates or dishes and allowed to adhere overnight. For 6 well plates, 3×10^6 cells were usually seeded in 3 ml DMEM culture media (Dulbecco's Modified Eagles Medium, Sigma) supplemented with 25 mM HEPES, 24 mM sodium hydrogen carbonate, 4.5g/L glucose, 10% Fetal Bovine Serum and depending on application, 0.005% gentamicin or 0.1% penicillin/streptomycin antibiotics).

2.8.2 Cell Lines

2.8.2.1 JEG-3

JEG-3 cells were obtained from Dr Carolyn Scott, Kolling Medical Research Institute, University of Sydney, NSW. It is a human placental choriocarcinoma cell line (ATCC # HTB-36). Cells were maintained in DMEM (Dulbecco's Modified Eagles Medium, Sigma) supplemented with 25 mM HEPES, 24 mM sodium hydrogen carbonate, 4.5g/L glucose, 10% Fetal Bovine Serum and depending on application, 0.005% gentamicin or 0.1% penicillin/streptomycin antibiotics.

2.8.2.2 Cos-7

Cos-7 cells are an African green monkey kidney fibroblast-like cell line suitable for transfection by vectors requiring expression of SV40 T antigen. Cells were obtained from Dr Rick Thorne, University of Newcastle, NSW (ATTC # CRL-1651). Cells were maintained in DMEM supplemented with 25 mM HEPES, 24 mM sodium hydrogen carbonate, 4.5g/L glucose, 10% Fetal Bovine Serum and depending on application, 0.005% gentamicin or 0.1% penicillin/streptomycin antibiotics.

2.8.2.3 *AtT-20*

AtT-20 cells (D16–16) were obtained from Karen Sheppard, Baker Medical Research Institute, Melbourne, Australia, (ATCC# CCL-89). AtT-20 are an ACTH secreting mouse cell line derived from a pituitary tumour. They were maintained in DMEM supplemented with 25 mM HEPES, 24 mM sodium hydrogen carbonate, 4.5g/L glucose, 10% Fetal Bovine Serum, 5% Horse Serum and depending on application, 0.005% gentamicin or 0.1% penicillin/streptomycin antibiotics.

2.8.2.4 *Normal Human Fibroblasts*

Normal human fibroblast cells were obtained from Professor Rodney Scott, Medical Genetics Research Centre, John Hunter Hospital, Newcastle, NSW. The fibroblasts were grown in DMEM media plus 10% FBS and gentamicin antibiotics.

2.8.2.5 *Additional Cell Lines*

Additional cell lines were obtained from Dr Rick Thorne, Cancer Research Unit, University of Newcastle, Australia. The cell properties are listed in Table 2.4.

Cell Line	Origin	Medium
Keratinocytes	Human foreskin primary cells	DMEM
MV3	Human melanoma	DMEM
ThP1	Human acute monocytic leukemia	RPMI
U937	Human histiocytic lymphoma	RPMI

Table 2.4. Additional Cell Lines, Origin and Culture Media

2.9 Transfections

2.9.1 *Calcium Phosphate Transfection*

The calcium phosphate transfection of primary placental cells was optimised by a previous student, Dr Bruce King. This is a chemical transfection method where a precipitate forms between DNA and calcium phosphate, and the complex is taken up by endocytosis. Calcium phosphate transfections were carried out on primary placental cells in 6 well plates that were seeded at a density of 3×10^6 cells per well the day before transfection. For each transfection, 400 μ l calcium DNA solution (DNA in 250 mM CaCl_2) and 400 μ l 2x HBS (274 mM NaCl, 10 mM KCl, 11.1 mM dextrose, 50 mM HEPES, 1.7 mM Na_2HPO_4 , pH 7.06-7.18). The 2x HBS is continually slowly vortexed while the DNA calcium mixture is added dropwise. The precipitate was allowed to form for 30min at room temperature before being added dropwise to each well. Cells were incubated for 18 h before changing the media, and further incubation until cell harvesting.

2.9.2 *Lipofectamine 2000 Transfection*

Lipofectamine 2000 (Invitrogen) is a proprietary formulation for the transfection of nucleic acids (DNA and RNA) into eukaryotic cells. It is based on cationic lipid-mediated transfection where the negatively charged DNA is complexed with positively charged lipid allowing it be taken up by the cell. Cells were seeded in 24 well plates at a density of 1×10^5 cells per well in 0.5ml DMEM culture medium, the day before transfection so that 80-95% confluency would be achieved on the day of transfection. 1

µg of plasmid DNA is diluted in 50 µl Opti-MEM® I Reduced Serum Medium (Invitrogen) while 2 µl Lipofectamine 2000 is diluted in 50 µl Opti-MEM to give a DNA to transfection reagent ratio of 1:2. The two mixtures are combined and incubated for 20min at room temperature. The complexes were then pipetted onto the cells in the wells and incubated overnight. The media was changed the next day.

2.9.3 β-Galactosidase Transfection Efficiency Assay

X-gal in situ staining for transfected β-galactosidase allows assessment of the transfection efficiency of a set of conditions. Cells were transfected with an expression vector for β-galactosidase (pSV-β-galactosidase control vector, Promega) with different amounts or ratios of plasmid. Cells were cultured for different time points and then washed with phosphate buffered saline (PBS) and fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5min. The fixed cells were washed three times with PBS and the cells stained with 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆.X3 H₂O, 1.5 mM MgCl₂, 1mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) and incubated for 2-24 h at 37°C in a 5% CO₂ incubator until strong blue staining developed. Cells were rinsed with PBS and assessed for positive blue staining.

2.9.4 Dual Luciferase Reporter Assay

2.9.4.1 The Dual Luciferase Reporter Assay

The Dual Luciferase Assay System is a reporter assay for gene promoter activity. ‘Dual reporters’ refers to the simultaneous expression and measurement of two separate reporter enzymes within a single system. The firefly (Photinus) luciferase reporter is

used to measure experimental activity, while renilla (sea pansy) luciferase is used as an internal control, normalising for cell number, transfection efficiency or assay efficiency.

Photinus firefly luciferase is a 61 kDa monomeric protein that does not require post-translational processing for enzymatic activity. Light emission occurs through oxidation of beetle luciferin in a reaction that requires ATP, Mg^{2+} and oxygen. The presence of coenzyme A stabilises the luminescent signal. Renilla luciferase is 36 kDa, and also does not require post-translational modification for activity. The luminescent reaction consists of oxygen and coelenterate-luciferin to produce light.

2.9.4.2 *Assay Conditions*

Cells were seeded in 6-well tissue culture plates the day before transfection in media containing DMEM, 10% heat inactivated fetal bovine serum (FBS), 100 μ g/ml streptomycin and 50 μ g/ml gentamycin. Calcium phosphate transient transfection was performed using 5 μ g control renilla reporter plasmid, pRLTKV, 30 μ g firefly luciferase reporter plasmid and 2 μ g transcription factor expression plasmid, plus PUC19 plasmid to make the amount of DNA equal between transfections. Cells were incubated for 18 h at 37°C before the transfection agent was removed and replaced with FBS-containing media. The cells were cultured for a further 48 h before being lysed with 400 μ l Passive Lysis Buffer (PLB) (Promega) and assayed with a Dual Luciferase Assay Kit (Promega) and TD-20/20 Luminometer (Turner Designs). Treatments were carried out 16-24 h before lysis.

Cells were also transfected with the dual luciferase vectors using Lipofectamine 2000 in 24 well plates. Cells were seeded at a concentration of 1×10^5 cells per well the day before transfection. 500ng of firefly luciferase reporter, 25ng renilla pRLTKV and 10ng-1 μ g of expression plasmid, with PUC19 plasmid to make the total amount of DNA equal were transfected with a 1:2 ratio of DNA to Lipofectamine 2000 reagent. Cells were incubated overnight and then the media changed. Cells were harvested using 100 μ l PLB per well.

The Dual Luciferase Assay reaction consists of 100 μ l Luciferase Assay Reagent (LAR II) and 20 μ l cell lysate. The sample is pipetted to mix and placed in a luminometer and the luminescence measured for 10s. The firefly signal is quenched with 100 μ l Stop and Glo reagent, and the renilla luciferase activated and measured for a further 10s. The ratio of firefly to renilla is calculated and recorded.

2.10 Protein Extractions

2.10.1 Nuclear Protein Extraction

Nuclear protein extractions were performed based on the method of Schreiber *et al.* and Andrews *et al.* Basically cells in culture plates were washed in cold TBS and scraped off plates with enzymatic inhibitor wash (TBS, 10 mM EDTA, 10 mM EGTA, 10 mM DTT, Complete protease inhibitor cocktail). Cells were pelleted by centrifugation at 1500g and resuspended in Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, complete protease inhibitor cocktail) and incubated on ice for 15 min to allow cells to swell. Nonidet NP-40 was added to a final

concentration of 0.5%, and the samples vortexed for 10 s before centrifugation. The nuclear pellet was resuspended in high salt Buffer B (20 mM HEPES pH 7.9, 0.4M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, complete protease inhibitor cocktail) and rotated at 4°C for 15 min. Tubes were centrifuged and the nuclear protein supernatant collected.

2.10.2 Cytosolic Protein Extraction

Cells were washed with cold PBS and covered with cold lysis solution (10 mM Tris pH 7.4, 1.6 mM EDTA, 0.5% Tween-80, complete protease inhibitor cocktail tablet). The cells were scraped into tubes and incubated on ice for 15 min. Lysates were microfuged at 12,000 rpm for 10 min at 4°C and the supernatants were collected.

2.10.3 Total Protein Extraction

Cells were washed with cold PBS and covered with 2D protein extraction buffer (7M Urea, 2M Thiourea, 30 mM Tris, 4% CHAPS, pH 8, complete protease inhibitor cocktail). Cells were scraped into tubes and incubated on ice for 30 min. Lysates were microfuged at 13,000rpm for 10 min at 4°C and the supernatants collected.

2.11 Polyacrylamide Gel Electrophoresis

2.11.1 NuPAGE® Gel System

The Invitrogen NUPAGE Gel System was used for all protein electrophoresis (Invitrogen). NuPAGE® lithium dodecyl sulphate (LDS) 4x Sample Buffer and

NuPAGE® 10x Reducing Agent (for reduced samples only) were added to each protein sample to 1x concentration before heating at 70°C for 10min. Samples were then loaded onto a 10% NuPAGE Bis-Tris Gel. Gels were run in either NuPAGE MES or MOPS SDS running Buffer at 200V for 35 or 50min respectively. MagicMark™ XP Western Protein Standards or SeeBlue® Plus2 Pre-Stained Standard (Invitrogen) were run on the gels for size reference. After electrophoresis gels were either stained with coomassie blue (50% methanol, 10% acetic acid, coomassie blue) or transferred to nitrocellulose.

2.12 Western Blotting

After electrophoresis, gels were transferred to Hybond-C Extra nitrocellulose membranes (Amersham Biosciences) using NuPAGE Transfer Buffer. Transfer were conducted at 25-45V for 1hour. After transfer, the membranes were air dried and then blocked in 5% skim milk/ TBST (25 mM Tris, 150 mM NaCL, 0.1% Tween 20). Primary antibody in blocking solution was incubated at room temperature for at least 1 hour. Membranes were washed in TBST and then incubated for 1 hour with HRP conjugated secondary antibody. After washing in TBST, the membranes were incubated with ECL reagent (Amersham Biosciences) for 1 minute. Chemiluminescence was detected by the LAS-3000 imaging system (Fuji Photo Film Co; Tokyo, Japan). Densitometric analyses were performed using Fujifilm MultiGauge v 2.3 software (Fuji Photo Film Co). The antibodies used are listed in Table 2.5.

Antibody	Cat #	Dilution	Company
CREAP anti-sera	NA	1:5000	NA
Actin (I-19)	Sc-1616	1:1000	Santa Cruz
Goat anti-rabbit IgG-HRP	Sc-2004	1:2000	Santa Cruz
Donkey-anti-goat IgG-HRP	Sc-2020	1:2000	Santa Cruz
ERK-1 (K-23)/MAPK1	Sc-94	1:1000	Santa Cruz

Table 2.5. Western Blotting Antibodies

2.13 Indirect Immunofluorescence

Cells were grown on 13 mM glass cover slips inside 24 well tissue culture plates and fixed in 5% paraformaldehyde, 2% sucrose in PBS. For intracellular staining the cells were permeabilised with 0.1% Triton-X, before blocking with 10% new born calf serum in PBS. Cells were incubated with either pre-immune or anti-CREAP polyclonal rabbit serum (1:1000) (or other appropriate antibody such as SC35 (Sigma) 1:5000) for 30 min and washed in PBS. The cells were then incubated with Alexa Fluor-488 or -594 conjugated secondary (Invitrogen Molecular Probes) antibody and post-fixed for 5 min. The cells were also stained with the nuclear stain 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen) to highlight the nucleus. The cells were mounted with Vectashield or Slowfade (Invitrogen) and epifluorescence examined using Zeiss Axioplan 2 microscope fitted with an Apotome optical sectioning device. The cells were examined under oil immersion using the PlanApochromat 63x objective. When using the Apotome, 0.3µm optical sections in the Z-plane are produced. XY

coincidence of red (Alexa-594), green (Alexa-488) and blue (DAPI) was validated using the TetraSpeck Calibration Slide Kit (Invitrogen).

2.14 Immunoprecipitation

Immunoprecipitation involves incubating a protein extract with specific antibody and using Protein A beads to capture the targeted protein and any proteins that are bound to it. JEG-3 nuclear extracts were incubated with 2 µg antibody (sera and ascites without known concentration were used at 1-5 µl) and 20-50 µl of Protein A agarose (Santa Cruz #sc-2001) at 4°C for 4 hours to overnight. The agarose beads were pelleted with microcentrifugation and washed several times with PBS. The bound proteins were eluted from the protein A using reduced LDS samples buffer and heating at 70°C. The samples were run on a 10% Bis-tris gel and western blotted according to Section 2.11 and 2.12.

2.15 Transcription Factor (TF) Array Screen

The TranSignal TF Protein Array IV (Panomics) is a membrane spotted with transcription factor proteins which are expressed from full length TF cDNAs, with an N-terminal His tag. A protein of interest can be used as a probe to search for interactions with the immobilised transcription factors and detected with an antibody. For the CREAP screen, purified CREAP protein and CREAP polyclonal antibody prepared according to Section 2.5 and 2.7 respectively were used. The TF Array membrane was blocked in Blocking Buffer then incubated with 5 µg purified recombinant CREAP protein for 2 h at room temperature. The membrane was washed

in Wash Buffer before incubation with 1:5000 dilution of CREAP antisera for 2 hours. The membrane was washed again and incubated with 1:5000 anti rabbit HRP-conjugated IgG for 1 hour. The membrane was washed and interactions detected using ECL Reagent (Amersham Biosciences) and the LAS-3000 imaging system (Fuji Photo Film Co). Densitometric analyses were performed using Fujifilm MultiGauge v 2.3 software (Fuji Photo Film Co).

2.16 Small Interfering RNA (siRNA)

siRNA is a means of knocking down expression of a target protein by causing degradation of its mRNA. HP Guaranteed siRNA was obtained from QIAGEN. Four custom designed siRNA against CREAP were synthesised (see Table 2.6) with the guarantee that at least two sequences would give efficient gene silencing. Scrambled Alexa-488 negative control siRNA and validated MAPK1 positive control siRNA were also purchased. 5×10^4 cells were seeded in 24-well plates the day before transfection in 500 μ l DMEM plus 10% FBS. 150ng siRNA (40 nM final) (QIAGEN) was combined with 3 μ l HiPerfect transfection reagent (QIAGEN) in 100 μ l DMEM per 24 well. Complexes were allowed to form for 10 min before being pipetted dropwise onto cells. Cells were incubated overnight and 1ml medium was added the next day and the cells incubated for 72 to 96 h to allow protein knockdown.

siRNA Name	Target Sequence
CREAP1	CTCGTTTGGCATTATCTCAA
CREAP2	CACGAGAGCGTTTGTAATAT
CREAP3	CCGGGATCGAAAGTCATATA
CREAP4	CTGCGGAATTGTTCAAAATA

Table 2.6. CREAP siRNA Target Sequences.

2.17 RNA Extraction

RNA was extracted from cells grown in culture plates using QIAGEN's RNeasy Mini Kit according to the manufacturers' protocol. Cells were grown in 24 or 6 well plates and lysed directly in the plates with Buffer RLT. Lysates were collected into tubes and homogenised by passing through a 21 gauge needle 5-10 times. One volume of 70% ethanol was added and the sample spun through an RNeasy spin column. The column was washed with Buffer RW1 and on column DNase 1 digestion performed. After digestion the column was washed with RW1 and RPE before the RNA was eluted with RNase-free water. RNA samples were quantified using the NanoDrop 1000 (Thermo Scientific) and stored at -80°C till use.

2.18 Microarray

2.18.1 Illumina Whole Genome BeadChip procedure:

The microarray RNA biotinylation, hybridisation, scanning and analysis assistance was performed by Dr Nikola Bowden of the Medical Genetics Research Centre, John Hunter

Hospital, Newcastle, Australia. If necessary the RNA was pooled, concentrated and desalted using, the RNeasy MinElute Cleanup Kit (QIAGEN). The RNA was then quantified using a fluorometer and RiboGreen reagent according to manufacturer's recommendations (Invitrogen).

2.18.1.1 Amplification and Biotinylation of RNA

RNA was amplified and biotinylated using the Ambion Illumina TotalPrep RNA Amplification Kit (Ambion Inc). Briefly, 500ng of total RNA in a volume of 11µl was used for the reverse transcription reaction. To each RNA sample, 9µl Reverse Transcription Master Mix was added before a 2 hour incubation at 42°C. The reactions were kept on ice until the Second Strand cDNA Synthesis step.

Second strand cDNA strand synthesis was conducted on each sample by adding 80µl Second Strand Master Mix, before an incubation of 2 h at 16°C. The reactions were kept on ice until the cDNA Purification step.

To each cDNA sample, 250µl cDNA Binding Buffer was added and mixed thoroughly. Each sample was applied to an individual cDNA Filter Cartridge. Tubes were centrifuged for 1 minute (10,000 x g) and the flow-through discarded. The cDNA was washed with 500µl Wash Buffer. The cDNA was eluted from the Filter Cartridge with 10µl of pre-heated, nuclease-free water (50-55°C). Filters were eluted with a second 10µl of preheated Nuclease-free water. The eluted cDNA was stored at -20°C.

To synthesise biotinylated cRNA from the cDNA, each sample was completely dried in a DNA Speedvac at a medium heat (40-50°C). Each cDNA sample was resuspended in 10µl of IVT (In vitro transcription) Master Mix containing Biotin-16-UTP, before being incubated for 14 h overnight at 37°C. The IVT reaction was stopped by adding 90µl of nuclease-free water to each reaction. The cRNA was then purified.

To each cRNA sample 350µl cRNA Binding Buffer and 250µl 100% ethanol was added and mixed thoroughly. Each mixture was transferred to a cRNA filter cartridge and centrifuged for 1 minute (10,000 x g). The cRNA was washed with 650µl Wash Buffer. The biotinylated cRNA was eluted from the filter cartridge with 100µl preheated nuclease-free water (50-60°C). The eluted cRNA was either stored overnight at -20°C, or stored at -80°C for longer periods.

2.18.1.2 BeadChip Hybridisation

After quantification of the amplified RNA using a fluorometer and RiboGreen reagent (Invitrogen) 1-2 µg of biotinylated cRNA was hybridised to a Whole Genome Gene Expression Human Ref8 V2 BeadChip (Illumina) containing approximately 24,000 transcripts. The hybridisation mix for each BeadChip consisted of 125µl Hyb E1 buffer (preheated to 55°C) and 75µl formamide. To each cRNA sample, 22.7µl of hybridisation mix was added and the samples preheated for 5m at 65°C. The samples were applied to the centre of each array on the BeadChip. The Hyb Cartridge was fully assembled and shaken to ensure air bubbles within were moving freely. The Hyb Cartridge was placed on the BeadChip Hyb Wheel and incubated for 16-20 h with rotation on at 55°C.

2.18.1.3 Washing and Staining

The BeadChip was washed in 250ml Wash E1BC solution and shaken on an orbital shaker at highest possible speed for 15min. The chip was then washed in 100% ethanol for 10min, then Wash E1BC for a further 2min. The BeadChip was transferred to 4ml Block E1 buffer and placed on a rocker for 10min at a medium-high speed. The chip was then stained in Block E1 buffer with streptavidin-Cy3 (2ml Block E1 buffer, 2 μ l streptavidin-Cy3) for 10min before washing with Wash E1BC solution for 5min. The BeadChip was centrifuged in the slide rack for 4min (275 rcf) and scanned immediately using the Illumina BeadArray Reader and Illumina BeadScan software before analysis.

2.18.2 Analysis and comparison of gene expression profiles:

The gene expression results were initially analysed by cubic spline normalisation using BeadStudio 2.0 software (Illumina, USA), the remaining analyses were performed using GeneSpring GX 7.0. To account for bias or skewing of expression results all the gene expression profiles and each individual gene were normalized to the median resulting in two way normalisation. For visualisation of the results the data was log transformed. To identify the unique set of genes altered in response to siRNA knockdown, the Kruskal-Wallis non-parametric test was used to exclude genes with a p-value greater than 0.05 and Benjamini and Hochberg false discovery rate (5%) was utilised. In addition to statistical significance, a-fold change cut-off of greater than 2 will be used to accurately identify the most significantly altered genes. Cluster analyses were then performed on the duplicate groups of all samples.

Chapter 3. CREAP Sequence Analysis

3.1 Identification and Sequence Analysis of CREAP

3.1.1 Background

Placental CRH is regulated by cAMP and glucocorticoids through the cAMP Response Element (CRE). To determine additional factors that may be regulating placental CRH expression through the CRE, a placental cDNA library was screened using a yeast one-hybrid strategy and a novel CRE-binding protein discovered. In this study, this new cDNA was analysed at both the DNA and protein sequence levels to further elucidate potential functional roles and classify this novel protein. Much of the data presented in this Chapter has been published in Shipman *et al.* 2006 (Shipman et al. 2006).

3.1.1.1 The One-Hybrid Screen

The yeast one-hybrid assay was performed by Dr Richard Nicholson of the Mothers and Babies Research Centre, Newcastle, Australia (Nicholson et al. 2004). This assay consisted of using three tandem copies of the CRE as bait in screening a MATCHMAKER placental cDNA library (Clontech). In this way, cDNAs encoding proteins capable of binding a CRE are isolated using growth selection. A cDNA encoding a novel protein was selected and used as a probe to screen a λ -TripleEX2 human placental cDNA library and several near identical and overlapping clones were obtained (Shipman et al. 2006). These encoded a novel protein that was named CREAP for cAMP Response Element Associated Protein.

3.1.2 Identification of a Novel CRE-Binding Protein, CREAP.

The clones isolated from the placental cDNA screen were analysed to try and annotate the full length sequence for CREAP. The initial cDNA isolated from the MATCHMAKER screen was 0.8kbp and encoded a protein sequence with potential leucine zipper motifs. A similar clone, CREAP-SV was identified in the λ -TriplEX2 placental screen and was identical to the 1-hybrid clone except that it contained an additional 90bp at the 5' end (Figure 3.1).

Two other clones from the λ -cDNA library, CREAP-LI and CREAP-LV, were much longer than, and overlapped, the CREAP-SV cDNA. The CREAP-LV cDNA sequence was very similar to the CREAP-LI cDNA sequence except for a 105 nucleotide deletion just prior to the stop codon. This deletion is flanked by DNA sequences commonly occurring in association with spliced introns, suggesting that CREAP-LV may be a splice variant (Breathnach and Chambon 1981). DNA sequence from all the clones was compiled to generate the full-length CREAP-1 coding sequence, the first 1450 nucleotides of which, and the predicted 432 amino acid sequence, is shown in Figure.

3.2. Homology with DNA sequences from the databases indicates that the CREAP-1 gene is located on human chromosome 17 (accession number AC005921).

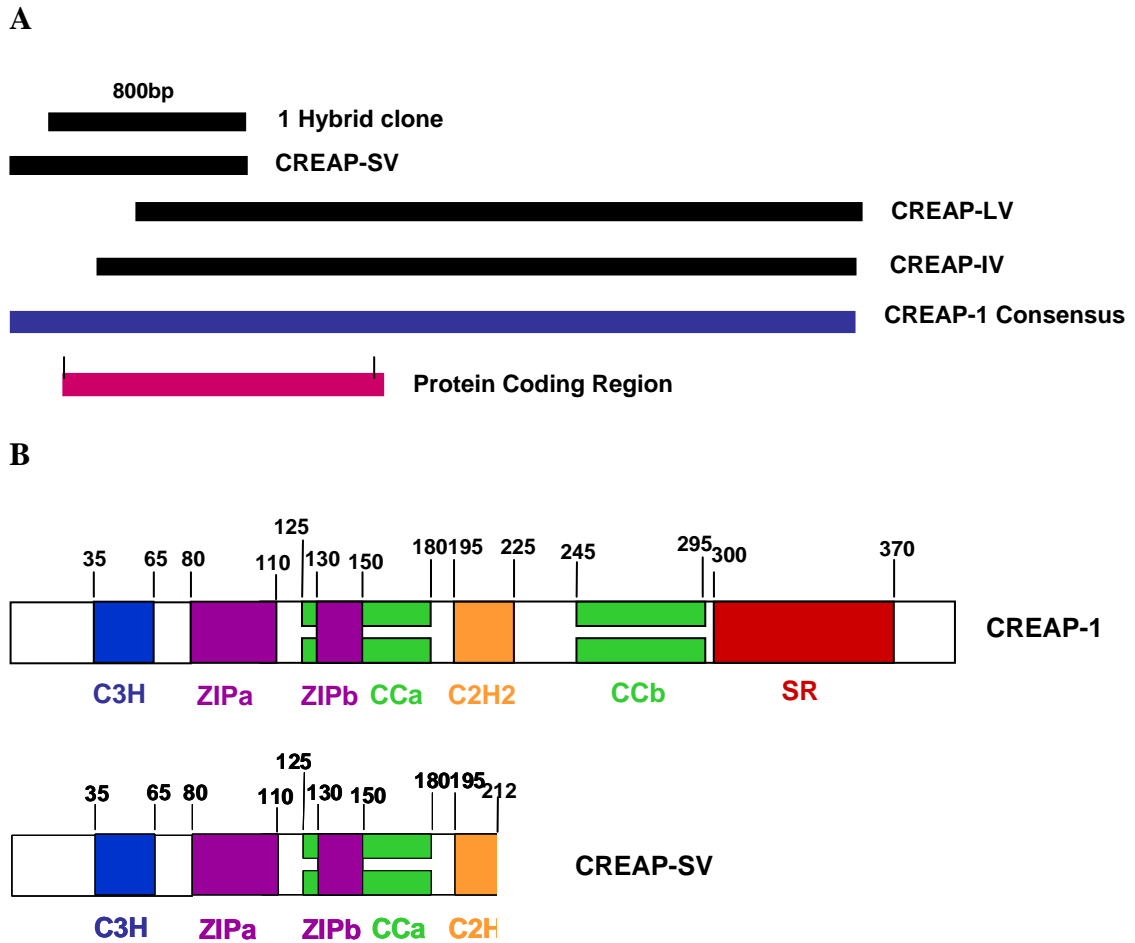


Figure 3.1. Schematic showing the overlapping clones and predicted protein domains used to determine the consensus DNA sequence for CREAP-1.

A. CREAP-SV, -LV and -IV were isolated from a λ library of human placental cDNA using the 1-hybrid clone as probe. The location of the CREAP-1 open reading frame (protein coding region) is shown. **B.** Schematic showing the full length and the truncated CREAP-SV summarising the predicted domain structures: C3H, represents the C3H zinc-finger domain; C2H2, represents the C2H2 zinc-finger domain; ZIPa & b, represent the leucine-zipper domains; CCa and CCb, represent coiled-coil domains; SR, represents the RS-rich domain. The truncated CREAP-SV clone stops towards the end of the C2H2 zinc finger, before the CCb.

3.1.2.1 Sequence Analysis of CREAP-1 Protein Domains

Examination of the predicted CREAP-1 peptide sequence shows that it consists of several known modular domains (Figure 3.1 and 3.2). There are two coiled-coil domains, typically found in transcription factor proteins, with the second coiled-coil domain being very rich in arginine and glutamate (RE) residues. The CREAP-1 protein has two potential leucine zipper domains, typical of bZIP transcription factors that bind to the CRE regulatory DNA sequence. There is another potential DNA binding domain, a zinc-finger domain of the C2H2 type, located between the coiled coil domains and a zinc-finger of the C3H type, often found in RNA-binding proteins, located upstream (5') of the first leucine-zipper-like motif. The C-terminal domain is very rich in arginine and serine residues typical of the SR family of proteins involved in RNA splicing. Other than the presence of leucine-zipper-like motifs the CREAP-1 sequence shows no similarity to other proteins known to bind to the CRE.

3.1.2.2 Possible Splice Variants of CREAP

The sequencing analysis indicated the presence of at least two splice variants and a potential single nucleotide polymorphism (SNP). The truncated or short variant of CREAP-1, called CREAP-SV, terminates with a stop codon followed by a poly-A tail at the location shown by a black arrow in Figure 3.2, and thus the predicted protein from this cDNA would be missing the SR domain, and the second coiled-coil domain as depicted in the schematic in Figure 3.1. The 1-hybrid and CREAP-SV clones were isolated from different cDNA libraries, terminated at the same nucleotide position and

were polyadenylated at their 3' ends, indicating that they represent a genuine splice variant.

The 105bp deletion in the long variant (CREAP-LV) of CREAP-1 represents a second splice variant that would result in the C-terminal 6 amino acids of CREAP-1, being replaced by 63 amino acids: (VQRKYAQMKMELSRVRRHTKASSEKDSVVLQNI LRYIVLSQLFCSRLVPPLVCLFGNYRPHL). In addition, the predicted CREAP-LV protein contains a TTG to GGG polymorphism causing a Leucine to Glycine change in amino acid 86. The peptide analysis of CREAP-1 and CREAP-SV proteins is listed in Table 3.1 showing predicted sizes and composition characteristics.

```

1      GGGATTTTCGG CCTGAGAGCG GGGCGAGGAG ATTGGCGACG GTGTGCCCCG
51     TGTTCCTGTT GGCAGGCTGCC TGGGCTGGTG GGAACAGCCG CCCGAAAGGAA
101    GCACCATGAT TTCGGCCGCG CAGTTGTTGG ATGAGTTAAT GGGCCGGGAC
        M I S A A Q L L D E L M G R D 15
151    CGAAACCTAG CCCCAGGACGA GAAAGCGAGC AACGTGCGGT GGGACACGA
        R N L A P D E K R S N V R W D H E 32
201    GAGCGTTTGT AAATATTATC TCTGTGGTTT TTGTCTGCG GAAATTGTTCA
        S U C K Y Y L C G F C P A E L E 48
251    CAAATACACG TTCTGATCTT GGTCCGTGTG AAAAAATTCA TGATGAAAAAT
        T N T R S D L G P C E K I H D E N 65
301    CTACGAAAAC AGTATGAGAA GAGCTCTCGT TTCATGAAAAG TTGGCTATGA
        L R K Q Y E K S S R F M K U G Y E 82
351    GAGAGATTTT TTGCGATACT TACAGAGCTT ACTTGACAGAA GTAGAACGTA
        R D F L R Y L Q S L L A E V E R 98
401    GGATCAGACG AGGCCATGCT CGTTTGGCAT TATCTCAAAA CCACAGTCT
        R I R R G H A R L A L S Q N Q Q S 115
451    TCTGGGGCCG CTGGCCCAAC AGGCAAAAAT GAAGAAAAAA TTCAGGTTCT
        S G A A G P T G K N E E K I Q V L 132
501    AACAGACAAA ATTGATGTAC TTCTGCAACA GATTGAAGAA TTAGGGTCTG
        T D K I D V L L Q Q I E E L G S 148
551    AAGGAAAAGT AGAAGAAAGCC CAGGGGATGA TGAATTAGT TGAGCAATTA
        E G K V E E A Q G M M K L V E Q L 165
601    AAAGAAAGAG GAGAACTGCT AAGGTCCACA ACGTCGACAA TTGAAAGCTT
        K E E R E L L R S T T S T I E S F 182
651    TGCTGCACAA GAAAAACAAA TGGAAAGTTG TGAAGTATGT GAGGCCTTTT
        A A Q E K Q M E V C E V C G A E 198
701    TAATAGTAGG AGATGCCCCAG TCCCAGGTAG ATGACCATTG GATGGGAAAA
        L I V G D A Q S R V D D H L H G K 215
751    CAACACATGG GCTATGCCAA AATTAAAGCT ACTGTAGAAG AATTAAAAAG
        Q H M G Y A K I K A T V E E L K E 232
801    AAAGTTAAGG AAAAGAACCG AAGAACCTGA TCGTGTGAG CGTCTAAAAA
        K L R K R T E E P D R D E R L K 248
851    AGGAGAAACA AGAAAAGAGG GAAAGAGAAA AAGAACGGGA GAGAGAAAGG
        K E K Q E R E E R E K E R E R E R 265
901    GAAAGAAAGG AAAGGAAAAG ACGAAGGGAA GAGGAAAGAA GAGAAAAAGA
        E E R E R K R R R E E E E R E K E 282
951    AAGGGCTCGT GACAGAGAAA GAAAGAAAGG AAGTCGTTCA CGAAGTAGAC
        R A R D R E R R K R S R S R S R 298
1001   ACTCAAGCCG AACATCAGAC AGAAGATGCA GCAGGTCTCG GGACCACAAA
        H S S R T S D R R C S R S R D N K 315
1051   AGGTCACGAA GTAGAGAAAAG AAGGCGGAGC AGAAGTAGAG ATCGACGAAG
        R S R S R E R R R S R S R D R R R 332
1101   AAGCAGAAGC CATGATCGAT CAGAAAAGAAA ACACAGATCT CGAAGTCGGG
        S R S H D R S E R K H R S R S R 348
1151   ATCGAAGAAG ATCAAAAAGC CGGGATCGAA AGTCATATAA GCACAGGAGC
        D R R R S K S R D R K S Y K N R S 365
1201   AAAAGTCGGG ACAGAGAACCA AGATAGAAAA TCCAAGGAGA AAGAAAAGAG
        K S R D R E Q D R K S K E K E K R 382
1251   GGGATCTGAT GATAAAAAAA GTAGTGTGAA GTCCGGTAGT CGAGAAAAGC
        G S D D K K S S V K S G S R E K 398
1301   AGAGTGAAAG CACAAACACT GAATCGAAGG AAAGTGATAC TAAGAATGAG
        Q S E D T N T E S K E S D T K N E 415
1351   GTCATGAGGA CCAGTGAAGA CATTAAATCT GAAGGTGACA CTCAGTCCAA
        V N G T S E D I K S E G D T Q S N 432
1401   TTAAACTGTA TCTGATAAGA CCTCAGATCA GACAGAGGTA AGTGATTGTT

```

Figure 3.2. DNA sequence, predicted amino acid sequence and domain structures of CREAP-1.

The coiled-coil domains are boxed, leucine zipper domains are thickly underlined, the zinc-finger domains are in underline & bold font, and the SR-rich domain is in italicised bold font. The black arrow shows the 3' end of the splice variant clone, CREAP-SV.

	CREAP-1	CREAP-SV
Number of residues	432	212
Predicted size	51.5 kDa	24.2 kDa
Predicted isoelectric point	10.56	5.15
Aliphatic residues	12.04%	20.75%
Aromatic residues	5.56%	7.55%
Non-polar residues	28.24%	46.70%
Polar residues	71.76%	53.30%
Charged residues	50.23%	33.49%
Basic residues	29.40%	16.04%
Acidic residues	20.83%	17.45%

Table 3.1. Pepstat Results for the Predicted CREAP-1 and CREAP-SV Proteins.

3.1.2.3 *Comparison of the CREAP Leucine Zippers with bZIP Proteins*

The CREAP leucine zipper-like regions were compared to common bZIP proteins to determine if the structure was likely to form a true bZIP domain. Leucine zippers are divided into heptad repeats and each amino acid residue position designated a letter from a-g, with the leucine residues at position (d). The a, e, and g positions are also important for proper helical turn structure and dimerisation and there are common amino acids found at those locations with hydrophobic residues at (a), and charged amino acids at (g) and (e) (for review see (Hurst 1995) and (Vinson et al. 2002). The comparison is shown in Table 3.2, with consensus with CREAP and the common

leucine zipper features highlighted in grey shading. There is complete consensus at position (d) with leucine or an accepted substitution present in every heptad repeat. The second zipper in CREAP contained a common residue at positions a, e and g, however, there was less consensus in the first zipper with only three positions with consensus over the five repeats.

	Residue Position	a	b	c	d	e	f	g
	Common Residue	V,T,I			L (V,I,M)	E,R,K,Q		E,R,K,Q
1ST ZIPPER	Heptad Repeat1	F	M	K	V	G	Y	E
	2	R	D	F	L	R	Y	L
	3	Q	S	L	L	A	E	V
	4	E	R	R	I	R	R	G
	5	H	A	R	L	A	L	S
2ND ZIPPER	1	I	Q	V	L	T	D	K
	2	I	D	V	L	L	Q	Q
	3	I	E	E	L	G	S	E

Table 3.2. Analysis of the Leucine Zipper Repeats of CREAP.

The first and second leucine zipper regions of CREAP were divided into heptad repeats and compared to the common residues found in other bZIP proteins. The leucine residues were designated as position (d). Positions (a), (e) and (g) have amino acid residues that are commonly found at those positions in most other bZIP proteins. Residues at positions b, c, and f are not generally conserved in the leucine zipper repeat. Consensus of the conserved residues with CREAP is highlighted in grey.

The DNA binding, basic region of bZIP proteins contains a sequence of N-x-x-AA-x-x-(C/S)R which is conserved across many proteins (Hurst 1995). Neither the first or second basic/zipper regions of CREAP contain this sequence, though the first region contains around 30% basic residues. This comparison is shown in Figure 3.3

CREB	EEAARKREVRLMKNREAAARECRRKKKEYVKCEENRVAVIENQNKTIEELKAKDLYCHKSD
CREM	EEATRKRELRLMKNREAAARECRRKKKEYVKCEENRVAVIENQNKTIEELKAKDLYCHKAE
ATF-1	DDPQLKRELRLMKNREAAARECRRKKKEYVKCEENRVAVIENQNKTIEELKTKDLYSNKSV
JUN	PEEEKRRIRREFNKMMAAKCRNRRRELTDITQAETDQLEDEKSALQTEIANLKEKEKEEF
FOS	SQERIKAEKRMNRIAASKCRKKLERIAKTEEKVKTKAQNSEASTANMREQVAQLKQ
CREAPa	FTNTRSDLGPCEKIHDENLRKQYEKSSRFMKVGYERDFIRYLQSLLAEVERRIRRGHARLAL
CREAPb	RRGHARLALSQNCQSSGAAGPTGKNEEKIQVITDKIDVILLQQIEELGSEGKVVEEAQGMMKLIV

Figure.3.3. Comparison of the CREAP-1 basic region and zipper-like domains.

CREAPa and CREAPb (1st and 2nd zipper like regions) were compared with the basic and leucine zipper amino acid sequences of some other bZIP proteins. The leucine residues are highlighted in black, the basic amino acid residues are highlighted in grey, and the conserved basic region consensus sequence is boxed. (Shipman et al. 2006).

3.1.3 Nuclear Localisation Signals

The CREAP sequence contains many motifs suggestive of it playing a role in the cell nucleus, possibly in transcription or splicing. Nuclear localisation signals (NLSs) are short stretches of amino acids that mediate the transport of proteins to the nucleus. The protein sequence was analysed using the PredictNLS program (Cokol et al. 2000) to identify the existence of any nuclear localisation signals. Five potential NLS were detected and the location and characteristics of the NLS's are shown in Figure 3.4. The 'experimental' NLS have been experimentally verified while 'potential' signals have been generated *in silico*.

A

```

1  TMISAAQLLD ELMGRDRNLA PDEKRSNVRW DHESVCKYYL CGFCPAELFT
51  NTRSDLGPCE KIHDENLRKQ YEKSSRFMKV GYERDFLRYL QSLLAEVERR
101 IRRGHARLAL SQNQSSGAA GPTGKNEEKI QVLTDKIDVL LQQIEELGSE
151 GKVEEAQG MM KLVEQLKEER ELLRSTTSTI ESFAAQEKQM EVCEVCGAFL
201 IVGDAQSRVD DHLMGKQHMG YAKIKATVEE LKEKLRKRTE EPDRDERLKK
251 EKQEREEREK EREREREERE RKRRREEER EKERARDRE RKRSRSRSRH
301 SSRTSDRRCS RSRDHKRSRS RERRSRSD RRRSRSHDRS ERK HRSRD
351 RRRSKSRDRK SYK HKSRD REQDRKSKEK EKRGSDDKKS SVKSGSREKQ
401 SEDTNTEske SDTKNEVNGT SEDIKSEGDT QSN

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B

Nuclear Localisation Signal	Type	No. proteins with NLS	% Nuclear Proteins
RKRRR	Experimental	20	85
RRREEEREKERARDRERRKR	Potential	193	97.92
KRSRSR	Potential	27	100
RRRSRSDR	Potential	74	97.29
RSRSDRRRSR	Potential	52	100

Figure 3.4. Potential Nuclear Localisation Signals in the CREAP Protein Sequence.

The CREAP protein sequences was analysed using the PredictNLS program. Panel A shows the location of the NLS in bold and underline. Panel B tabulates the NLS sequences and describes the type of signal, how many proteins have been found with the NLS and the percentage of these proteins located in the nucleus.

3.1.4 CREAP is a Member of a Family of Human Proteins

To determine if there were any related, homologous proteins, the CREAP-1 peptide sequence, with and without the SR domain, was compared to the protein sequence databases. Two proteins were found that were highly related, which we call CREAP-2 and -3 (accession numbers: CREAP-2 (AE006462 or AAG22846), CREAP-3 (NP_057103). These two proteins have no previously recognised function. CREAP-1 is located on chromosome 17, CREAP-2 is on chromosome 16, and CREAP-3 is on chromosome 7. The three proteins are 95% similar to each other over the N-terminal two-thirds, and very similar (60%) overall (Figure 3.5), with the highest degree of variation being in the C-terminal one-third of the protein. All three proteins share the same structural features of two coiled-coil domains, two zinc-finger domains, a leucine-zipper-like domain and an SR-domain.

The CREAP family of proteins contains a string of amino acids that are uniquely descriptive of this new family. The sequence TRxDLGxCxK[LVMI]HDxxLRxxYE is a putative motif, located in the C3H zinc-finger-like domain, which is a unique characteristic of the CREAP protein family. Using PROTEININFO to search the human protein databases, no other known proteins are related, even to 80% identity for any amino acid substitution. Another signature sequence of amino acids is located at the start of the C2H2 zinc-finger-like domain, VCEVCxA[FYW]L. Similarly, there are no other proteins in the database with a motif even closely related in sequence to this. These protein sequence and domain similarities suggest that the proteins represent a new family of proteins that may have similar functions.

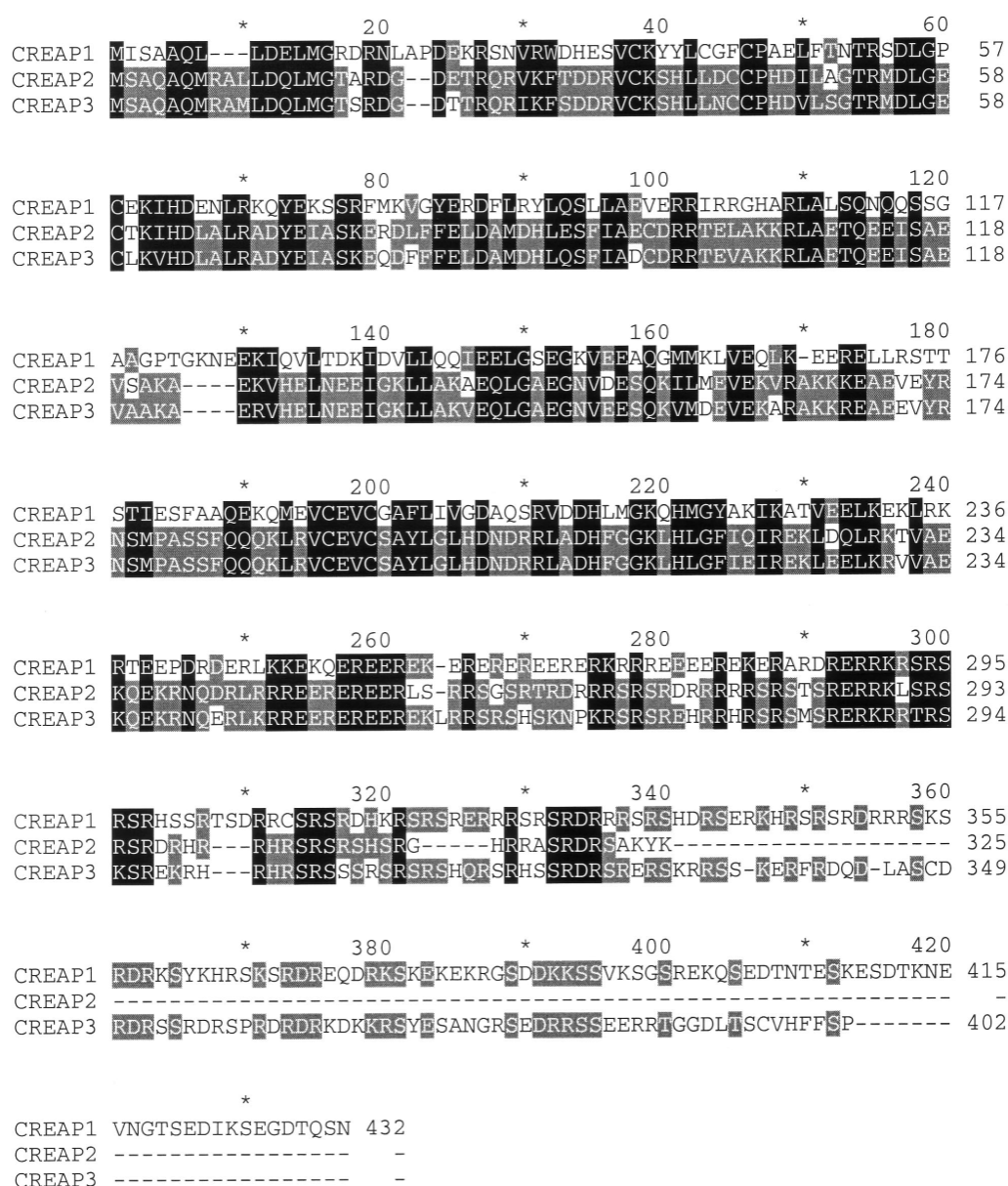


Figure 3.5. Alignment of the CREAP family of proteins.

Absolute amino acid identity between the family members is shaded in black, >75% is in dark grey shading, >50% is in light grey shading and <50% is unshaded. CREAP-1 is the protein presented in Figure 3.1 while the other two are proteins of unknown function found in the protein sequence databases.

3.1.5 CREAP is another human homologue of Luc7p

Since CROP and hLuc7A are homologous with a yeast U1 snRNP protein (See Chapter 1.9.5), the CREAP sequence was compared with yLuc7p. The predicted protein sequence of CREAP has considerable similarity to the protein sequence (accession # Q07508) of the yeast Luc7p. The sequence alignment of Luc7p and CREAP-1 shows a high degree of protein sequence conservation between this yeast protein and the human protein CREAP-1 (Figure.3.6). This includes high conservation of regions containing the C3H (58 %) and C2H2 (68 %) zinc-finger motifs and most of the first leucine-zipper motif of CREAP-1. However, Luc7p is shorter and lacks the second coiled coil domain and SR domain at the C-terminal of CREAP-1. This very high sequence identity between such distant species suggests that CREAP-1 is also a human homologue of yeast Luc7p, which is expected as CREAP and CROP have identical sequences.

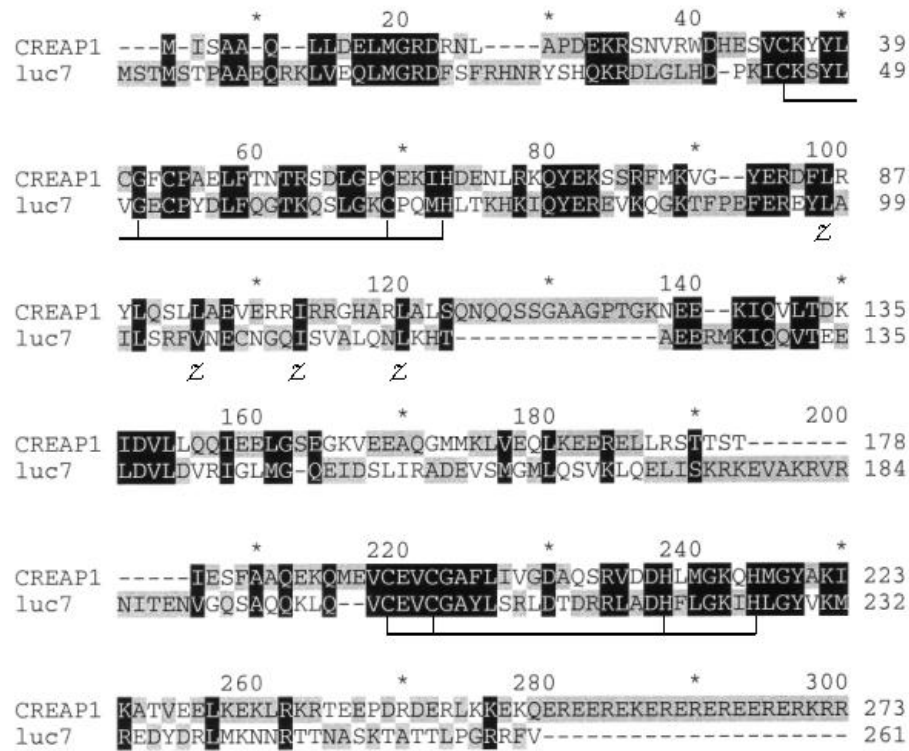


Figure 3.6. Comparison of the protein sequences of yeast Luc7p and human CREAP-1.

Absolute amino acid identity between the family members is shaded in black, >75% is in dark grey shading, >50% is in light grey shading and <50% is unshaded. The conserved zinc-finger motifs are underlined, whereas the conserved leucine zipper motif is indicated by stylised Z's. CREAP-1 is presented only to residue 273 of its full length of 432.

3.2 Discussion

CREAP was isolated as a novel CRE-binding protein and represents a new protein family. It contains several protein domains that suggest roles in DNA binding/transcription, protein-protein interactions and RNA binding/splicing. Indeed the yeast homologue, Luc7p is an essential splicing factor. These findings are discussed in detail below.

3.2.1 *CREAP Protein Domains*

3.2.1.1 *Leucine Zippers*

The leucine zipper motif is a defining feature of the bZIP family of transcription factors. These factors tend to bind the cAMP Response Element (CRE) and/or related sequences such as AP1 and C/EBP, of promoters to regulate transcription. Since CREAP contains leucine zipper-like repeats and was discovered by its ability to bind to a CRE in a one-hybrid assay, it suggests that CREAP may have functions as a bZIP transcription factor.

The leucine zipper-like regions of CREAP are of a similar sequence to those of other members of the bZIP group of proteins (Shipman et al. 2006). The number of leucine residues in the zippers of bZIP proteins can vary from three to six (Hurst 1995). It is not unusual for the leucine to be substituted by alternative hydrophobic amino acids such as methionine, isoleucine or valine. In the first of the leucine zipper motifs of CREAP-1 the fourth leucine (of five) is replaced by isoleucine, whereas the second leucine zipper motif is a repeat of only three leucine residues. The bZIP proteins characteristically contain a domain of 3 to 6 leucine amino acids preceded by a region rich in basic amino

acids, which binds to DNA. The first zipper-like region in CREAP-1 follows a fairly basic region (about 30 % basic residues) and contains leucine at locations corresponding to 3 of 5 leucine residues in the zippers of Fos and Jun. CREAP-1 contains conservative substitutions of valine and isoleucine in place of the other two leucines. The second zipper-like region in CREAP-1 contains leucine at locations corresponding to the first 3 of the leucine residues in the corresponding region of Fos and Jun, as well as CREB, CREM and ATF-1, but this zipper region follows a region containing very few basic amino acids. Therefore the first leucine zipper but not the second more closely resembles the classical bZIP structure. The basic region is preceded by the C3H zinc finger-like region, which has been shown to bind both DNA and RNA so could possibly substitute for the basic region in DNA binding (Section 3.2.1.3).

The heptad repeat in the leucine zipper region contains conserved residues to allow helix formation and dimerisation stability. The (a), (e) and (g) positions are important for proper helical turn structure and dimerisation and there are common amino acids found at those locations with hydrophobic residues at (a), and charged amino acids at (g) and (e). Dimerisation stability results from side-by-side packing of the leucines at position (d) and the hydrophobic residues at (a), with extra stability provided by intra- and inter-helical salt bridges between the charged residues at positions e and 'g and e' and g in the proteins trying to dimerise (Hurst 1995). The lack of conservation at the a, e, and g positions of CREAP may potentially influence its ability to form dimers or have protein-protein interactions.

The basic region varies considerably between all bZIP proteins, but there is a conserved sequence, N-x-x-AA-x-x-(C/S)R, which appears to be present in many bZIP proteins.

Notably, the CREAP basic regions do not contain this sequence. However, the bZIP protein, CHOP (C/EBP homologous protein), also diverges completely from the consensus and is not predicted to be able to bind DNA. CHOP has been reported to act as a dominant inhibitor of transcription by forming heterodimers with C/EBP proteins (Ron and Habener 1992), although it has been found that CHOP can specifically bind DNA in certain circumstances, such as when heterodimerising with C/EBP (Barone et al. 1994). There are several examples of proteins which have leucine zipper motifs but no associated basic region, including c-Myb, HSF and c-Ski, however these proteins are not members of the bZIP family. It is thought that the leucine zippers may act as general dimerisation domain (Hurst 1995). The CREAP identical protein, CROP, has been shown to form homodimers by interacting with itself in a yeast two-hybrid assay and it was found that the protein-protein interaction occurred when the leucine zippers were absent and was dependent on the RE (overlaps with the second coil-coil region of CREAP) and RS domains (Umehara et al. 2003). So it is possible that CREAP's leucine zippers-like domains may not function as DNA binding domains or dimerisation domains or that it may be inhibitory.

It has also been shown that a number of bZIP factors are regulated by naturally occurring truncated variants which retain the bZIP domain but may lack other functional domains (Foulkes and Sassone-Corsi 1992). CREAP appears to have a naturally occurring truncated splice variant that is missing the SR region of the protein but retains the other domains. It is therefore possible that the truncated splice variant CREAP-SV may be regulating the full length CREAP variant.

3.2.1.2 *Coiled-Coils*

CREAP contains two coiled-coil domains. The coiled-coil is a common structural motif, formed by approximately 3-5% of all amino acids in proteins (Wolf et al. 1997). It consists of two to five α -helices wrapped around each other into a left-handed helix to form a supercoil. Left-handed helices are made up of heptad repeats that are designated (a-b-c-d-e-f-g). The residues in the (a) and (d) positions must be hydrophobic to facilitate dimerisation along one face of each helix (O'Shea et al. 1993). The residues (e) and (g) must be charged to form interhelical electrostatic interaction, while the remaining positions must be hydrophilic as they will form the helical surfaces that are exposed to the solvent (O'Shea et al. 1993). The coiled-coil motif is adaptable to many biological processes such as skeletal proteins, motor proteins, molecular recognition systems and ion channels (Burkhard et al. 2001)

Leucine zippers are a type of coiled-coil and the first coiled-coil region of CREAP overlaps with the second leucine zipper-like region of CREAP. Coiled-coils are one of the principal subunit oligomerisation motifs in proteins (Burkhard et al. 2001). Coiled-coil proteins have a broad range of functions related to the specific characteristics of their domains such as oligomerisation state, rigidity and function as a molecular recognition system (Burkhard et al. 2001). It is most probable that the coiled-coil regions of CREAP are acting to help dimerisation or oligomerisation. The second coiled-coil region of CREAP overlaps with the KE (lysine/glutamate) and RE (arginine/glutamate) rich regions identified in CROP and the RE was found to be important in CROP homodimerisation (Nishii et al. 2000; Umehara et al. 2003).

3.2.1.3 *Zinc Fingers*

CREAP contains two different zinc finger-like domains, a C3H- and C2H2-like domain. Zinc fingers are small protein domains in which zinc plays a structural role. The structure of very small domains is usually stabilised by disulfide bond formation or by binding to a metal ion such as zinc (Kutko et al. 2003). The C2H2 zinc finger motif is the classic zinc finger that has been found in many transcription factors and DNA binding proteins. This domain usually consists of a repeated 28-30 amino acid sequence with two conserved cysteines and histidines, though other Cys/His combinations are possible (Kutko et al. 2003). This amino acid sequence motif forms an independent, DNA-binding mini-domain-folded around a central zinc ion with a tetrahedral arrangement of cysteine and histidine metal ligands (Miller et al. 1985). The C2H2 motif is usually repeated, with the number of repeats ranging from 2 to 37 in different proteins (Klug and Schwabe 1995). It has been estimated that between 300 and 700 human genes encode zinc finger proteins (Hoovers et al. 1992) and thus this motif appears to be a ubiquitous structural motif for DNA binding. CREAP contains a single C2H2-like motif.

C2H2 zinc fingers are usually associated with DNA binding but a number of C2H2 zinc fingers that bind to RNA have been identified. TFIIIA is necessary for transcription and contains nine C2H2 domains. Fingers 1-3 of TFIIIA bind strongly to DNA while fingers 4-6 bind RNA (Searles et al. 2000). The mammalian C2H2 zinc finger proteins wig-1 (Mendez-Vidal et al. 2002) and JAZ (Chen et al. 2004) show RNA binding activity and are localised to the nucleolus, while hZFP100 is involved in histone pre-

mRNA processing (Dominski et al. 2002). The C2H2 motif seems to be a common structure in multifunctional proteins (Cassiday and Maher 2002).

Many RNA binding zinc fingers are of the C3H type. Two copies of the C3H finger domain are present in the immediate early response proteins Nup475 and TISIIId and these proteins bind the AU-rich elements of tumour necrosis factor α mRNA (Brown 2005). C3H zinc finger proteins across many species show RNA binding activity such as the viral HIV-1 nucleocapsid (Tisne, 2003), plant *Arabidopsis* HUA1 nuclear protein (Cheng et al. 2003) and parasite trypanosome tcZFP1 (Espinosa et al. 2003). C3H zinc fingers can have between one and six zinc finger domains (Hall 2005) with a single C3H zinc finger being shown to be able to weakly bind to AU-rich element RNA (Michel et al. 2003).

CREAP has both a C2H2- and C3H-like zinc finger domain suggesting that it may be able to function in both DNA and RNA binding. Since CREAP lacks an RNA recognition motif (RRM) it is possible that perhaps the RNA binding characteristics of one or both zinc fingers could substitute for a RNA recognition motif (RRM) in any SR protein related activity. Alternatively, the DNA binding characteristic of the C2H2 may contribute to any transcriptional activity of CREAP.

3.2.1.4 RS Domains

CREAP has an RS domain similar to the SR protein family. Serine-arginine (SR) proteins are involved in pre-mRNA splicing. They contain one or two N-terminal domain RNA binding domains (RRM-RNA Recognition Motif) and a C-terminal

arginine-serine (RS) rich domain (Phillips, Celotto 2003). The RRM's determine RNA binding specificity while the RS domain mediates protein-protein interactions and directs localisation to nuclear splicing speckles (Caceres et al. 1997). The RS domains mediate protein-protein interactions with other RS domain containing proteins (Wu and Maniatis 1993). The SR family of proteins function in early spliceosome formation and in multiple steps of the splicing reaction (Staknis and Reed 1994)

The nuclear import pathway for SR proteins differs from the classical nuclear import pathway. A specific import receptor for SR proteins exists and is termed transportin-SR (TRN-SR) (Kataoka et al. 1999). TRN-SR is a novel member of the importin β /transportin family and it binds directly to the RS domains of SR proteins including ASF/SF2 and SC35 to mediate nuclear import (Kataoka et al. 1999).

CREAP does not contain any known RRM's; this however does not preclude it from being an SR protein or having the same nuclear targeting. The *Drosophila*, Tra and SWAP splicing regulators have RS domains but lack RRM's, and the RS domain is necessary and sufficient to target reporter proteins to nuclear speckles (Li and Bingham 1991). There is a class of RS domain-containing proteins, called SR protein-related polypeptides or SR-like proteins, that are also involved in splicing regulation (Fu 1995). So given that CREAP contains an RS domain but no RRM, it is more probable that it may be an SR-like protein and will still be targeted to the nucleus and nuclear speckles via its RS domain.

3.2.2 Nuclear Localisation Signals

CREAP contains several nuclear localisation signals, as well as the alternate possible nuclear targeting by its RS domain. Transport into the nucleus occurs via nuclear pore complexes (NPCs) (Stoffler et al. 1999; Allen et al. 2000; Fahrenkrog and Aeby 2003) and soluble carrier proteins called importins (Gorlich et al. 1994). During classical nuclear import, the NLS of the target protein is directly bound by importin α , which is part of a heterodimeric import receptor with importin β , which mediates interaction with the NPC (Lange et al. 2007).

Although there is not a strict consensus sequence, classical NLSs are typically small stretches of positively charged amino acids, arranged as either monopartite (a single cluster) or bipartite (two clusters separated by a 10–12-amino acid spacer) sequences (Kalderon et al. 1984; Robbins et al. 1991). In general, positively charged amino acids are abundant in NLSs, though there are glycine-rich motifs with a few positive charges (Bonifaci et al. 1997). It has been found that monopartite classical NLSs require a lysine followed by basic residues to give a loose consensus of K(K/R)X(K/R) (Hodel et al. 2001). Two of the five NLS found in CREAP fit this pattern, RKRRR and KRSRSR, suggesting that they may function as monopartite NLSs.

3.2.3 Multifunctional Proteins

Multifunctional regulatory proteins with the ability to perform different functions in the nucleus or cytoplasm, or with the ability to bind both DNA and RNA, are known and have been reviewed (Wilkinson and Shyu 2001; Cassiday and Maher 2002). Several of these proteins function as transcription factors but also have functions requiring RNA

binding ability. p53 has a second function as an RNA anti-helicase (Oberosler et al. 1993), TRA-1 has a role in RNA nuclear export (Graves et al. 1999; Segal et al. 2001), and splice variants of WT1 have been shown to localise to nuclear regions involved in RNA splicing (Larsson et al. 1995). Many of the known multifunctional proteins contain C2H2 zinc-finger motifs for nucleic acid and RNA binding (Cassiday and Maher 2002). Thus, although multifunctionality is an emerging feature of some nucleic acid binding proteins, no instance of a protein that can interact specifically with the CRE and also act as a RNA binding protein or splicing factor has previously been described (Cassiday and Maher 2002).

3.2.4 Splice Variants

The screen of the placental cDNA library generated several overlapping clones. It seems likely that at least one splice variant of CREAP-1 exists and is the truncated CREAP-SV. This sequence lacks the regions encoding the second coiled coil and the RS domain. In this way, this variant more closely resembles the yeast protein Luc7p. Puig et al, also identified possible splice variants in their Luc7p homologue, Luc7A (Puig et al. 2007). They used sequence analysis to define three splice variants of 109, 452 and 462 amino acids, however they detected smaller bands during western blotting but do not indicate the size. It is possible that the smaller bands they detected during Western blotting correspond to CREAP-SV.

3.2.5 The CREAP Protein Family

Database searching revealed that CREAP belongs to a novel family of proteins with three members. The CREAP family of proteins contains a string of amino acids that are uniquely descriptive of this new family. The sequence TRxDLGxCxK[LVMI]HDxxLRxxYE is a putative motif, located in the C3H zinc-finger-like domain, which is diagnostic of the CREAP protein family. Review of the literature and EST databases shows that CREAP-2 and -3 correspond to the hLuc7B1 and B2, respectively, found by Fortes et al and Puig et al (Fortes et al. 1999a; Puig et al. 2007). The databases designate these proteins as Luc7-like (LUC7L) and place them in the Pfam LUC7 N-terminus family. This family contains the N-terminal region of several LUC7 protein homologues and only contains eukaryotic proteins. The family also contains human and mouse LUC7 like proteins and human CROP (<http://pfam.sanger.ac.uk/family?entry=luc7>).

3.2.6 CREAP is a Luc7p Homologue

The LUC7 gene was identified by a mutation that causes synthetic lethality in a yeast strain lacking the nuclear cap binding complex (CBC) (Fortes et al. 1999b). The LUC (lethal unless CBC is produced) family includes genes that encode several components of the yeast commitment complex (the first step of splicing), including Mud2p/Luc2p and several components of the yeast U1-snRNP (Fortes et al. 1999a). The LUC7 gene is essential for yeast survival, as disruption of a LUC7 allele stops colony formation and growth (Fortes et al. 1999a).

The LUC7 gene encodes the protein Luc7p. The sequence encodes two putative zinc finger motifs, the first a C3H type and the second is a C2H2 type (Fortes et al. 1999a). Examination of protein databases revealed several metazoan relatives of Luc7p in which the zinc finger regions are highly conserved. All of the metazoan relatives have C-terminal extensions containing RS and RE repeats (Fortes et al. 1999a). Luc7p has been found to be a component of the yeast U1 snRNP and involved in mRNA splicing (Fortes et al. 1999a). Recent work on Luc7p has shown that it contacts the RNA mainly through the C3H zinc finger and that Luc7p is required for CBC-U1 snRNP interactions during splicing (Puig et al. 2007). CREAP appears to be another human homologue of Luc7p with conservation of the zinc finger domains and leucine zipper-like domain, suggesting that CREAP may have a similar function in splicing in humans.

3.3 Summary

Analysis of the CREAP nucleotide and protein sequence reveals that CREAP contains a unique set of modular domains. There are domains related to protein-protein interactions (coiled-coils, leucine zippers and RS domain), DNA binding and transcription (C2H2 zinc finger, leucine zippers), and mRNA splicing (RS domains and zinc fingers). CREAP also appears to be a member of a new protein family with two other proteins being found in the databases and with two unique putative motifs found to describe the family. There is a yeast homologue of CREAP, Luc7p, which is essential for yeast growth and involved in splicing. CREAP also contains five NLS, two of which appear to be classical monopartite signals, and also contains an RS domain which has been shown to be sufficient for nuclear targeting and localisation to

intranuclear speckles. These properties of CREAP will be investigated in the following Chapters to elucidate the function of these unique modular domains.

Chapter 4. CREAP Antibody Production and Localisation

4.1 Introduction

The production of custom antibodies was necessary as no commercial antibodies for CREAP were available. These antibodies could then be used for techniques such as western blotting, indirect immunofluorescence and immunoprecipitation to determine the size and number of immunoreactive proteins in cell lysates, the sub-cellular localisation of CREAP and binding/interacting partners. In this chapter CREAP was expressed as a GST fusion protein to produce large amounts of recombinant protein. Expression as a GST-fusion protein is a common method to produce large amounts of protein and enable affinity purification. The CREAP protein was then used to create polyclonal antibodies.

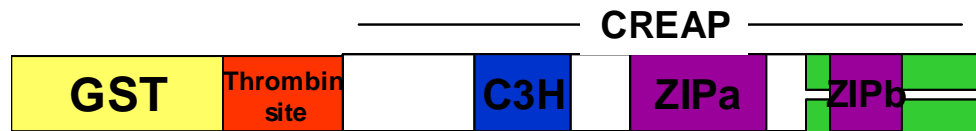
4.2 The GST Fusion Protein

4.2.1 Preparation of the GST-CREAP Fusion Protein for Immunisation

Expressing the protein of interest as a GST fusion protein is a well established and useful technique for producing large quantities of purified protein. The glutathione S-transferase (GST) tag allows affinity purification using glutathione Sepharose 4B (Amersham Biosciences). A 455bp section of the CREAP N-terminal DNA sequence encoding the C3H zinc finger, leucine zipper domains and most of the first coiled-coil were cloned into the pGEX-AHK, GST vector (see Section 2.4.4) to create the GST-CREAP fusion protein vector (see Figure 4.1A). The GST-CREAP fusion vector was transformed into bacteria, on a small scale, and protein expression is induced by IPTG,

with the optimal concentration for protein induction determined empirically as shown in Figure 4.1B. Concentrations from 100 μ M to 1 mM IPTG were used to induce protein expression in BL21 *Escherichia coli* transformed with GST-CREAP, and the bacteria lysed in loading buffer, electrophoresed and stained. An approximately 43 kDa protein is induced at all IPTG concentrations but is particularly induced by 250 μ M and 750 μ M IPTG. The GST-CREAP fusion protein was expected to be around 43 kDa as the GST protein is 27 kDa and the CREAP protein moiety around 15.6 kDa.

A



B

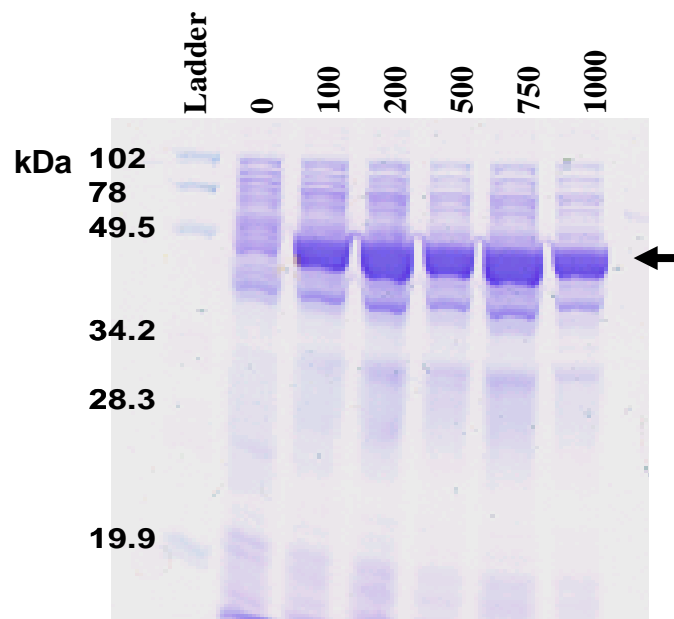


Figure 4.1. IPTG Induction of GST CREAP Fusion Protein.

A. Schematic showing the structure of the GST-CREAP fusion protein. There is the GST sequence (yellow) followed by a thrombin cleavage site (orange) and then the truncated CREAP sequence containing the C3H zinc finger (blue), two leucine zippers (purple) and most of the first coiled-coil (green). B. BL21 cells were transformed with GST-CREAP and protein expression induced with 0-1000 μ M IPTG. 10 μ l of protein sample was run on a 10% Tris-Glycine gel and stained with coomassie blue. The black arrow indicates GST-CREAP protein induction.

It is quite common for eukaryotic proteins to be expressed in *E.coli* as insoluble aggregates termed inclusion bodies, that can make affinity purification difficult (Yasukawa et al. 1995). To test whether GST-CREAP is present in inclusion bodies, the proteins were extracted into soluble and insoluble fractions. As can be seen in Figure 4.2, the majority of GST-CREAP is present in the insoluble fraction. There are several strategies to resolubilise protein but one option is to co-express *E.coli* thioredoxin. Thioredoxins are low molecular weight disulfide oxidoreductases that are capable of reducing disulfide bonds on proteins (Arner and Holmgren 2000). It has been shown that not only does co-production of thioredoxin increase solubility it also allows expression in the native protein conformation (Yasukawa et al. 1995). After co-expressing thioredoxin with GST-CREAP the fusion protein became largely soluble, appearing predominately in the soluble fraction. This approach was therefore used in subsequent work.

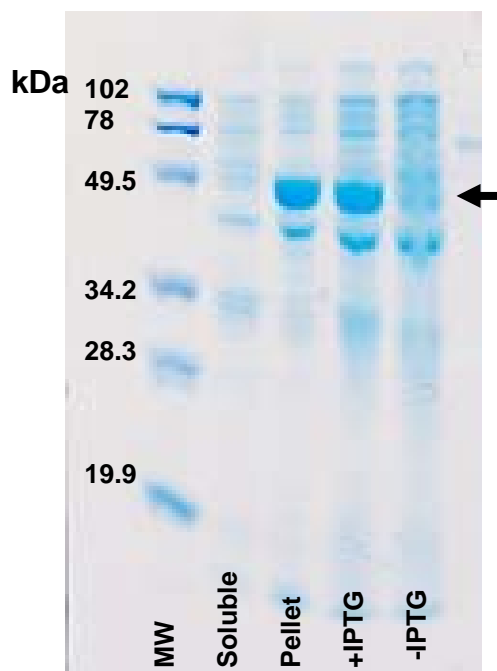


Figure 4.2. GST-CREAP is Produced as Exclusion Bodies.

A small scale transformation of BL21 with GST CREAP was induced with 250 μ M IPTG. The bacteria pellets were processed into soluble and insoluble fractions and 5 μ l ran on a 10% SDS-PAGE gel. Lane 1 is marker, lane 2 soluble fraction, lane 3 insoluble fraction, lane 4 +IPTG crude lysate, lane 5 -IPTG crude lysate. The fusion protein (indicated by a black arrow) appears to be present in the insoluble pellet fraction only.

With confirmation that the fusion protein could now be obtained in a soluble form, larger scale cultures were then prepared to affinity purify the CREAP protein using Glutathione Sepharose 4B. Thrombin cleavage would then be used to obtain the pure CREAP protein moiety for use as an immunogen.

4.2.1.1 GST Purification

GST Purification is an affinity-based technique. The GST tag binds to glutathione which is immobilised on the solid support of Sepharose, allowing the GST-tagged protein to be separated away from the other bacterial proteins. The soluble fraction of the bacterial lysate is incubated with the glutathione Sepharose beads to capture the GST-CREAP protein, while the other protein contaminants are washed away. The GST protein can be competitively eluted from the Sepharose using 50 mM reduced glutathione, left attached to Sepharose, or the non-GST part of the protein can be cleaved away from the bound GST using thrombin.

4.2.1.2 Thrombin Cleavage

The GST-CREAP fusion protein contains a thrombin cleavage site that allows the GST tag to be separated from the CREAP protein. To determine the optimal thrombin digest time, 200 µg of eluted affinity purified fusion protein was incubated with 2U thrombin for 1-24 h and then ran on a gel and stained. Figure 4.3A shows the thrombin digest gel. Efficient lysis appears to occur from 1 hour on, though by 24 hour there appears to be some degradation or over-digestion as the amount of protein present appears to be less.

Overnight digestions were carried out at room temperature to produce CREAP protein for further work. Large scale fusion purifications and overnight thrombin digests were performed to increase protein yields. To determine the efficiency of large scale digests, a proportion of the sample was analysed by SDS-PAGE. As can be seen in Figure 4.3B, the digested sample contains a mixture of un-cleaved fusion protein and cleaved GST and CREAP. To overcome the problem of un-cleaved protein and contamination from the cleaved GST moiety, the technique of on-column thrombin digestion was performed for further work.

The on-column digestion technique allows easy and rapid purification of CREAP away from the GST-tag. After thrombin digestion, the GST-tag and any un-cleaved GST-CREAP remains bound to the glutathione Sepharose in the column. Only the cleaved CREAP flows through the column and is collected. Using the optimised parameters, highly purified CREAP protein lacking the GST-tag was then produced. This purified CREAP protein was then used as an antigen in the subsequent polyclonal antibody production.

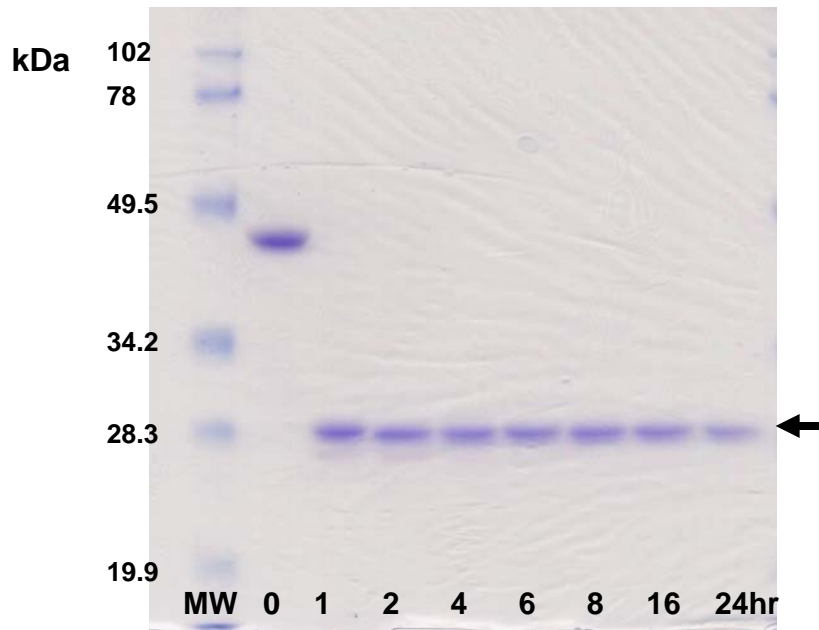
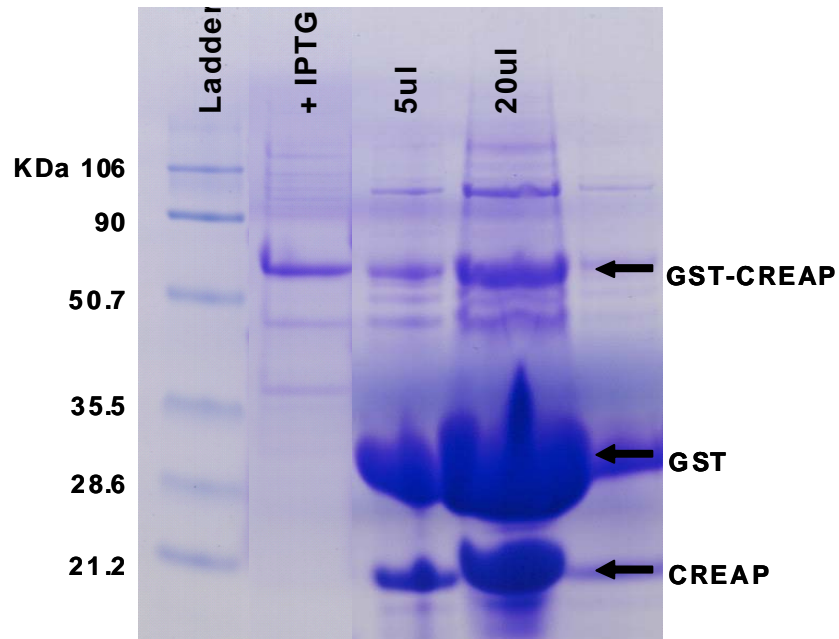
A**B**

Figure 4.3. Optimisation of Thrombin Cleavage of GST-CREAP Fusion Protein.

A. 200 μ g affinity purified GST CREAP fusion protein was digested with Thrombin for 1- 24 h to cleave the GST. Samples were then subjected to SDS-PAGE a 10% gel and stained with coomassie blue. The efficiency of the cleavage is detected by the appearance of the 27 kDa GST indicated by the black arrow. B. After a large-scale preparation, GST CREAP was digested overnight with thrombin and 5 and 20 μ l was run on a gel. The +IPTG lane is a positive control for the size of un-cleaved GST-CREAP.

4.3 Antibody Production and Characterisation

4.3.1 Rationale

Since CREAP is a novel, uncharacterised protein, no commercial antibodies were available. With a lack of established reagents, especially antibodies, molecular and biochemical assays would be extremely difficult. The production and characterisation of an antibody for CREAP would allow techniques such as western blotting, immunolocalisation and immunoprecipitation to be conducted.

4.3.1.1 Immunisation Protocol

Affinity purified and cleaved CREAP protein was used as an antigen to immunise rabbits to produce polyclonal CREAP sera. 100 µg of CREAP protein initially in complete Freund's adjuvant was injected subcutaneously, and subsequently boosted in incomplete Freund's every month. Six rabbits (A-F) were immunised over a six month period with blood collected pre-immunisation and monthly post-immunisation after the second boost. Rabbit sera collected on the third bleed was used to conduct antibody tests and optimisations, along with pre-immunisation sera used as a negative control.

4.3.2 Western Blotting

Western blotting specifically detects a protein of interest in a sample after it has been electrophoretically separated and transferred to a membrane (see Section 2.11 and 2.12). Western blotting analysis was first used to characterise the sera collected from the

different animals. Both the pre- and post- immunisation serum was tested by conducting western blotting on total placental protein as CREAP was isolated from a placental cDNA library. The predicted size of the full length and truncated splice variants of CREAP is 51.5 kDa and 24.2 kDa, respectively (see Table 3.1).

As can be seen in Figure 4.4, the pre-immune serum detects no major bands, which means that there were no endogenous non-specific antibodies present in the serum. After immunisation, all six immune sera detected a band between 50 and 60 kDa, which is within the predicted size range for the full length CREAP protein. The background was slightly clearer on serums A, C and D.

Further characterisation of the antibodies was performed on JEG-3 total protein (Figure 4.5). JEG-3 cells are derived from a choriocarcinoma so are placenta-related. A major band of approximately 58 kDa was detected by all sera and at higher protein loadings a fainter band of around 50 kDa was also detected, especially by sera B, C, E and F. Pre-absorption of the antibody with CREAP protein was also carried out. The specific 58 kDa CREAP band disappeared when the sera was pre-absorbed (data not shown) indicating that the band being detected was specific. Serum A appeared to give the clearest background and had a strong specific signal so it was used for all further antibody applications.

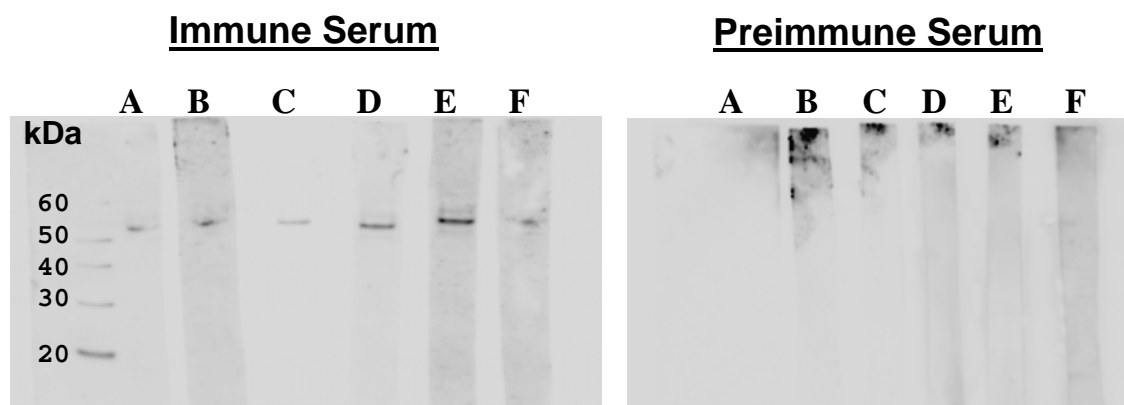


Figure 4.4. Rabbit Sera Reactivity Against Placental Protein Extract.

30 μ g total placental protein was run on a 10% Bis-tris gel and transferred to nitrocellulose. The membrane was cut into strips and individual strips incubated with a different serum (A-F) diluted 1:5000 either following immunisation or as the pre-immune serum.

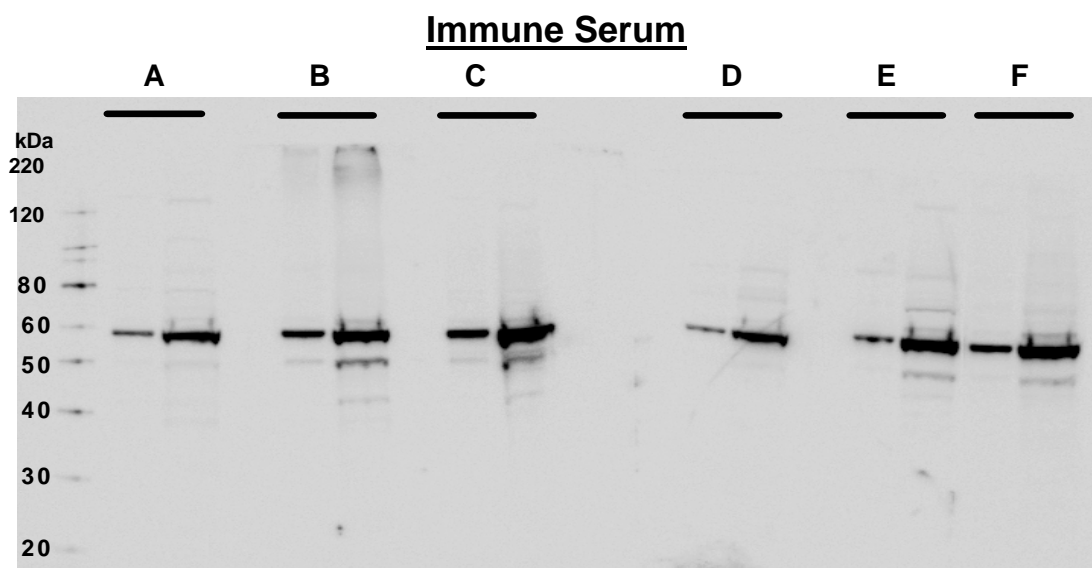


Figure 4.5. Rabbit Sera Reactivity Against JEG-3 Protein Extract.

JEG-3 cells were lysed in 2D buffer and 5 μ g and 30 μ g protein loaded onto a 10% Bis-Tris gel. Membranes were cut into individual strips and probed with sera from six rabbits, A-F, after immunisation with CREAP. A 58 kDa protein was detected in all samples, with a fainter ~50 kDa band at higher protein loadings.

4.3.2.1 Possible Splice Variants Present in Human Placenta and Mouse Pituitary

The placental cDNA screen suggested that there may be a truncated splice variant of CREAP, which lacks the RS region (see Section 3.1.2.2). To investigate this finding, further, western blots were conducted on placenta and the mouse pituitary cell line, AtT-20. At higher protein concentrations, faint approximately 25 and 30 kDa bands were detected in the AtT-20 cells while a strong ~25 kDa band was detected in the placental cells (see Figure 4.6).

Therefore these results do suggest that the truncated splice variant of CREAP does occur as a protein, especially in placental cells. However, the splice variant expression appears to be cell-type dependent but the full-length CREAP protein is quite strongly expressed in both cell types.

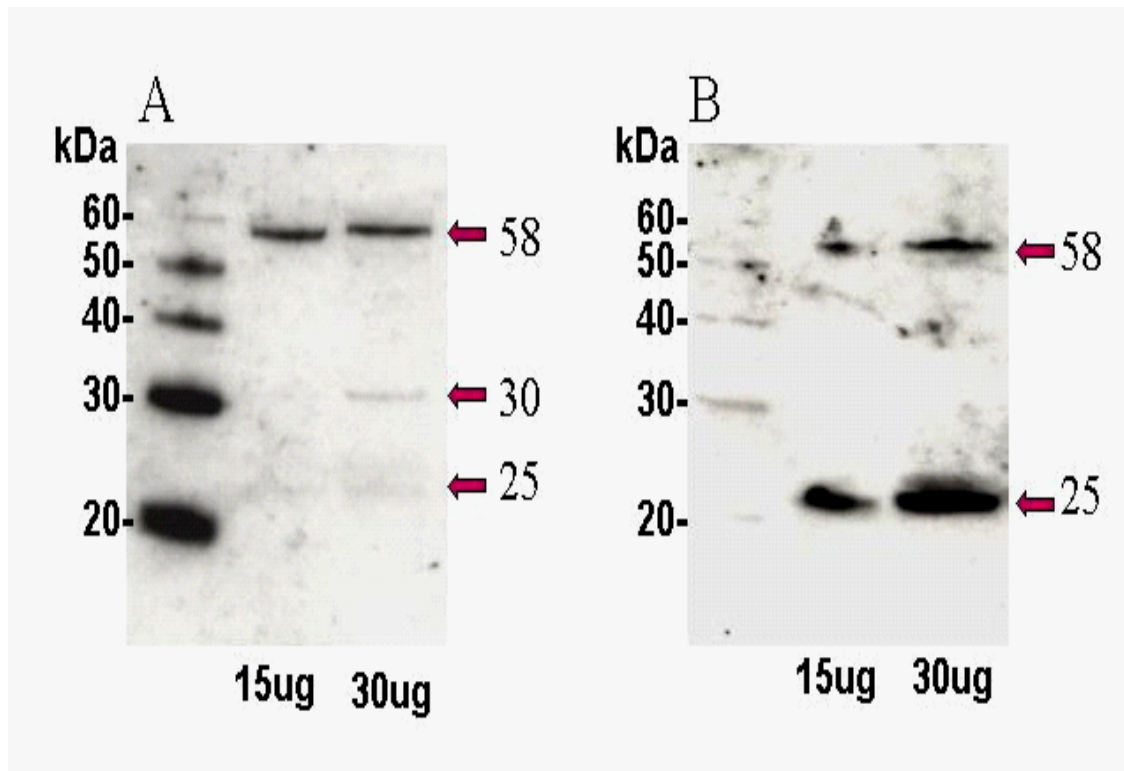


Figure 4.6. Probable Splice Variants in Placenta and AtT-20.

Anti-CREAP antibodies were used to probe nuclear protein extracts from mouse AtT-20 cell lines (panel A) or the human primary placental cells (panel B). Western blot analysis showed that both extracts contained a protein at 58 kDa corresponding to full length CREAP-1. The placental cells also contained a prominent protein of around 25 kDa corresponding to the expected size of CREAP-SV. The AtT-20 cells also contained two less prominent protein bands at 25 kDa protein and at 30 kDa, (Shipman et al. 2006).

4.4 Localisation of CREAP

4.4.1 *Presence of CREAP in Nuclear, Cytosolic and Whole Cell Protein Extracts.*

The results of Chapter 3 suggest that CREAP is related to the bZIP family of transcription factors and that it contains several nuclear localisation signals. Therefore it would be expected that CREAP would be found in the nucleus. To determine the localisation of CREAP and assess the best protein extraction method, western blotting was carried out on total, nuclear and cytoplasmic protein fractions from Jeg-3, Cos-7 and AtT-20 cells (see Section 2.10 for extraction protocols). Figure 4.7 shows the western blot of the nuclear and cytoplasmic proteins. Clear bands are detected at 58 kDa in the nuclear enriched fractions from the three cell lines but the bands are barely present in the cytosolic fractions. This strongly suggested that CREAP was indeed a nuclear protein as would be expected if it has a role as a transcription factor.

As nuclear extractions can be quite time consuming, a total protein lysis buffer was tested. Figure 4.8 shows the western blot of the total protein from the three cell lines. This 2D lysis buffer was successful at extracting CREAP, as a 58 kDa protein was detected in all samples. This method is a simpler technique for obtaining protein lysates for western blotting and is employed for most future western blots. Additionally, the antibody was able to detect both reduced and non-reduced protein samples (data not shown).

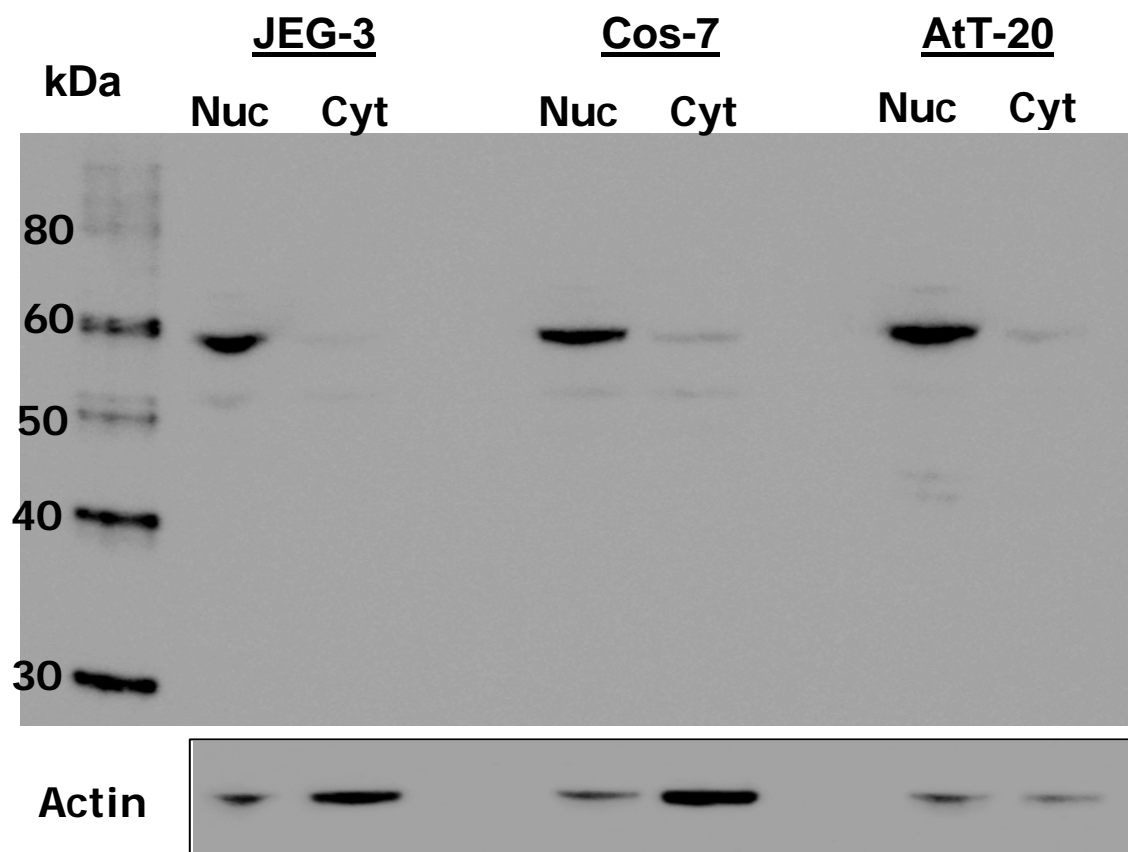


Figure 4.7. Nuclear and Cytosolic Protein Extracts Western Blot.

10 μ g of nuclear (Nuc) or cytosolic (Cyt) protein from three different cell lines was transferred to nitrocellulose and probed with anti-CREAP antibody. A band of approximately 58 kDa was detected in the nuclear extracts but was barely detectable in the cytosolic extracts. The blot was reprobed for actin as a loading control.

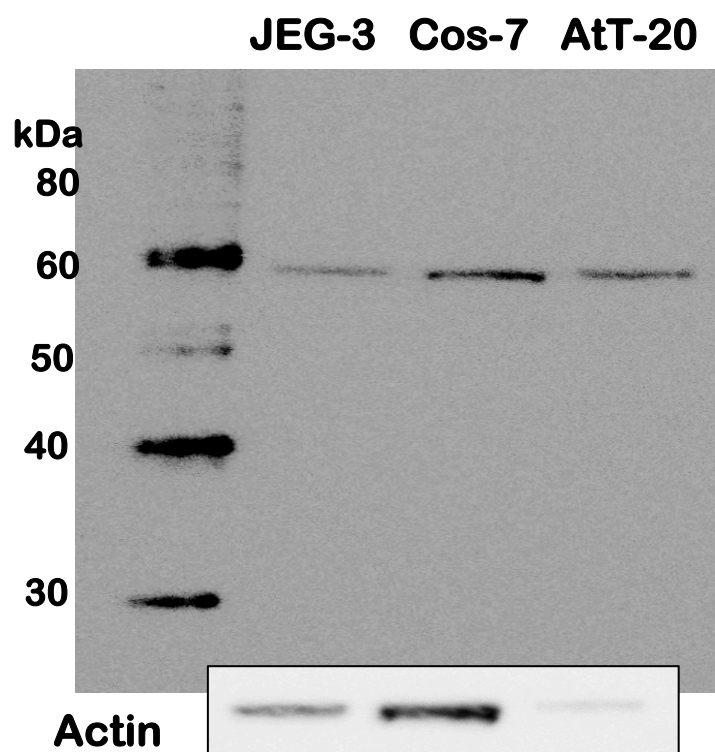


Figure 4.8. Total Protein Extracts from JEG-3, Cos-7 and AtT-20 Cells.

10 μ g of total protein extract was transferred to nitrocellulose and probed with anti-CREAP antibody. A band of approximately 58 kDa was detected in all cell lines. The blot was reprobed for actin as a loading control.

4.4.2 CREAP Localisation by Indirect Immunofluorescence Microscopy.

4.4.2.1 CREAP Nuclear Localisation in Different Cell Types

To further characterise the new CREAP polyclonal antibodies and to determine the sub-cellular localisation of CREAP, indirect immunofluorescence staining was conducted on cells grown on glass coverslips. The cells are fixed, permeabilised with Triton-X100 and then incubated with primary antibody and Alexa Fluor-labelled secondary antibodies with the nucleus counter-stained with DAPI, and examined using epifluorescence microscopy (see Section 2.13 for detailed methodology).

Both pre-immune and immune rabbit sera A were tested on JEG-3, Cos-7 and AtT-20 cells. Figures 4.9-4.11 show the immunofluorescence results for the three cell lines with CREAP fluorescence shown in green and DAPI in blue. The pre-immune serum didn't give any specific staining pattern except for some very faint, diffuse background staining, similar to the secondary antibody alone (data not shown). The reactivity shown by the CREAP antibody was specifically localised to the nucleus in all three cell lines tested. The reactivity appeared speckled, partially overlapping with chromatin staining (DAPI). However the nucleoli were devoid of CREAP fluorescence.

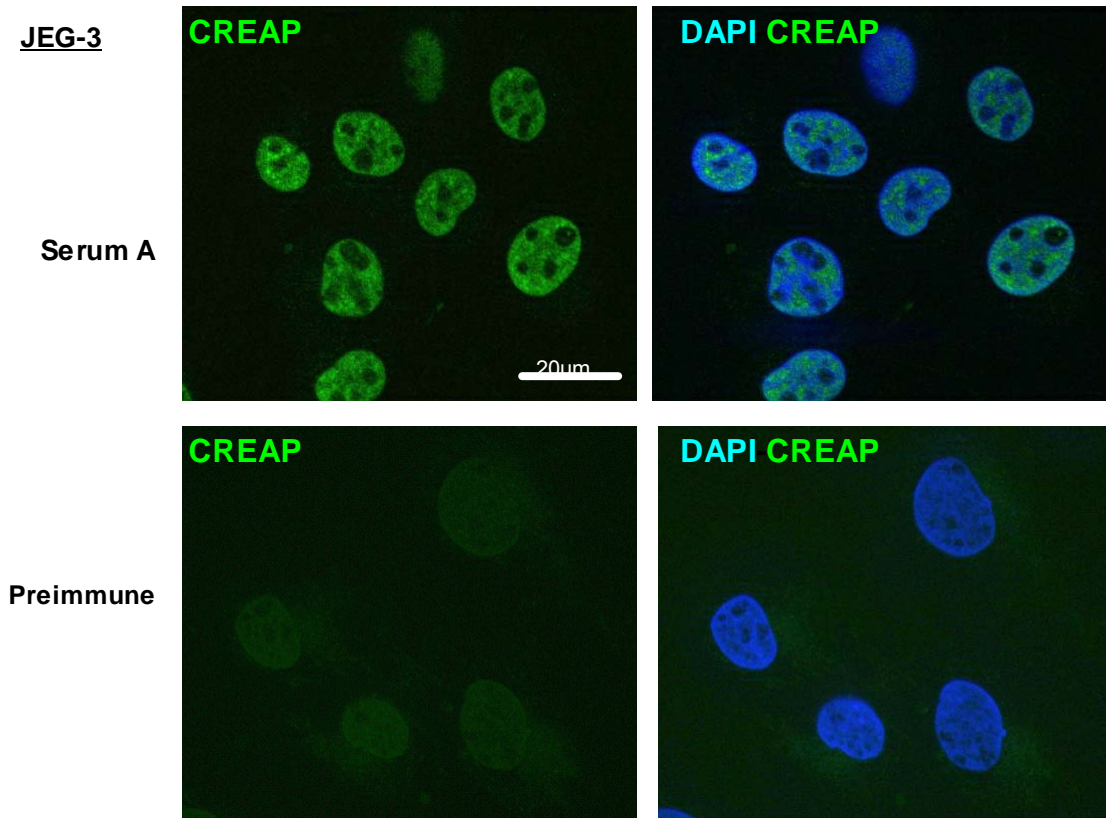


Figure 4.9. CREAP Localisation in JEG-3 Cells.

JEG-3 cells were probed with anti-CREAP serum or pre-immune serum and detected with Alexa Fluor-488 secondary antibody. The nuclei were stained with DAPI. CREAP shows nuclear localisation and a speckled intranuclear pattern, with no staining of the nucleolus. There is little non-specific immunostaining observed in the pre-immune samples.

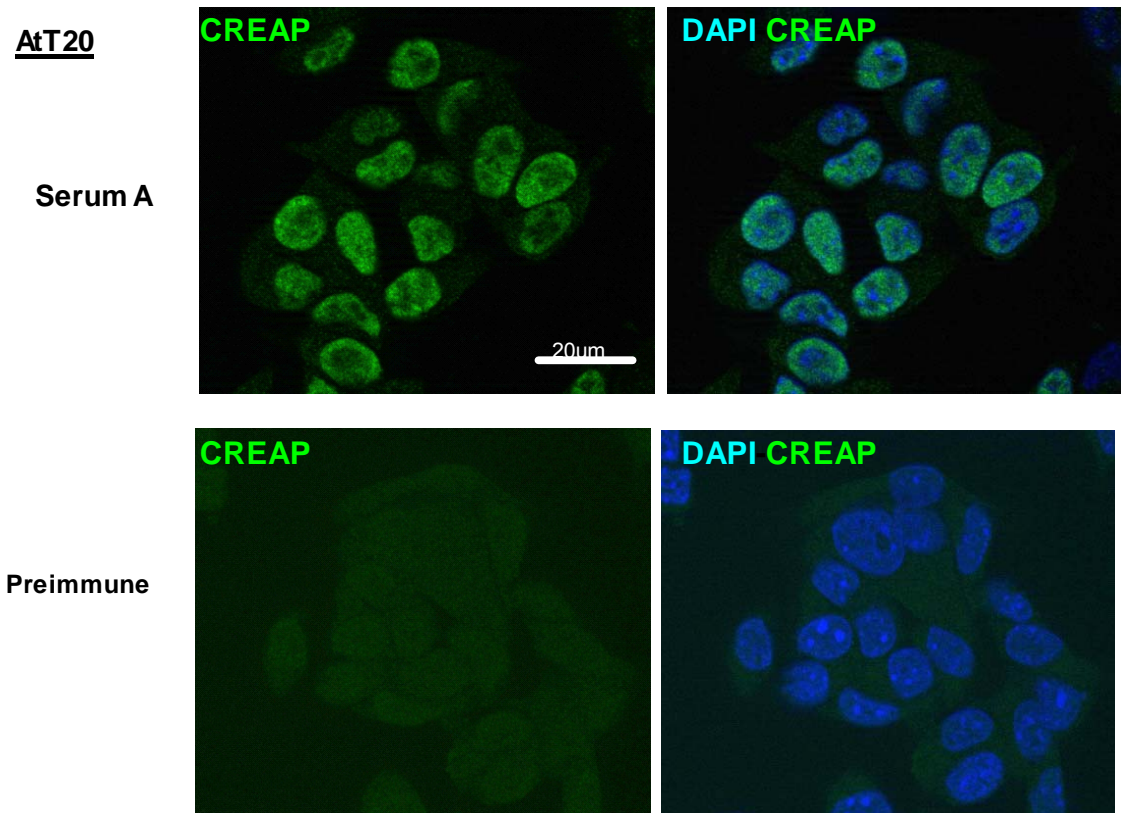


Figure 4.10. CREAP Localisation in AtT-20 Cells.

AtT-20 cells were probed with anti-CREAP serum or pre-immune serum and detected with Alexa Fluor-488 secondary antibody. The nuclei were stained with DAPI. CREAP shows a speckled intranuclear pattern, with no staining of the nucleolus. There is little non-specific immunostaining observed in the pre-immune samples.

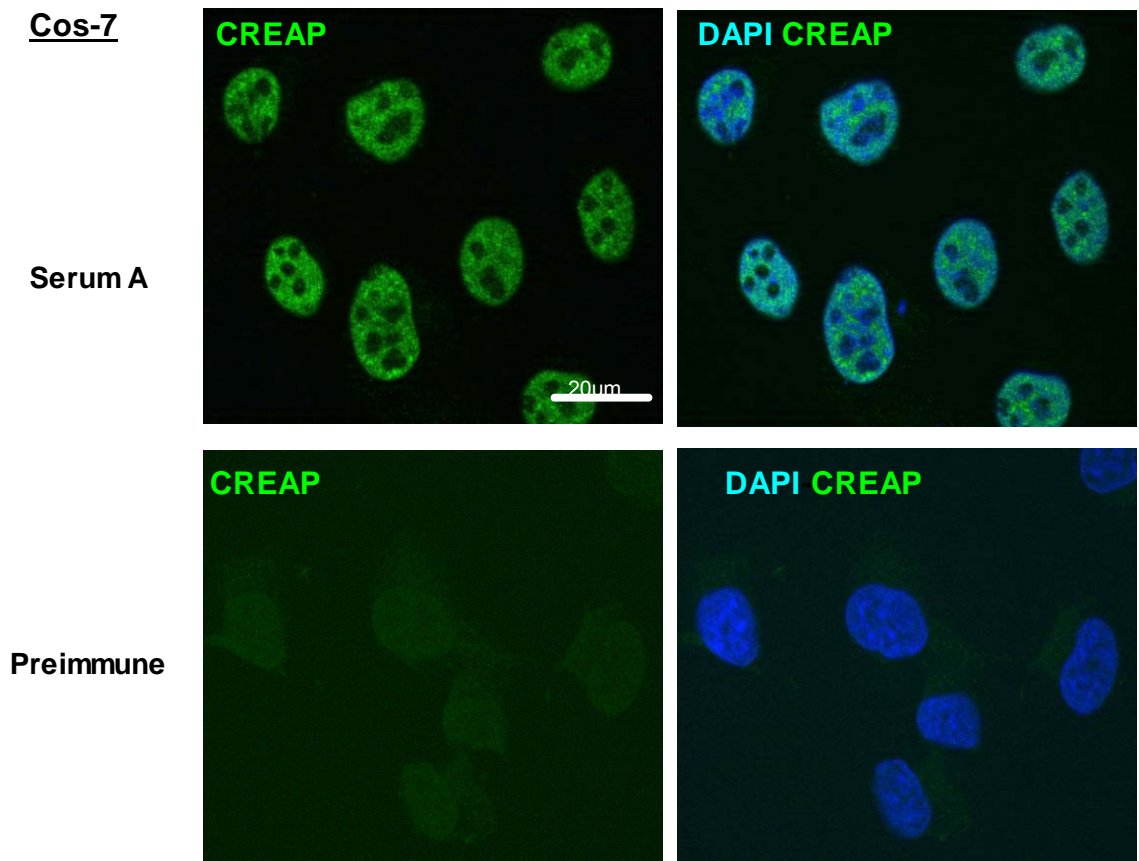


Figure 4.11. CREAP Localisation in Cos-7 Cells.

Cos-7 cells were probed with anti-CREAP serum or pre-immune serum and detected with Alexa Fluor-488 secondary antibody. The nuclei were stained with DAPI. CREAP shows a speckled intranuclear pattern, with no staining of the nucleolus. There is little non-specific immunostaining observed in the pre-immune samples.

Indirect immunofluorescence was used to determine the CREAP localisation in several other cell lines that were available. Human primary keratinocytes (K3), human melanoma (MV3), human acute monocytic leukaemia (ThP1) (Tsuchiya et al. 1982), and histiocytic lymphoma (U937) (Sundstrom and Nilsson 1976) cells were immunolabelled with CREAP. The CREAP localisation is shown in Figure 4.12. All cells showed the same nuclear and speckled intranuclear distribution for CREAP. The human breast adenocarcinoma cell line, MDA-MB-468 (Cailleau et al. 1978) was also western blotted and CREAP protein detected (data not shown). These results correlate with the earlier MTE RNA Array results which showed that CREAP was present in a variety of tissues and cell lines, and a nuclear localisation seems to exist for all cells.

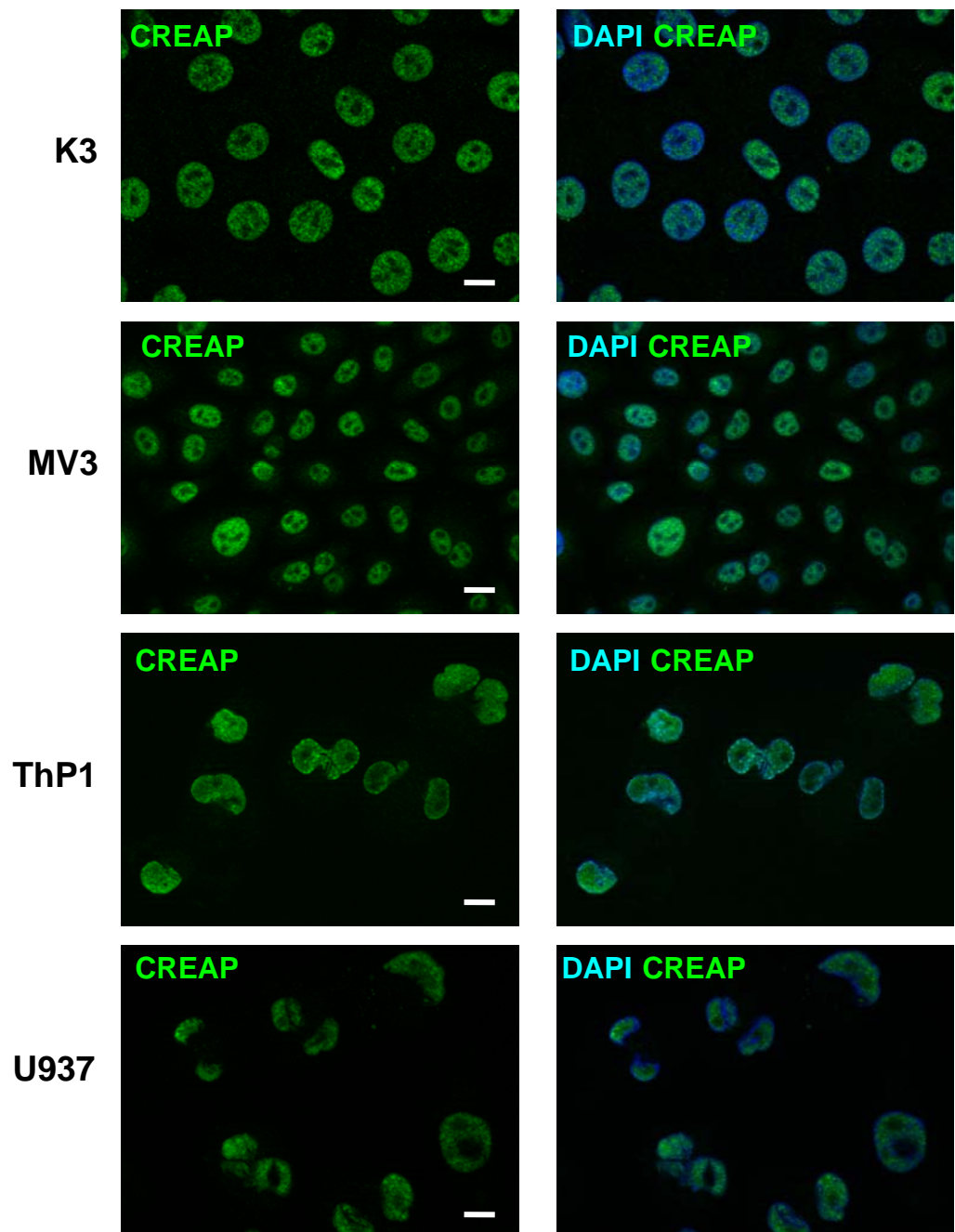


Figure 4.12. CREAP Immunofluorescent Staining of a Cell Panel.

CREAP immunofluorescent staining was conducted on four cell lines. K3 (human primary keratinocytes), MV3 (melanoma), ThP1 (monocytic leukemia) and U937 (histiocytic lymphoma). CREAP consistently gave a pattern of nuclear staining with intranuclear speckles in all cell lines (green staining). The merge image shows CREAP and blue DAPI nuclear staining. The white bar represents 20µm.

4.4.2.2 CREAP is Predominantly Localised in Nuclear Speckles.

Nuclear speckles are known to contain splicing proteins. To determine if CREAP was localising within the nuclear splicing speckles immunofluorescent cell staining was used with the splicing factor, SC35. Both polyclonal CREAP and monoclonal SC35 (Sigma) antibodies were used to co-stain the same cells using Alexa-488 and Alexa-594 mouse and rabbit secondary antibodies, respectively. In Figure 4.13, SC35 (green) was localised to distinct intranuclear speckles, with no staining of the nucleolus. CREAP (red) was also localised in the intranuclear speckles but also had a general nuclear staining excluding the nucleolus. Co-localisation of SC35 and CREAP is indicated by areas of yellow fluorescence in the merge images. It can be clearly seen that there is indeed co-localisation of SC35 and CREAP in the intranuclear speckles, suggesting that CREAP has a role in splicing. Figure 4.13 also shows the co-localisation staining in JEG-3 cells treated with and without 0.5 mM 8-Br-cAMP treatment for 24 hours. The SC35 and CREAP speckles appear more rounded and defined in the untreated cells than the cAMP-treated cells.

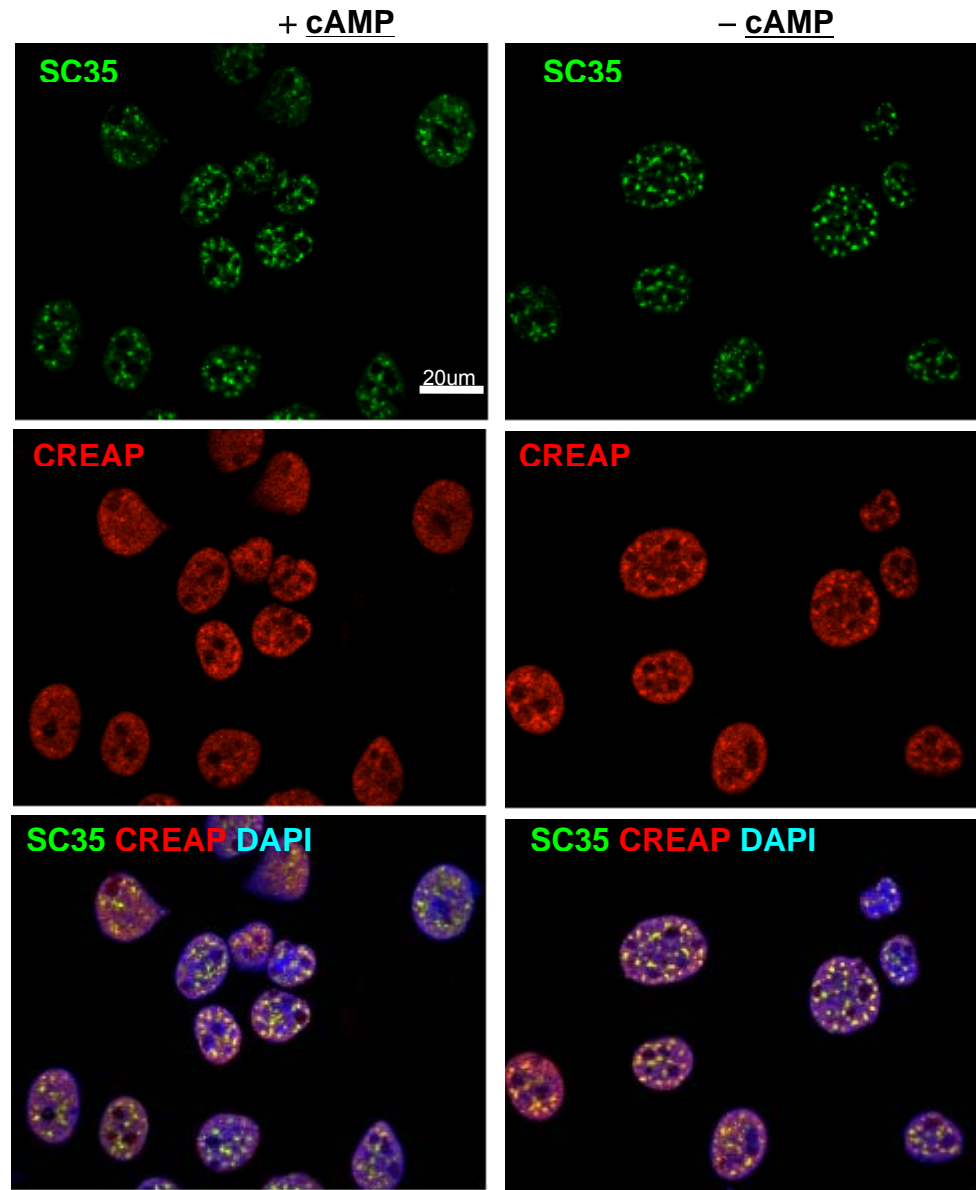


Figure 4.13. CREAP and SC35 Co-Localisation in JEG-3 Cells.

JEG-3 cells were grown on coverslips and treated with or without 0.5 mM 8-Br-cAMP. Immunofluorescent cell staining for SC35 (green) and CREAP (red) was conducted and co-localisation indicated by yellow fluorescence in the merged images. Nuclei were stained blue with DAPI. SC35 and CREAP were co-localised in intranuclear speckles which contain slicing proteins. The SC35 and CREAP speckles look more rounded and defined in non-treated cells than in cAMP-treated cells.

The effect of cAMP treatment on Cos-7 cells was also tested using immunofluorescence. Cos-7 cells were plated on coverslips and treated with 0.5 mM 8-Br-cAMP or left untreated. Cells then underwent indirect immunofluorescent staining with CREAP antibody. Figure 4.14 shows that in the untreated cells the CREAP speckles look more rounded and defined than cAMP-treated cells. Several mitotic cells were captured in these images as shown by the chromosome clustering highlighted in blue with DAPI-DNA fluorescent stain. During mitosis CREAP appears to redistribute to the cytoplasm as the nuclear organisation is lost.

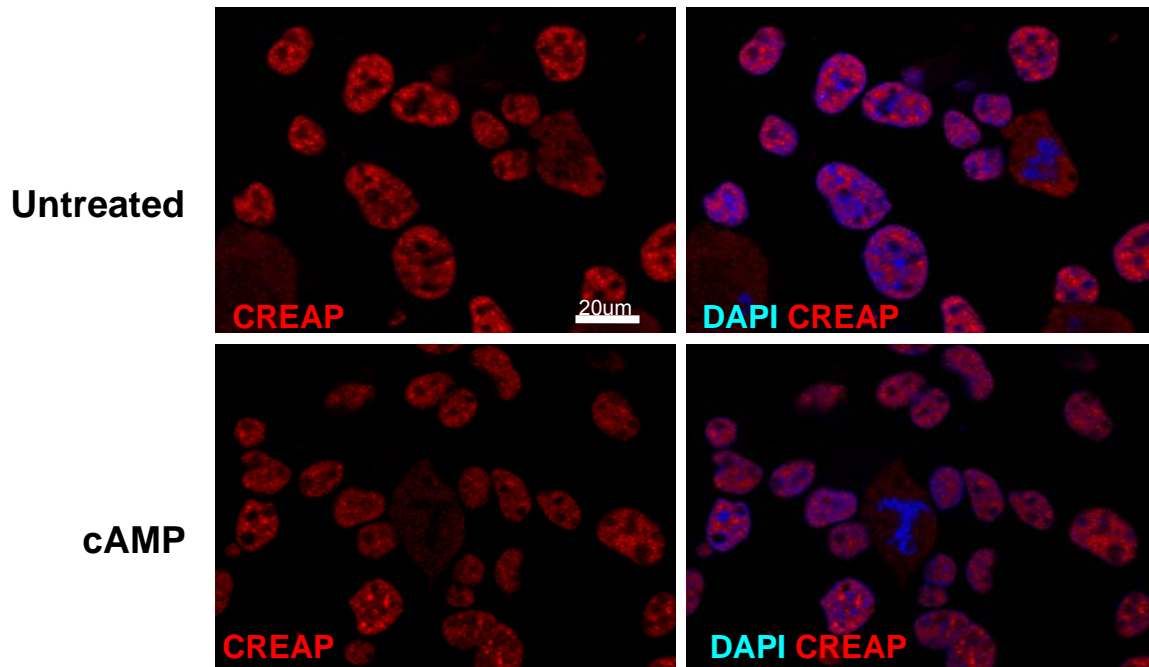


Figure 4.14. Localisation of CREAP After cAMP Treatment.

Cos-7 cells were plated on coverslips and treated for 24 h with 0.5 mM 8-Br-cAMP or left untreated. The cells were fixed and immunofluorescent staining for CREAP conducted. The nuclei were stained blue with DAPI. The CREAP Alexa-594 (red) results as well as the DAPI-CREAP merge are shown. In general the speckles in the untreated cells are more rounded and defined than the cAMP-treated cells.

4.5 Immunoprecipitation of CREAP with Splicing Complexes

4.5.1 Co-Immunoprecipitation of CREAP and SC35

In the previous section, CREAP was shown to physically co-localise within nuclear speckles with the splicing factor SC35. Nuclear speckles are known to represent sites of splicing factor storage and of gene splicing itself. To determine whether CREAP formed part of a splicing complex, co-immunoprecipitation (Co-IP) experiments were used. Nuclear extracts of JEG-3 cells were incubated with either CREAP or SC35 antibody. Any proteins that are bound to the antibodies are captured using Protein A/G agarose, which combines an IgG binding protein with a solid agarose support. In this way, proteins and protein complexes that can be bound by the antibodies can be separated away from the other proteins in the extract. The captured proteins can then be released from the antibody/protein A/G using heat and denaturing sample buffer. The sample can then be electrophoresed and western blotted. The results of a Co-IP experiment are shown in Figure 4.15, proteins captured by CREAP or SC35 antibodies were western blotted and probed using CREAP antisera.

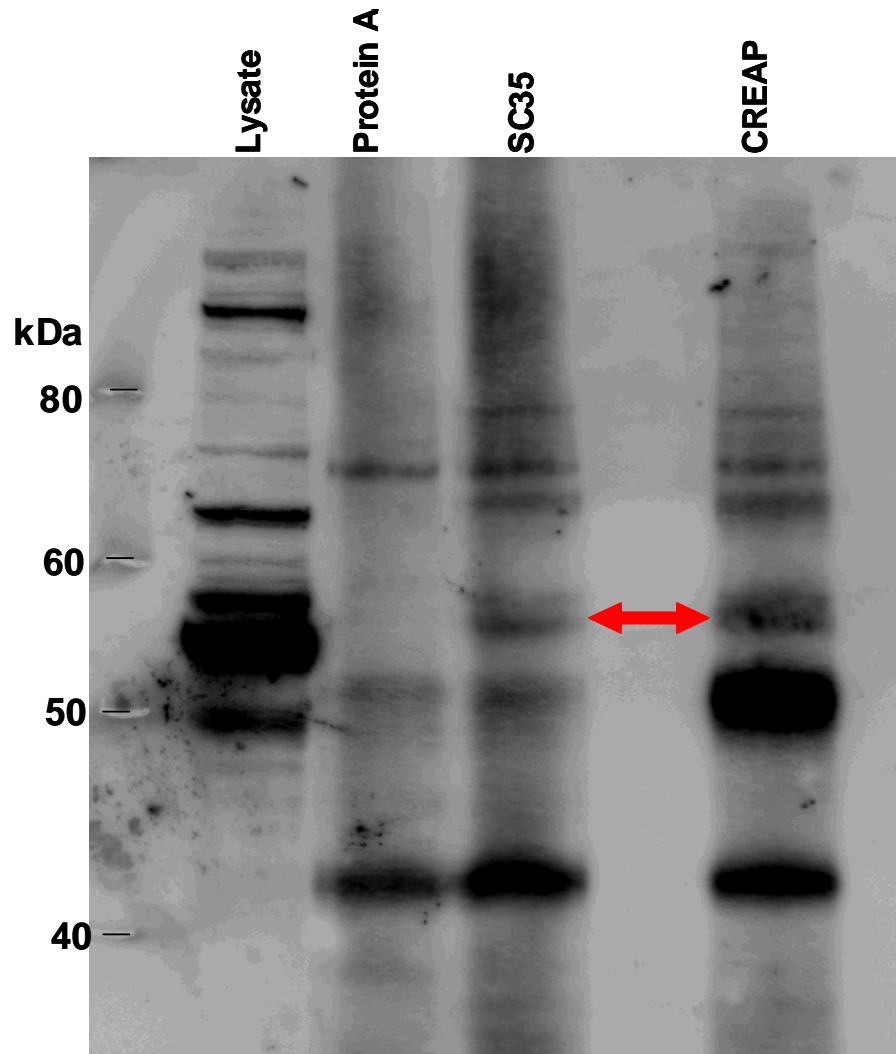


Figure 4.15. CREAP and SC35 Co-Immunoprecipitation of JEG-3 Nuclear Protein.

CREAP serum A or SC35 monoclonal antibody and protein A agarose or protein A agarose alone were incubated with JEG-3 nuclear protein. The beads were washed and the captured proteins eluted with LDS sample buffer to form the IP samples and western blotted. The membrane was blotted with 1:5000 CREAP antisera. As can be seen CREAP protein was detected in the lysate, the control CREAP IP and the SC35 IP sample (red arrow), but not the negative control Protein A/G sample. This indicates that CREAP and SC35 are physically interacting within the cell, probably in the form of a gene splicing complex.

As can be seen, the CREAP Co-IP sample blotted with CREAP antibody serves as a positive control, in that CREAP antibody should bind CREAP protein and be detected when western blotted. The SC35 Co-IP sample also has an immunoreactive band at the same molecular weight as the CREAP Co-IP and JEG-3 nuclear lysate. This indicates that CREAP and SC35 physically interact with each other, probably in the form of a splicing complex and the complex is captured by SC35 antibody.

4.6 Discussion

4.6.1 GST Fusion Protein and Antibody Production

Polyclonal antibodies often have the advantage of better specificity as they are produced by a number of B cell clones each with a different epitope so that the serum is a combination of antibodies against the antigen of interest (Lipman et al. 2005). Their reactivity is more tolerant when small changes occur in antigens such as polymorphisms, glycosylation, denaturation or changes that occur from processes such as fixation. They are also more stable over a broader pH range and salt concentrations (Lipman et al. 2005). Therefore when working with an uncharacterised protein such as CREAP, a polyclonal antibody is preferable as it will be more likely to detect all isoforms of the protein and would be more amenable to different experimental conditions. Moreover, the resources to make monoclonal antibodies were not available.

The GST-tagged fusion protein provided a convenient way to produce and purify CREAP recombinant protein. A truncated version of CREAP was used to produce the GST fusion protein as the cDNA was available from the previous yeast work and

contained the leucine zipper-like domains, which are important for CRE binding. We were initially interested in CREAP as a CRE-binding transcription factor and gene regulation through the CRE. This region is also common to all the splice variants meaning that the antibodies should be able to detect all the splice variants that may exist. The placental cDNA library screen revealed at least two possible splice variants of CREAP, a full-length and truncated version. The truncated version is very similar in structure to the yeast homologue, Luc7p. However, the detection of a truncated CREAP protein with western blotting was variable and it would appear the full-length CREAP is more likely to be the major form as it is consistently detected in many cell types.

4.6.2 Nuclear Localisation of CREAP

4.6.2.1 CREAP is Detected in Nuclear and Whole Cell Protein Extracts

CREAP has a nuclear localisation. Proteins extracted from the cytoplasmic fraction do not contain CREAP in any abundance, but the nuclear fraction and total cellular proteins do contain significant amounts of CREAP protein according to western blotting. This was to be expected as transcription factors and splicing factors tend to localise to the nucleus as this is the place where transcription processes occur. An approximately 58 kDa protein was detected in nuclear and total protein extracts from primary placental cells, JEG-3, Cos-7 and AtT-20. This protein represents the full length CREAP. The predicted size of CREAP was 51 kDa but the larger size detected by western blotting could be due to post translational modifications such as phosphorylation and glycosylation, and to slight inaccuracies in size prediction using pre-stained markers. In this study, CREAP expression was detected in the nucleus of

cells from placenta, JEG-3, AtT-20, Cos-7, MV3, keratinocytes, ThP1 and U937 cells. Coupled with the previous results from a MTE Array (Chapter 1), where CREAP was expressed in a large variety of adult and fetal tissues, CREAP expression appears to be quite ubiquitous.

Two truncated proteins were detected in placental and AtT-20 cells which most likely represent a truncated splice variant which is missing part of the C-terminal domain. This splice variant was discovered in a placental cDNA library using a yeast one hybrid assay with the CRE as bait. The truncated protein seems to be expressed at much lower levels as it is not usually detectable in most western blots of cell lysates. Puig and colleagues used computer analysis to detect three alternative splice site variants, of 109, 452 and 462 amino acids in length, however, none of these are the correct size for CREAP-SV (Puig et al. 2007). They also detected a shorter protein variant in some cell lines, though the size is not described, it is likely that this protein variant corresponds to CREAP-SV. Nishii and colleagues also detected a shorter mRNA in their Northern Blot analysis of CROP. The size was not described but it could also be representing a truncated splice variant such as CREAP-SV (Nishii et al. 2000). Overall, it seems very likely that CREAP does have alternative splicing to produce a truncated protein.

Alternative splicing of CRE-binding bZIP proteins is common. CREB is spliced to produce two isoforms, CREB341 and CREB327 (Yamamoto et al. 1990). The CREM gene produces a large family of alternatively spliced transcripts, some which are stimulatory and others that are repressors (Foulkes et al. 1992; Foulkes and Sassone-Corsi 1992; Laoide et al. 1993).

4.6.2.2 Nuclear Localisation Signals Dictate the Sub-Nuclear Distribution of CREAP

CREAP was detected diffusely throughout the nucleus and also within intranuclear speckles. The basic domain of most bZIP proteins is involved in nuclear localisation. The nuclear localisation signal (NLS) of several bZIP proteins has been found to be located in the basic region (Waeber and Habener 1991; Chida and Vogt 1992; Hurst 1994). In Chapter 3, the PredictNLS program (cubic.bioc.columbia.edu/predictNLS/), was used to screen CREAP for NLS's and several possible signals were found (Cokol et al. 2000). They were found in the C-terminal domain within the RS and RE rich regions with lysine (K) being an important residue.

To determine the domains of CROP responsible for nuclear localisation, deletion constructs were used (Umehara et al. 2003). They found that the RE and RS domains of CROP were necessary for speckled distribution, while N-terminal deletions had no effect on speckles. Deletion of the C-terminal 187 amino acids (RE, RS and SKE rich regions) of CROP resulted in a loss of both speckled and nuclear localisation. Therefore the CROP deletion construct results and the CREAP PredictNLS analysis suggest that the nuclear and intranuclear speckles localisation signals reside in the RS and RE domains of CREAP.

Mostly it has been found that the RS region is responsible for the nuclear localisation and targeting of SR proteins to speckles (Cazalla et al. 2002). It is thought that the alternating positive and negative charge in the RS domain is important for nuclear localisation as alternative arginine and aspartic or glutamic acid can also direct proper

subcellular localisation (Cazalla et al. 2002). The RS domain can confer nuclear targeting to a NPc protein reporter, showing that the RS domain can function as a nuclear targeting signal (Caceres et al. 1997). CREAP has both an RS domain and a RE domain at the C terminal and both appear to be directing the protein to nuclear speckles.

4.6.3 Speckles and Splicing

The pre-mRNA splicing machinery is localised to intranuclear speckles, also known as 'SC35 domains' (Wansink et al. 1993) or splicing factor compartments (Phair and Misteli 2000). They contain small nuclear ribonucleoprotein particles (snRNPs), spliceosome subunits and other non-snRNPs splicing factors such as SR proteins. The speckles correspond to interchromatin granule clusters (IGC's) that are seen by electron microscopy (Lamond and Spector 2003). Perichromatin fibrils also contain splicing proteins and are fibres that are visible by electron microscopy and can occur on the periphery of IGC's and also in the nucleoplasm (Fakan 1994).

The majority of pre-mRNA splicing factors are enriched in speckles and not at the sites of active transcription (Zimber et al. 2004). Nuclear speckles are thought to function in the storage and assembly of spliceosomal factors (Lamond and Spector 2003) while perichromatin fibrils are the sites of transcription and pre-mRNA processing as they are rapidly labelled with [³H]uridine, suggesting that they represent nascent RNA transcripts (Fakan 1994; Cmarko et al. 1999). Pre-mRNA splicing factors are dynamic and are rapidly recruited from the speckles to sites of transcription after gene activation (Misteli et al. 1997). It has been shown that splicing factors are on average 5-10-fold more enriched in speckles than in the nucleoplasm (Wei et al. 1999).

4.6.3.1 Components of Speckles

Mintz et al. (1999) attempted to identify the proteins located in IGC's using mass spectrometry and protein microsequencing. They found approximately 75 proteins and were able to identify 33 known proteins, many that have been shown to be involved in pre-mRNA splicing, as well as numerous unknown proteins (Mintz et al. 1999). Therefore it is possible that they may have identified CREAP, but as the sequences had not been lodged in any databases at that time it remained unidentified. That study was updated in 2004 (Saitoh et al. 2004). This time they identified 360 proteins from IGC's- 146 known IGC proteins, 70 potential IGC proteins, 112 unexpected proteins and 32 proteins for which only sequence data is available. They detected proteins with RS motifs. 81% of the identified speckle proteins were involved in RNA processing and 3% were involved with transcription. They don't appear to have detected the CROP sequence which is identical to CREAP and was present in the databases at this time, though they did fail to detect some proteins that have been reported to localise to speckles, such as protein phosphatase I. This could be due to many factors such as protein dissociation from speckles during the purification process, phosphorylation state or the number of trypsin cleavage sites (Saitoh et al. 2004). They were also looking at IGC's from mouse liver nuclei. We have previously cloned the mouse CREAP homologue and have shown that it is lacking the SR region (unpublished data) so may not be strongly localised to speckles in mouse liver. However, we detected CREAP in speckles in the mouse pituitary cell line AtT-20 (Figure 4.10) although the speckles are not as prominent in these cells as the other human or primate derived lines, suggesting

that there may be a difference in distribution of CREAP in mouse versus human cells, and possibly between different mouse tissues.

4.6.4 SC35

CREAP and SC35 co-localised to intra-nuclear splicing speckles. This co-localisation was likely to be genuine as the alignment ability of the microscope filter sets had been calibrated and the immunofluorescence results were also confirmed by co-immunoprecipitation of CREAP and SC35, indicating that is probable that they physically interact.

4.6.4.1 SC35

The mammalian splicing factor SC35 is required for the first step in the splicing reaction and for spliceosome assembly (Fu and Maniatis 1992b). SC35 is an SR protein that consists of a single RRM and an RS rich region, and localises to nuclear speckles. It has been shown that U1 snRNP interacts specifically with both the 5' and 3' splice sites in the presence of ATP and that SC35 is required for these ATP-dependent interactions. Significantly, the SC35-dependent interaction between U1 snRNP and the 3' splice site requires U2 snRNP but not the 5' splice site. SC35 is also required for the ATP-dependent interaction between U2 snRNP and the branch-point sequence during splicing (Fu and Maniatis 1992a).

4.6.4.2 *SC35 and Speckles*

SC35 is a non-snRNP, SR protein and a classical marker of nuclear speckles. When HeLa cells are treated with the RNA polymerase II transcription inhibitor, α -amanitin, SC35 immunolabelled speckles round up into several large clusters while in actively transcribing cells the speckles are more irregular and may have connections between speckles (see Figure 4.16) (Spector et al. 1993; Mintz and Spector 2000). This supports the theory that speckles are acting as storage sites for splicing factors. The SC35 labelling in JEG-3 cells shows a similar pattern when treated with and without 8-Br-cAMP. The speckles in untreated cells have a rounder more defined appearance and in the cAMP-treated cells the speckles are more irregular. This fits with the α -amanitin results as 8-Br-cAMP stimulates the PKA pathway leading to increased transcription. A similar pattern is observed in the CREAP labelling of speckles though it is not as pronounced as SC35. The CREAP expression in cAMP-stimulated cells is more irregular than non-treated cells. It has been shown that speckle proteins become diffusely distributed throughout the cytoplasm during mitosis (Thiry 1995). Since, CREAP has a diffuse cytoplasmic distribution during mitosis, this again supports its designation as splicing related protein.

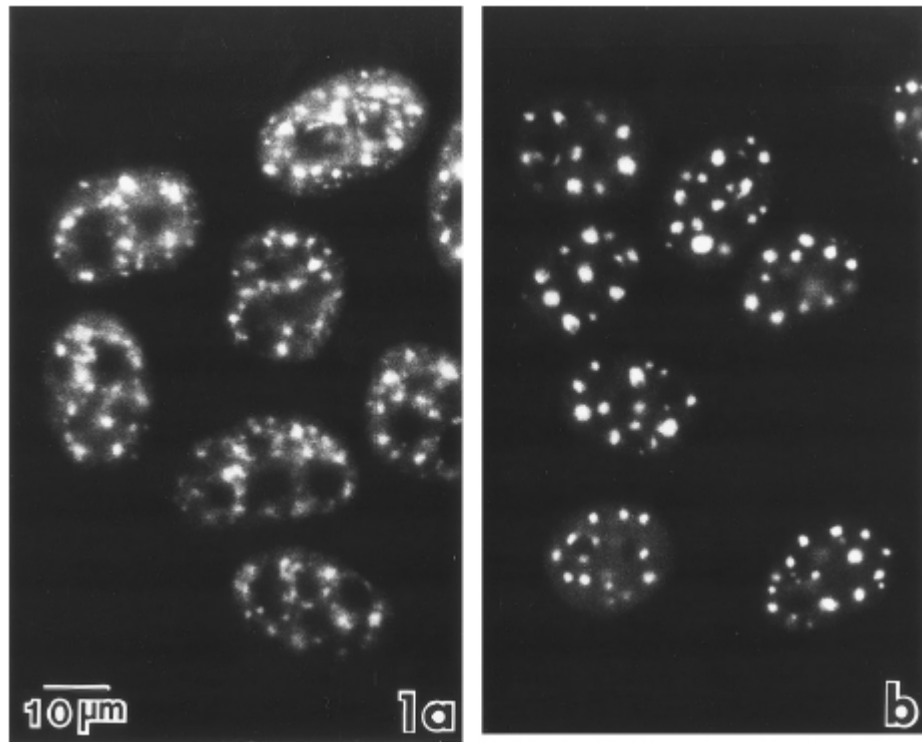


Figure 4.16. SC35 Localisation in HeLa Cells.

In actively transcribing HeLa cells, the splicing factor SC35 localizes in a speckled distribution pattern. The speckles are irregular in shape and connections are observed between some of the speckles (a). Upon inhibition of RNA polymerase II by α -amanitin (50 mg/ml, 6 h), the speckles become uniform in shape and increase in fluorescence intensity and connections between speckles are no longer observed (b). Reproduced from Spector, O'Keefe et al. 1993 (Spector et al. 1993).

4.6.4.3 SC35 Interactions

In vitro, SC35 has been found to interact with itself, the general splicing factor SF2/ASF, the splicing regulators Tra and Tra 2, and weakly with hnRNP protein A1 but it had no interaction with the basic-leucine zipper region of transcription factor C/EBP (Wu and Maniatis 1993). It also interacts with several unidentified proteins of various sizes from HeLa cell extracts. SC35 has also been shown to co-precipitate with the U1-70K component of U1 snRNP, which strengthens its role in mediating U1 and U2 snRNP interactions at the 5' and 3' splice sites (Fu and Maniatis 1992a; Wu and Maniatis 1993), and to interact with U2AF³⁵ in a yeast based assay (Wu and Maniatis 1993). All of the proteins shown to interact with SC35 have an RS domain. It could be thought that the RS domain mediates non-specific protein interactions between SR proteins, but the same study found no interaction between U1-70K and Tra and Tra2 (Wu and Maniatis 1993).

CREAP and SC35 co-localise in nuclear speckles and appear to physically interact as shown by co-immunoprecipitation experiments. As CREAP has a SR-like region and SC35 is a SR protein, it is likely that they may interact through the RS domains. Other SR protein interactions have been detected with the CREAP identical proteins CROP and hLuc7A. CROP was found to interact with the SR protein, SF2/ASF and with itself and the RE and RS domains are necessary for CROP-CROP protein interactions (Umehara et al. 2003). In another study, U1 snRNA was detected in precipitates from antibodies to hLuc7A and the U1 snRNP protein, U1-A. (Puig et al. 2007). This shows that Luc7A is able to associate with human U1 snRNP even though previous work has not been able to co-purify it (Luhrmann et al. 1990).

There are at least 300 proteins associated with splicing, so it is possible that CREAP is just another one that is part of the process. There seems to be definite evidence that CREAP/CROP/hLuc7A is capable of interacting with other SR or SR-like proteins involved in splicing, specifically SC35, SF2/ASF and U1 snRNP. SC35 and SF2/ASF and U1-70K and SF2/ASF are known to interact (Wu and Maniatis 1993; Cao and Garcia-Blanco 1998), suggesting an intricate protein-protein relationship and possible splicing factor complex between CREAP and these splicing proteins, that is most likely mediated through their RS domains.

4.6.5 Summary

The anti-CREAP polyclonal antibody is able to detect both the full length and truncated splice variants of CREAP using Western Blotting. CREAP is only present in nuclear or total cell protein lysates. The nuclear localisation of CREAP is further divided into nucleoplasmic and an intranuclear speckled distribution. CREAP has been shown to co-localise and co-immunoprecipitate with the non-snRNP, SC35, suggesting that CREAP does have a role in splicing. However, transcription factors have also been found in speckles as well as the nucleoplasm, signifying that CREAP may be acting in a multifunctional manner as both a transcription factor and as part of a splicing factor complex.

Chapter 5. CREAP Regulation of CRH/CRE Gene Promoters?

5.1 Does CREAP Regulate CRH or CRE-containing Reporters.

5.1.1 Rationale

CREAP was initially identified by its ability to bind to a CRE-bait in a yeast one-hybrid screen of a placental cDNA library. CREAP contains domains which implicate it in transcription and splicing. From Chapter 4 it is known that CREAP localises to the nucleoplasm and to splicing factors and interacts with the splicing factor SC35. To investigate whether CREAP has transcriptional activity and is capable of regulating CRE-containing promoters, especially the CRH promoter, co-transfection assays of CREAP and CRE-containing or CRH promoter reporters were conducted.

Transient transfection is a useful tool to study promoter regulation. Gene promoters fused to a reporter can be transfected into cells and regulatory mechanisms investigated. In this Chapter, cells were transiently transfected with sections of the CRH promoter and CRE containing promoters with a luciferase reporter along with CREAP expression plasmids to determine if CREAP regulates these promoters. Up- or down-regulation is detected by an increase or decrease in luciferase reporter activity. Two transfection protocols were utilised in this study, a calcium phosphate and liposome-based method, and these were combined with a Dual Luciferase Assay.

5.1.2 Dual Luciferase Assay

Genetic reporter systems are widely used to study gene expression. The term “dual reporter” refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. Dual reporters are used to improve experimental accuracy by providing an internal control that serves to normalise the activity of the experimental reporter to transfection efficiency and cell viability, lysis and assay efficiency. In the Dual Luciferase Reporter (DLR) Assay the activities of firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*, also called sea pansy) luciferases are measured sequentially from a single sample. Both reporters generate a stabilised luminescent signal and yield linear assays (Promega 2006).

5.1.2.1 Experimental Design and Analysis

Transfections were conducted in triplicate, in three independent experiments and the data pooled. The effect of the treatments and co-transfections were compared to the luciferase-reporter alone controls, or to CREB-mediated stimulation in some cases. The statistical significance of the upregulation or inhibition was assessed using the Students, two-tailed, unpaired t-test with a p value <0.05 considered significant.

5.2 Primary Placental Cell CRH Promoter Assays

5.2.1 Placental CRH-663 Luciferase Assays

The calcium phosphate method of transfecting primary placental cells was optimised by Dr B.R King during his doctorate at the MBRC. Since primary placental cells are not mitotically active they can be difficult to transfect and cannot be stably transfected. The

CRH-663 luciferase reporter consists of 663bp of the CRH promoter linked to the pGL3 firefly luciferase expression vector. This region of the promoter contains several important regulatory elements such as the CRE, 1/2GRE, CDXA, and EcRE (see Figure 5.1A). Previous work by Y.H Cheng and B.R King in our laboratory has shown that this region is responsive to both glucocorticoid and cAMP stimulation and that the CRE is necessary for both (Cheng et al. 2000b; Cheng et al. 2000a). Y.H Cheng has previously shown that 0.5 mM 8-Br-cAMP gave a 5-fold induction in CRH-663 promoter activity in primary placental cells.

5.2.2 Analysis of CREAP Activity in CRH-663 Assay

Primary placental cells were isolated and grown in culture for 24 h before transfection. 30µg of CRH-663 reporter was co-transfected with increasing amounts of CREB (as a positive control for CRH and CRE promoter induction), CREAPlv, CREAPsv or PCI neo vector as a negative control. Cells were treated with vehicle or 0.5 mM 8-Br-cAMP for 24 h before assay. 10 µg of Renilla (pRLTKV) luciferase plasmid was used as an internal control to which the experimental, firefly luciferase activity is normalised to give a relative luciferase unit of activity. As can be seen in Figure 5.1B, CREB co-transfection caused a significant 220-340% induction (basal levels given a value of 100%) over basal promoter activity in vehicle-treated placental cells and a 200-250% increase over cAMP basal levels. Treatment with cAMP caused a 280% induction over vehicle basal levels. These results are consistent with the CREB induction results obtained previously in this laboratory.

In contrast to CREB, transfection with either CREAP variant resulted in only basal levels of activity. CREAP_{lv} had no significant effect on promoter activity except a 37% reduction at the 5 µg concentration. There was no significant effect of CREAP_{sv}. The cAMP-treated CREAP_{lv} transfected cells also showed a significant, up to 40% inhibition of cAMP-treated basal levels at 2 and 5 µg concentrations while CREAP_{sv} had no effect. Therefore there was no stimulation of CRH-663 promoter activity by either CREAP isoform in placental cells and in some instances inhibition occurred. However, the PCI neo vector also caused inhibition of CRH-663 promoter activity and this could be responsible for the apparent inhibition by CREAP.

The PCI neo vector is the DNA vector backbone that the CREAP sequences were cloned into. It was tested in the luciferase assay to determine if the vector alone has any effect on promoter activity. In vehicle-treated cells, PCI neo gave a significant 10-50% inhibition of CRH-663 promoter activity. The highest inhibition was at 0.5 and 1 µg of vector. However at these concentrations CREAP_{lv} and CREAP_{sv} had the same promoter activity as basal levels. Treatment with cAMP resulted in greater significant inhibition by PCI neo. There was a 60-75% reduction in promoter activity which didn't seem concentration-dependent. Taken together, these results suggested some inhibitory effect of the vector alone, especially in cAMP-stimulated cells. It also suggests that the weak inhibitory actions of CREAP are probably attributable to the PCI vector backbone used.

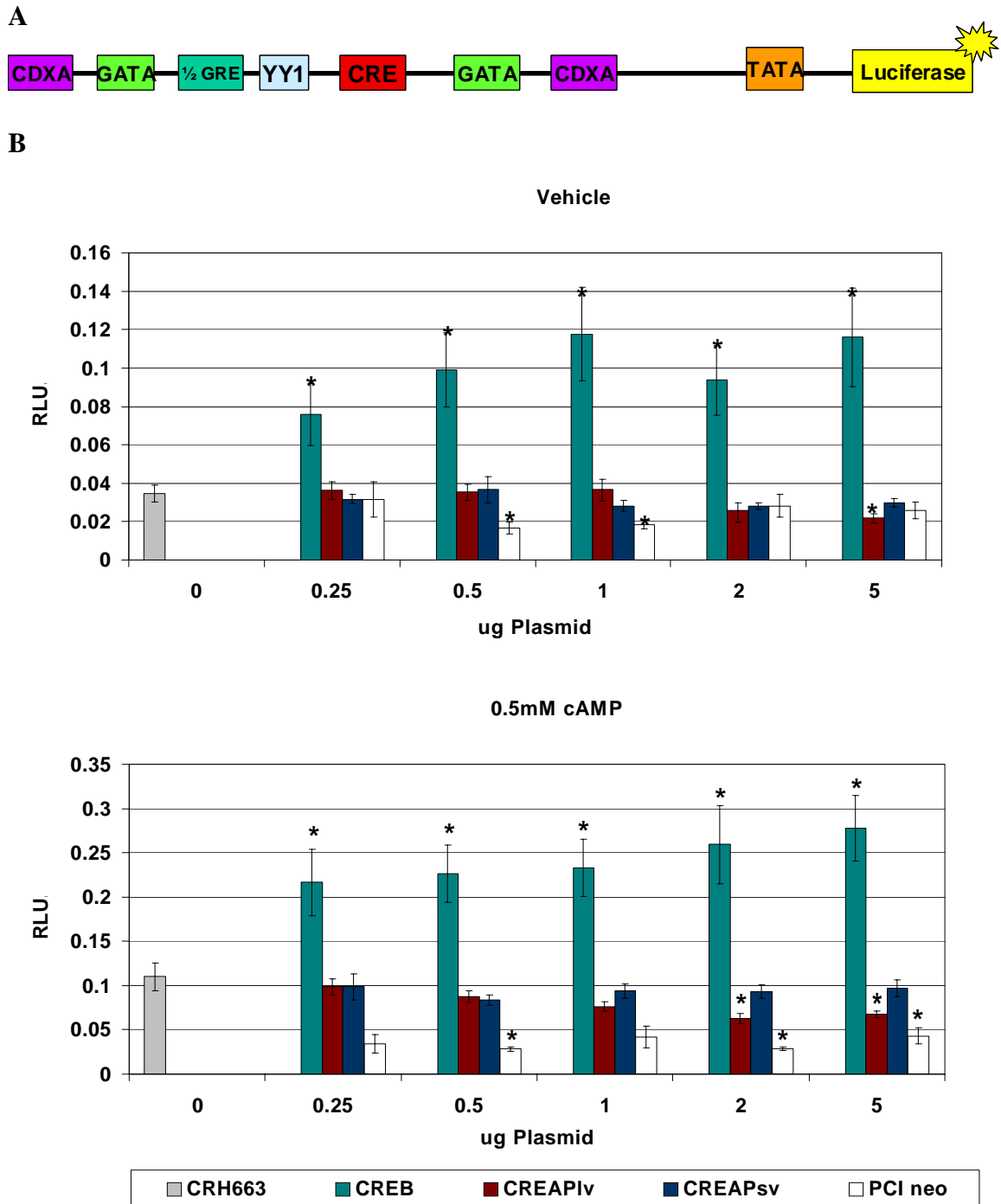


Figure 5.1. Placental CRH-663 Luciferase Assay.

A. Schematic of the CRH663 vector. B. Primary placental cells were transfected with 30ug CRH-663 luciferase reporter and increasing amounts of CREB, CREAP or PCI-neo expression plasmids using the calcium phosphate method. Cells were treated with vehicle or 0.5 mM 8-Br-cAMP for 24 h. Promoter activity is represented as relative luciferase units (RLU). Error bars are the standard error of the mean (SEM), n=3. (*) indicates significance (p<0.05) compared to the CRH663 control.

5.2.3 CREAP Does Not Synergise with CREB in Placental Cells.

Previously, CREAP was not observed to affect CRH-663 promoter activity so it was investigated if CREAP has any effect on CREB-mediated stimulation. To determine if CREAP activity could add to, synergise with or inhibit CREB mediated stimulation of CRH, 2 µg of both CREB and either CREAP or PCI neo were co-transfected with CRH-663. Figure 5.2 shows that in vehicle-treated cells, CREB alone transfection caused a significant 324% increase in CRH-663 promoter activity over basal levels, while CREAP_{lv}, -sv and PCI alone had no effect. When CREAP_{lv}, -sv and PCI were co-transfected with CREB, there was no significant difference in luciferase activity compared to CREB alone.

In cAMP-treated cells, CREB caused a 350% induction over cAMP-treated basal levels. When CREAP_{lv}, -sv or PCI was co-transfected with CREB there was a 26%-55% decrease in promoter activity compared to CREB alone, but this decrease was not statistically significant. Co-transfection with PCI neo resulted in a 55% reduction in CREB stimulated activity, and there was a significant reduction in activity in cAMP-treated PCI alone transfections suggesting that this vector definitely has inhibitory effects in cAMP-treated placental cells, indicating a possible interaction with PCI vector containing elements and the cAMP/PKA pathway in placental cells. Despite this complication, it is clear that CREB and CREAP do not act synergistically to regulate CRH-663 promoter activity.

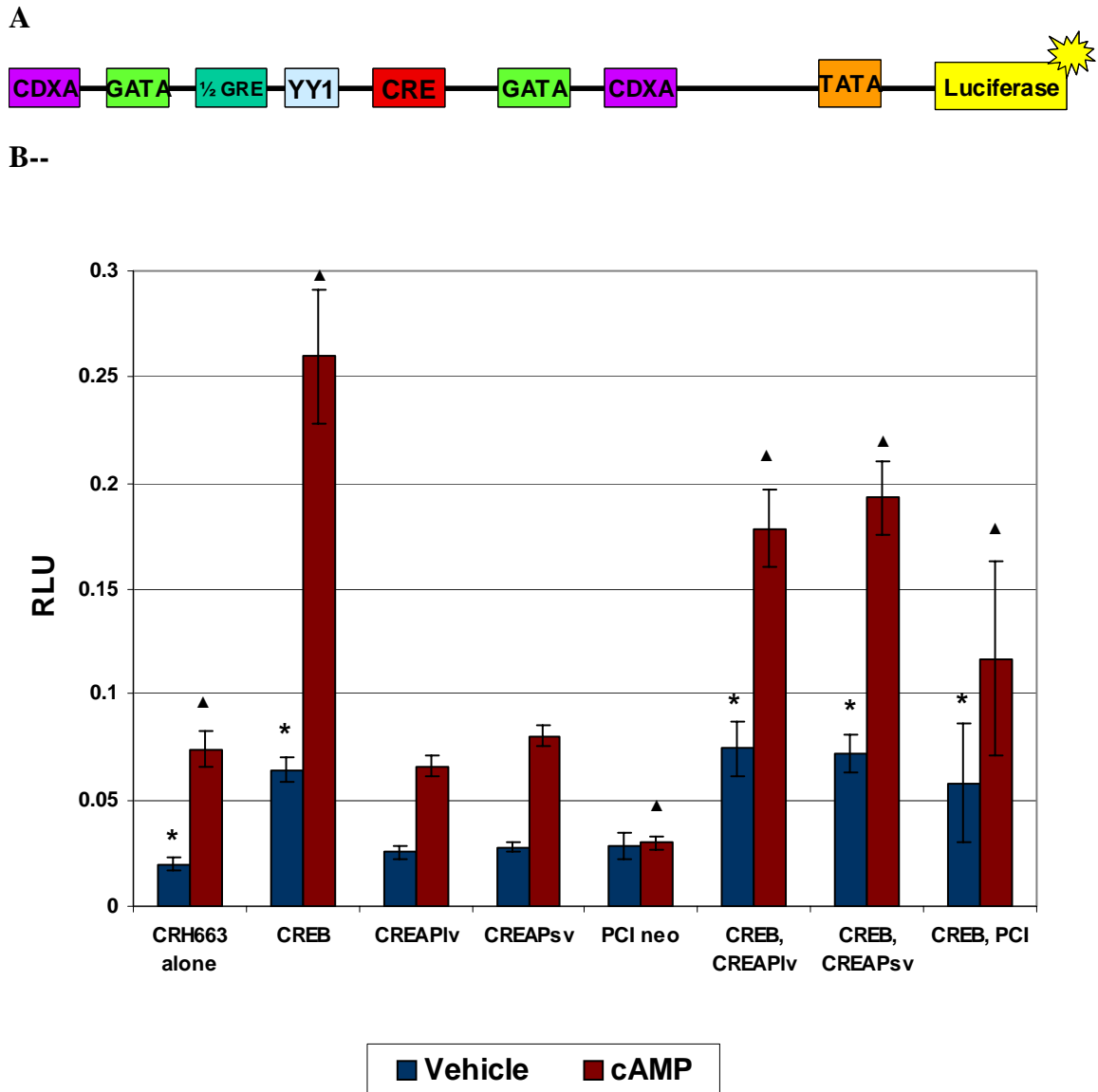


Figure 5.2. Placental CRH-663 Luciferase Assay with CREB and CREAP Co-expression.

A. Schematic of the CRH-663 vector. B. Primary placental cells were transfected with 30ug CRH-663 and 2ug each of CREB, CREAP or PCI plasmids. Cells were treated with vehicle or 0.5 mM cAMP for 24 h before analysis. CREB and CREAP were co-expressed in the same cells to see if there is a synergistic or antagonistic effect between them. Promoter activity was measured using the -relative luciferase units (RLU). Error bars represent the SEM, n=3. Asterisks (*) indicate significance between control and vehicle-treated samples, while (▲) indicates significance between cAMP-treated cells with the cAMP CRH-663 control (p<0.05).

5.3 AtT-20 CRH-663 Luciferase Assays.

5.3.1 Analysis of CREAP Activity in AtT-20 CRH-663 Assay

In an approach to compare the differences in CRH regulation between the placenta and the brain, the neuroendocrine cell line, AtT-20 is often used. These are a mouse pituitary cell line in which the regulation of CRH and the cAMP response has been well studied. These cells do not express endogenous CRH but transfection studies have been used extensively to characterise CRH regulation (Dorin et al. 1989; Van et al. 1990; Guardiola-Diaz et al. 1996; King et al. 2002; Nikodemova et al. 2003). To investigate the effect of CREAP in a pituitary cell line versus primary placental cells, the dual luciferase assay was used.

The CRH-663 luciferase reporter plasmid was transfected into AtT-20 cells using the calcium phosphate method. Increasing amounts of CREB, CREAP_{lv}, CREAP_{sv} and PCI neo vector were co-transfected into the AtT-20 cells and the luciferase activity measured. Figure 5.3 shows that CREB co-transfection resulted in a significant 166-605% increase in CRH-663 promoter activity over vehicle-treated basal levels in a dose-dependent manner. CREAP_{lv} only produced a statistically significant increase of 183% over basal levels when transfected at a concentration of 2 µg while CREAP_{sv} co-transfection resulted in a significant, up to a 187% induction with 0.5 and 1 µg plasmid, with neither result appearing to be dose-dependent. Transfection of the empty PCI neo vector was not significantly different to the basal activity, which indicates that PCI does not appear to have any inhibitory effect in vehicle-treated AtT-20 cells.

The transfected AtT-20 cells were also treated with 0.5 mM cAMP to keep the treatment conditions consistent with the primary placental cells. Treatment with cAMP gave a 13-fold induction with 5 µg CREB and a significant, dose-dependent induction at all concentrations of CREB over cAMP-treated basal levels. CREAP_{lv} transfection resulted in modest but significant increases in CRH promoter activity at all concentrations except 0.25 µg. There was no significant effect of CREAP_{sv} transfection over cAMP-treated basal promoter activity. Unlike CREB, CREAP_{lv} did not give a dose-dependent response. PCI neo co-transfection resulted in a small but significant induction at higher plasmid concentrations indicating that PCI neo is not inhibitory to CRH-663 promoter activity in cAMP-treated AtT-20 cells, unlike the placental cells. So in AtT-20 cells CREAP_{lv} had a tendency to stimulate CRH-663 promoter activity in contrast to the results seen in the primary placental cells. The reason for the difference in PCI neo activity in AtT-20 versus placenta may be a tissue or species-specific effect.

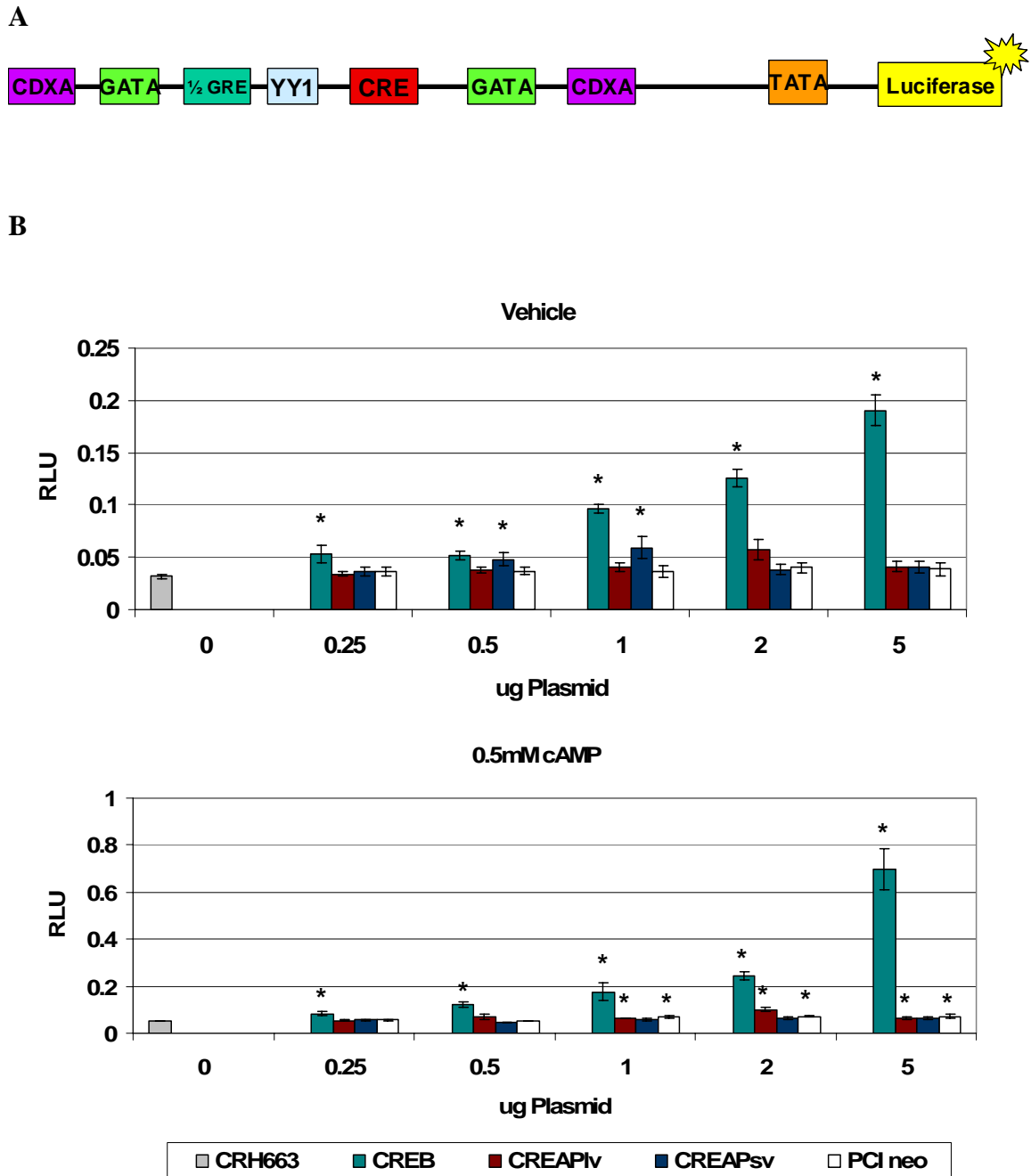


Figure 5.3. AtT-20 CRH-663 Luciferase Assay.

A. Schematic of the CRH-663 Luciferase Reporter. B. AtT-20 cells were transfected with 30 μ g CRH-663 luciferase reporter and increasing amounts of CREB, CREAP or PCI plasmids using the calcium phosphate method. Cells were treated with vehicle or 0.5 mM cAMP for 24 h before assay. Promoter activity was assessed by measuring the relative luciferase units (RLU). The error bars represent the SEM, n=2, asterisks (*) indicate significance compared to the reporter alone, p<0.05.

5.3.2 CREAP Does Not Synergise with CREB in AtT-20 Cells.

Similar to Section 5.2.3, it was tested whether CREAP could synergise with CREB on CRH-663 promoter activity. To determine if CREAP has any effect on CREB-induced CRH-663 promoter stimulation in AtT-20 cells, both CREB and CREAP were transfected into the same cells and the relative luciferase activity assayed. The results are shown in Figure 5.4. All transfections containing CREB gave a significant induction over vehicle-treated basal activity of up to 345%. There was no statistically significant difference between CREB alone and CREB co-transfected with CREAPlv, -sv or PCI neo. Therefore co-transfection with CREAPlv, CREAPsv and PCI neo had negligible effect on CREB mediated induction in vehicle-treated AtT-20 cells.

In cAMP-treated cells, CREB transfected cells had a significant 376% induction over cAMP-treated CRH-663 levels. There was no significant difference in cAMP-treated basal activity and transfection of CREAPlv-sv or PCI neo. Co-transfection with CREB and CREAPlv resulted in a significant 17% reduction in cAMP CREB alone levels, while CREAPsv and PCI co-transfections were not significantly different once again. In contrast to the placental cells, the CREAPsv and PCI neo vector did not inhibit cAMP CREB mediated stimulation of CRH-663 promoter activity, and CREAPlv only caused a 17% reduction in AtT-20 cells compared to a 31% reduction in placenta. These experiments therefore indicate CREB and CREAP do not synergise to increase stimulation of CRH-663 promoter activity in AtT-20 cells, and that PCI neo is not inhibitory in these cells either.

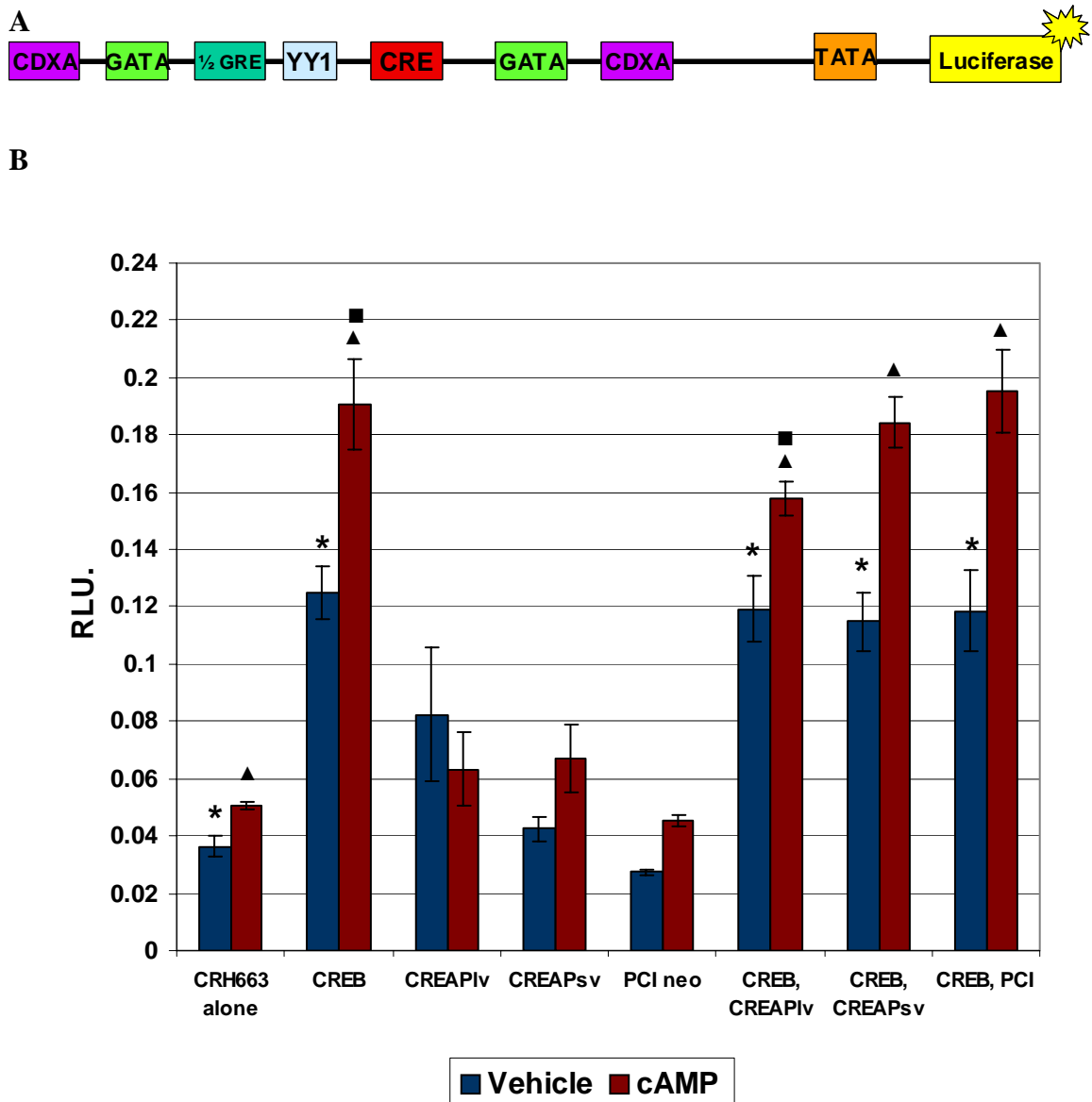


Figure 5.4. AtT-20 CRH-663 Co-Transfection of CREB and CREAP Assay.

A. Schematic of the CRH-663 reporter. B. AtT-20 cells were transfected with 30µg CRH-663 and 2µg of CREB, CREAP or PCI plasmids. Cells were treated with vehicle or 0.5 mM cAMP for 24 h before analysis. CREB and CREAP were co-expressed in the same cells to see if there is a synergistic or antagonistic effect between them. Promoter activity was measured using relative luciferase units (RLU). Error bars represent the SEM, n=3. (*) represents significant difference between vehicle CRH-663 control and transfectants, (▲) the significant difference between cAMP-treated control and transfectants. (■) represents a significant difference between cAMP CREB induction, and CREAPlv co-transfection, p<0.05.

5.4 JEG-3 Lipofectamine 2000 Transfections.

5.4.1 JEG-3 Luciferase Assays

Given the apparent inhibitory effect of PCI neo in primary placental cells, especially in cAMP-treated cells, a placental cell line was also investigated in order to fully confirm the activity of CREAP in the promoter assay. JEG-3 is a choriocarcinoma cell line that is often used as a model for the placenta. JEG-3 cells do not express CRH but have been used as a model for placental CRH gene regulation (Scatena and Adler 1996; Scatena and Adler 1998; Ni et al. 2002). Compared to primary placental cultures, JEG-3 are easier to obtain, culture and transfect and are amenable to lipid-based transfection techniques to greatly enhance transfection efficiency.

5.4.2 Lipofectamine 2000

Lipofectamine 2000 (LF2000), (Invitrogen), is a commercially available proprietary cationic lipid-based transfection reagent. It provides a quick, reliable and consistent method of cell transfection and has been shown to provide high level transfection efficiency across many cell types (www.invitrogen.com/celllines). When the transfection efficiency of calcium phosphate transfection was compared with LF2000 there was a significant increase in β -galactosidase transfected cells in the LF2000 transfected cells (see Figure 5.5). Therefore, LF2000 was used to transfect the JEG-3 cells in subsequent assays.

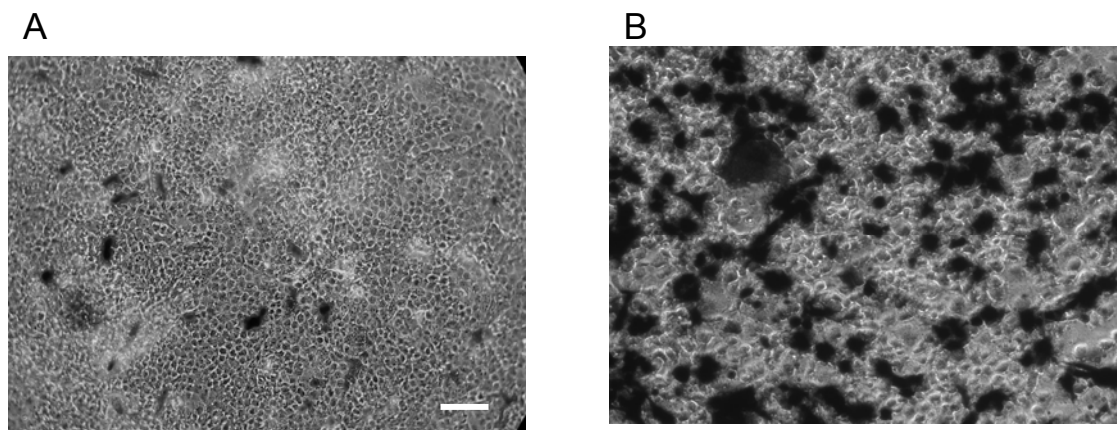


Figure 5.5. Comparison of Calcium Phosphate and LF2000 Transfection Efficiency in JEG-3 Cells.

A. JEG-3 cells were transfected with 30 µg β-galactosidase using calcium phosphate and stained 48 h after transfection. B. JEG-3 transfected with 1 µg β-Galactosidase and 2 µl LF2000 stained 48 h after transfection. Black staining shows cells that are positive for β-galactosidase protein. Scale bar represents 250 µm.

5.4.3 JEG-3 CRH663 and mCRE Luciferase Assays.

Both the CRH-663 luciferase reporter and the mCRE luciferase reporter vectors were used in these experiments. The mCRE reporter consists of the same 663bp of the CRH promoter as the CRH-663 reporter, but the CRE consensus sequence has been inactivated by mutation.

5.4.3.1 Vehicle- and cAMP-Treated CRH-663 and mCRE Results.

JEG-3 cells were transfected with either the CRH-663 or mCRE luciferase reporters and then co-transfected with CREB, CREAP or PCI neo expression plasmids. The comparison of the activity of the two promoters, expressed as relative luciferase units is

shown in Figure 5.6. In vehicle-treated cells the CRH-663 reporter had approximately the same basal activity as the mCRE vector with only a 3% difference. CREB transfection resulted in a modest but significant 166% increase over mCRE basal activity and a 179% increase over CRH-663 basal promoter activity. There was negligible effect of co-transfection of CREAP_{lv}, -sv or PCI neo over basal levels, and no statistical difference between CRH-663 and mCRE activities for each transfection condition.

Treatment with 500 μ M 8-Br-cAMP caused 173% stimulation over vehicle basal levels in mCRE transfected cells and a statistically significant 224% increase in CRH-663 cells. Co-transfection with CREB resulted in a significant 313% increase in CRH-663 activity but no significant effect in mCRE cells. The difference between CRE-663 and mCRE reporter activities was significant under cAMP-treated conditions for both the reporters alone and CREB co-transfectants, indicating that the mutant CRE reporter is not responsive to cAMP and CREB-mediated stimulation.

There was a significant decrease in cAMP-treated CREAP_{lv}, -sv and PCI neo co-transfections with CRH-663 compared to cAMP-treated CRH-663 reporter alone activity. CREAP_{sv} and PCI neo-transfected with mCRE were also significantly reduced compared to cAMP-treated mCRE-reporter-alone levels. There was no difference in promoter activity between CRH-663 and mCRE reporters in cells transfected with CREAP or PCI. Similar to the results seen in the primary placental cells, CREAP and PCI have little effect on CRH promoters in vehicle-treated cells but are inhibitory in cAMP-treated cells.

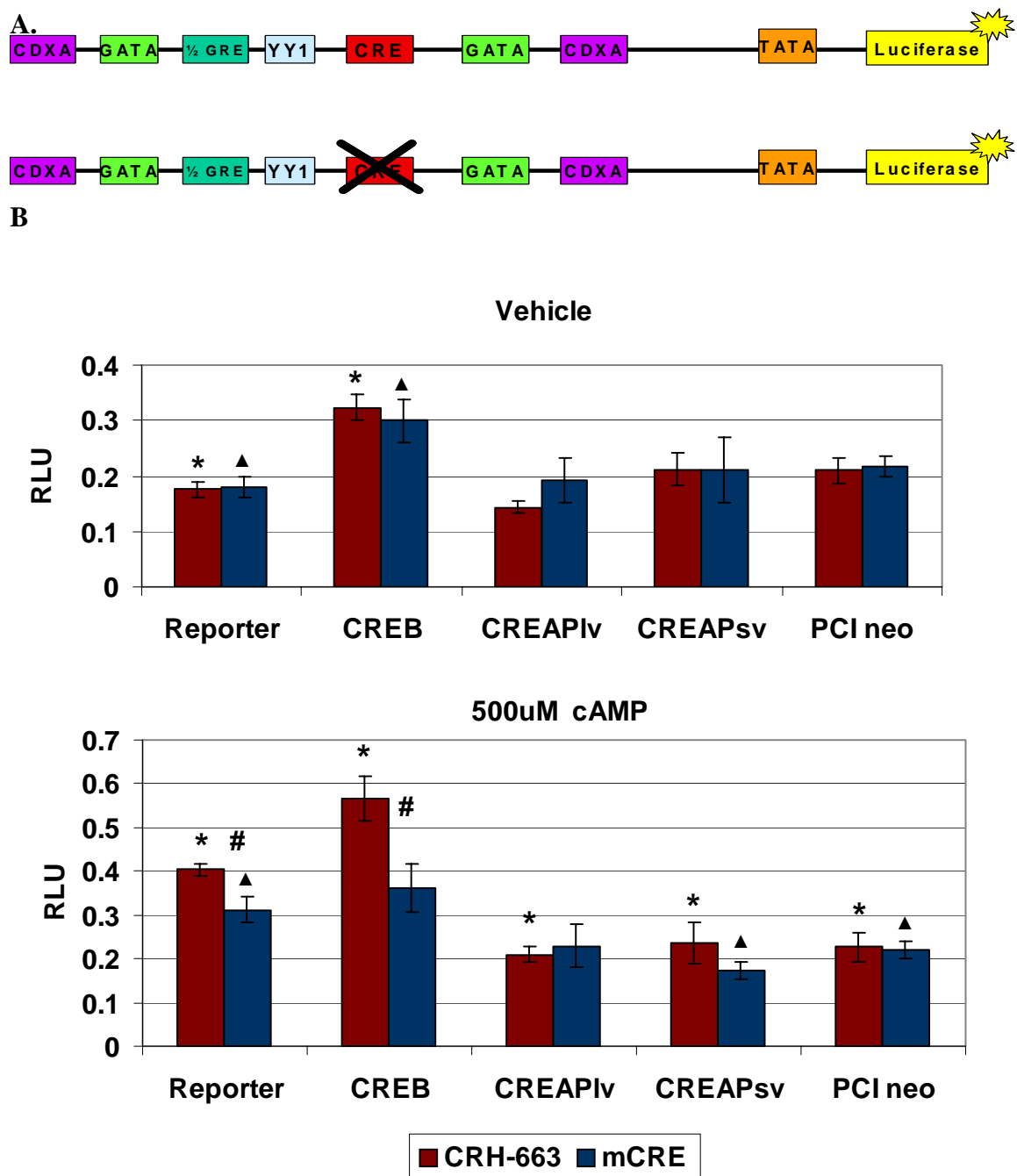


Figure 5.6. JEG-3 CRH-663 and mCRE Vehicle and cAMP Assay.

A. Schematic diagram of the CRH-663 and mCRE reporters. B. JEG-3 cells were transfected with 500ng CRH-663 and mCRE luciferase reporters and 100ng CREB, CREAP or PCI neo plasmid. Cells were treated with vehicle or 500μM cAMP for 24 h. Promoter activity was measured using relative luciferase units (RLU). Error bars represent the SEM, n=3. (*) represents significant difference between CRH-663 control and the transfectants, (▲) the mCRE control and transfectants, (#) represents a significant difference between CRH-663 and mCRE activities. $p < 0.05$.

5.4.3.2 *Dexamethason- and PMA-Treated CRH-663 and mCRE Results.*

The CRH-663 and mCRE Lipofectamine 2000 transfected cells were also treated with the synthetic glucocorticoid, dexamethasone (0.1 μ M) and 100 μ M phorbol ester (PMA). Dexamethasone is known to regulate the CRH gene even though it does not contain a glucocorticoid response element. The dexamethasone responsiveness is mediated through the CRE (Cheng et al. 2000b). Phorbol 12-myristate 13-acetate (PMA) stimulates the protein kinase C (PKC) pathway as opposed to the PKA pathway stimulated by cAMP. Phorbol ester has been shown to regulate CRH expression in hypothalamic cultures (Emanuel et al. 1990; Parkes et al. 1993).

Figure 5.7 shows there was no significant effect of dexamethasone treatment between CRH-663 and mCRE transfectants. Co-transfection with CREB produced a significant increase on both CRH-663 and mCRE activity of around 200%. Transfection of CREAPlv, -sv or PCI neo had negligible effect on either promoter when treated with dexamethasone. Overall there was very little difference between the mCRE and CRH-663 reporters and the CREAP and PCI transfections when treated with dexamethasone.

Phorbol 12-myristate 13-acetate (PMA) activates the PKC (protein kinase C) pathway. This lab has not previously looked at the effect of PMA or the PKC pathway on CRH regulation. PMA treatment resulted in increased promoter activity of CRH-663 and mCRE over vehicle levels, with the CRH-663 result being statistically significant. Co-transfection with CREB caused a significant increase in CRH-663 promoter activity. Other than a significant decrease of 29% in PCI neo and CRH-663 transfected cells,

there was negligible effect of CREAP and PCI transfections on either reporter when treated with PMA.

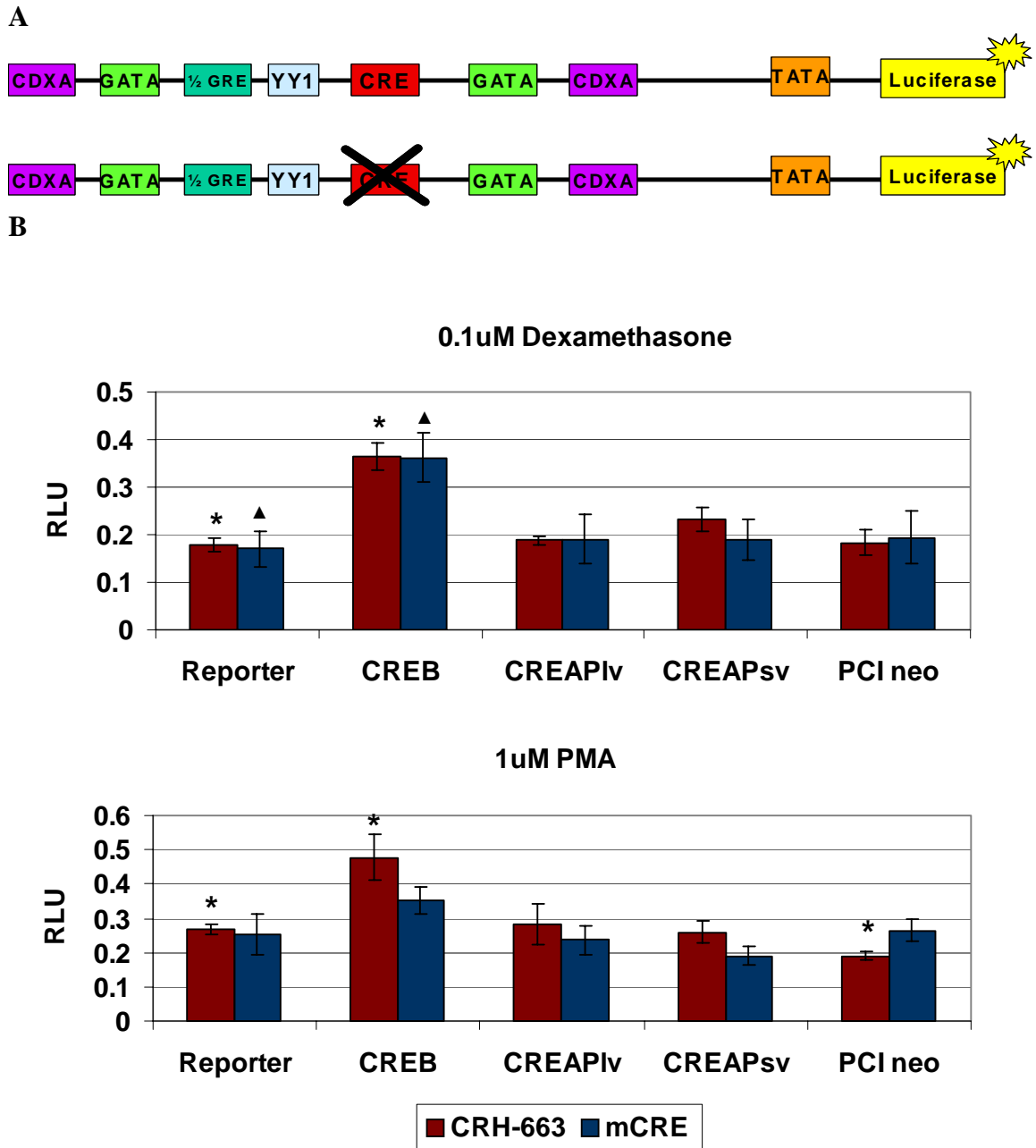


Figure 5.7. JEG-3 CRH-663 and mCRE Dex and PMA Assay.

A. Schematic diagram of the CRH-663 and mCRE Reporters. B. JEG-3 cells were transfected with 500ng CRH-663 and mCRE luciferase reporter and 100ng CREB, CREAP or PCI neo plasmid. Cells were treated with 0.1μM Dexamethasone or 1μM PMA for 24 h before assay. Promoter activity was measured using relative luciferase units (RLU). Error bars represent the SEM, n=3. (*) represents a significant difference between CRH-663 reporter control and the transfectants, and (▲) the mCRE reporter control and transfectants, p<0.05.

5.4.4 JEG-3 CRE-CRH-99 and CRH-99 Luciferase Assays.

The CRH-99 control construct consists of 99 bps of the CRH promoter which doesn't contain any regulatory elements linked to a luciferase reporter (Figure 5.8A). The CRH-CRE-99 construct consists of the same 99bps with a CRE in front so that any promoter activity is mediated by the CRE. These reporters provide greater insight into the effect on promoter activity due to the CRE. The CRH-663 and mCRE reporters contain additional regulatory elements to the CRE that are responsive to cAMP, or able to bind other cellular regulatory factors to alter reporter activity. The CRH-99 and CRE-CRH-99 are "bare-bones" promoters where any effect on promoter activity can only be attributable to the TATA box in the case of CRH-99 and the CRE and TATA box in CRE-CRH-99.

5.4.4.1 Vehicle and cAMP Treated CRH-99 and CRE-CRH-99 Luciferase Results.

JEG-3 cells were transfected with CRH-99 or CRE-CRH-99 luciferase reporters and co-transfected with CREB, CREAP or PCI neo and the relative luciferase activity measured. The results are presented in Figure 5.8B.

In vehicle-treated cells the CRH-99 promoter activity was given a value of 100% to which all other results are compared. The basal CRE-CRH-99 promoter activity was 2390% greater than the CRH-99 activity. CREB co-transfection resulted in a significant 251% increase in basal levels in CRH-99 cells and 6830% increase in CRE-CRH-99 cells. Transfection of CREAPlv, -sv and PCI neo resulted in no significant difference compared to the vector alone controls in vehicle-treated cells, but there was a trend towards slight reduction in activity in CRE-CRH-99 transfected cells and a slight

increase in CRH-99 transfected cells. The presence of a CRE in the reporter caused a very large increase in luciferase activity in vehicle-treated cells under all transfection conditions.

Treatment with 500 μ M 8-Br-cAMP led to a 151% increase in promoter activity in CRH-99 transfected cells which wasn't significant and a significant 4128% increase in CRE-CRH-99 cells over vehicle-treated CRH-99 levels. CREB co-transfection resulted in a significant 365% increase in CRH-99 cells and a significant 10520% increase in CRE-CRH-99 cells which was a 440% increase over cAMP-treated promoter alone activity. This suggests that CREB is increasing promoter activity without the presence of a CRE. There was no significant difference between either reporter alone and CREAPlv, -sv or PCI neo transfections in cAMP-treated cells.

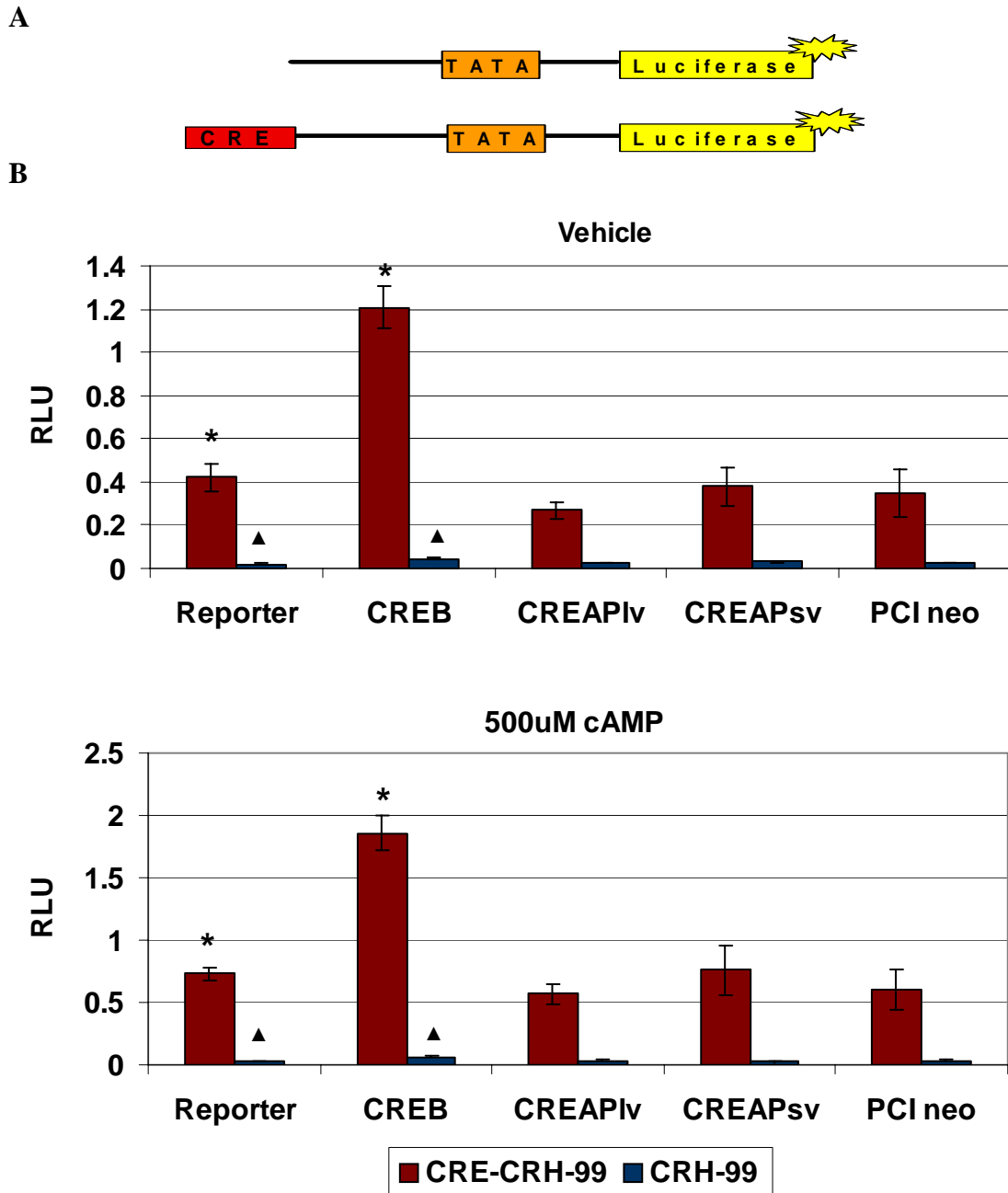


Figure 5.8. JEG-3 CRE-CRH-99 and CRH-99 Vehicle and cAMP Assay.

A. Schematic diagram of CRE-CRH-99 and CRH-99 reporters. JEG-3 cells were transfected with 500ng CRE-CRH-99 and CRH-99 luciferase reporter and 100ng CREB, CREAP or PCI neo plasmid. Cells were treated with vehicle or 500μM cAMP for 24 h. Promoter activity was measured using relative luciferase units (RLU). Error bars represent the SEM, n=3. (*) represents significant difference compared to the CRE-CRH-99 reporter control, and (▲) compared to the CRH-99 reporter control, p<0.05.

5.4.4.2 *Dexamethasone- and PMA-Treated CRH-99 and CRE-CRH-99 Results.*

CRH-99 and CRE-CRH-99 transfected JEG-3 cells were treated with 100 μ M dexamethasone for 24 h before assay for promoter activity, and the results presented in Figure 5.9. Treatment with dexamethasone slightly increased both CRH-99 and CRE-CRH-99 promoter activities compared to vehicle but the result was not statistically significant. CREB co-transfection resulted in significantly increased activity in CRH-99 and CRE-CRH-99 cells compared to vehicle- and cAMP-treated basal levels, indicating that CREB seems to be having an effect even when a CRE is not present. Similarly to the vehicle and cAMP results, transfection with CREAPlv, -sv and PCI neo vector had no significant effect on either luciferase promoter in dexamethasone-treated cells. However there was a slight trend towards CREAP and PCI neo being slightly inhibitory to the CRE-CRH-99 promoter. Overall, the dexamethasone results are very similar to the vehicle results.

Treatment of CRH-99 and CRE-CRH-99 transfected JEG-3 cells with PMA resulted in 171% and 3683% activity over basal CRH-99 vehicle-treated activity, however this increase was not statistically significant. Co-transfection with CREB produced 374% activity in CRH-99 cells and 6230% stimulation in CRE-CRH-99 cells which was significantly increased compared to vehicle CRH-99 levels, and was also significantly increased compared to the PMA-treated promoters alone. Once again, there was no significant effect of CREAPlv,-sv or PCI neo on either reporter but there was a slight trend for inhibition of CRE-CRH-99 by CREAP and PCI neo.

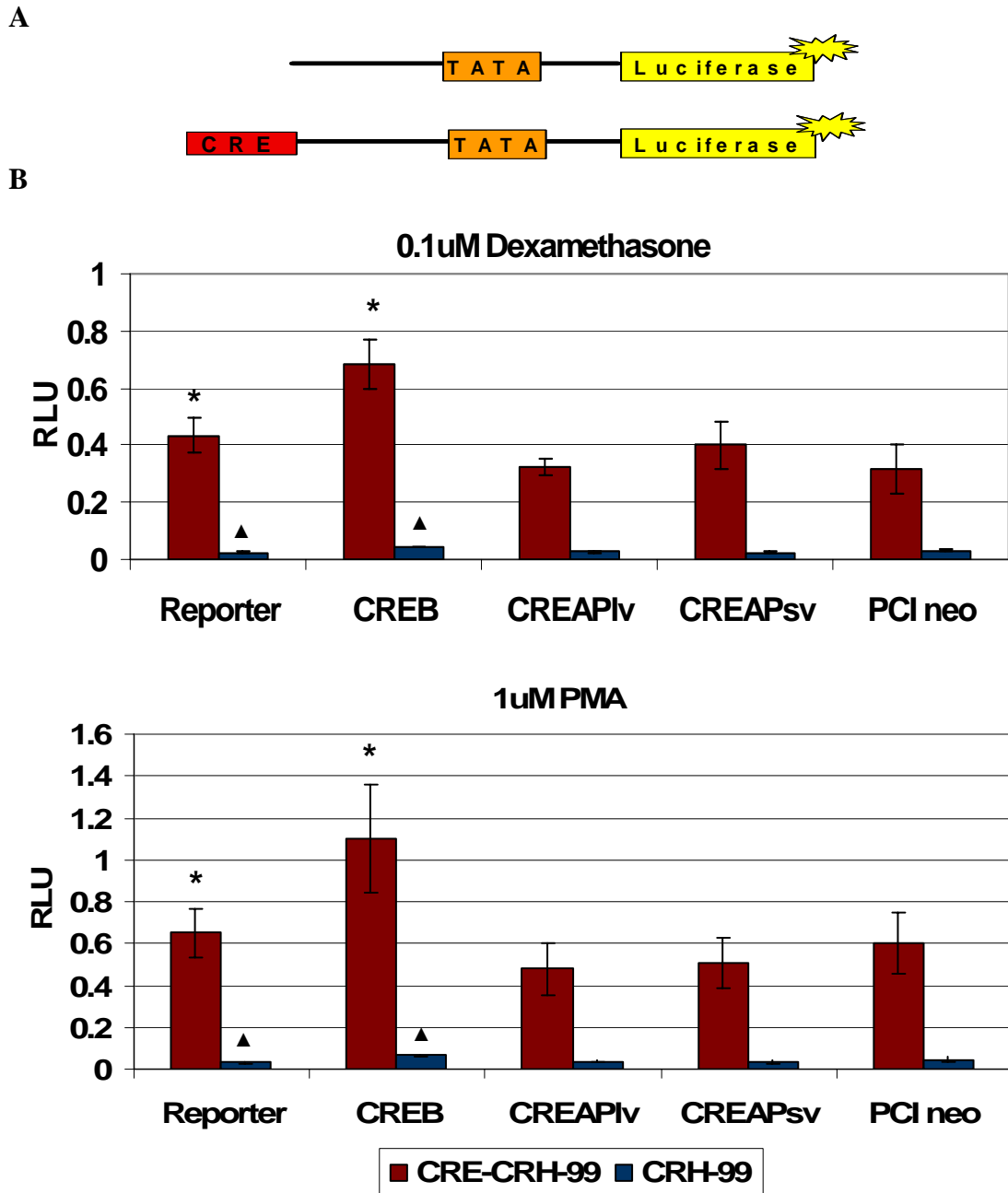


Figure 5.9. JEG-3 CRE-CRH-99 and CRH-99 Dexamethasone and PMA Assay.

A. Schematic diagrams of CRE-CRH-99 and CRH-99 reporters. B. JEG-3 cells were transfected with 500ng CRE-CRH-663 and CRH-99 luciferase reporter and 100ng CREB, CREAP or PCI neo plasmid. Cells were treated with 0.1 μ M Dexamethasone or 1 μ M PMA for 24 h before assay. Promoter activity was measured using relative luciferase units (RLU). Error bars represent the SEM, n=3. (*) represents a significant

difference compared to the CRE-CRH-99 reporter control, and (▲) a significance compared to the CRH-99 reporter control, $p < 0.05$.

5.5 Discussion

5.5.1 Placental Cells and CRH Regulation

5.5.1.1 The cAMP Response

The regulation of CRH in the placenta has been studied by this lab and others. Both cAMP and glucocorticoids stimulate placental CRH production through the CRE (Cheng et al. 2000b; Cheng et al. 2000a). The cAMP response unit consists of the palindromic CRE (TGACGTCA), the protein kinase, PKA, and bZIP transcription factors such as CREB. cAMP binds to two sites on the regulatory subunit of PKA, releasing the catalytic subunit (Roesler et al. 1988; Lalli and Sassone-Corsi 1994). The activated catalytic subunit can then phosphorylate both cytoplasmic and nuclear proteins on serine residues to influence transcription. PKA is required for cAMP to stimulate transcription. Cells that are deficient in PKA activity are unable to stimulate CRE-containing reporters (Montminy et al. 1986) and over-expression of the PKA inhibitor, PKI, abolished cAMP dependent transcription in transfected cells (Grove et al. 1987).

The first CRE-binding protein to be characterised was CREB (Hoeffler et al. 1988). At least an additional ten CRE-binding proteins have now been found. All CRE-binding proteins belong to the bZIP transcription factor family which is characterised by a leucine zipper motif which allows bZIP proteins to dimerise (Sassone-Corsi 1995). PKA phosphorylation activates CREB so it can dimerise and bind to the CRE to initiate

transcription (Gonzalez et al. 1989). CREB can be phosphorylated at Ser133 which is part of the consensus PKA phosphorylation site.

5.5.1.2 Primary Placental Cells and CRH-663

Cheng *et al.* used transfection of primary placental cell cultures to investigate the regulation of the CRH gene. They found that 8-Br-cAMP or forskolin increased CRH peptide levels and increased luciferase promoter activity up to five-fold in a dose-dependent manner (Cheng et al. 2000a). It was determined that the cAMP stimulation was mediated by the CRE and that the CRE was required for CRH expression (Cheng et al. 2000a). They also found that the CRE from the CRH promoter was able to transfer cAMP responsiveness to a heterologous promoter.

The results obtained here from the primary placental cell luciferase reporter transfections reproduced the cAMP effects in that 8-Br-cAMP treatment increased CRH-663 promoter activity by 280%. Co-transfection of CREB was used as a control for bZIP proteins, as it is known that CREB is part of the protein complex that forms on the CRE of the CRH promoter thus stimulating CRH promoter activity (King et al. 2002). CREB is required under normal conditions to mediate the effect of cAMP on CRH through the PKA pathway, therefore over-expression increases the number of CREB proteins available to bind to the CRE, and cAMP treatment further enhances the phosphorylation and activation of CREB. CREB significantly stimulated CRH-663 promoter activity by 220-340% under basal conditions and by 200-250% over cAMP-treated promoter activity, which was at least a two-fold increase over basal levels which indicated that the assay was able to reconstitute the CRH/CREB response.

Co-transfection with CREAPlv, CREAPsv or the vector backbone PCI neo generally resulted in luciferase activity similar to the promoter alone activity under basal conditions. However, under cAMP-treated conditions the backbone PCI neo vector appeared to be inhibitory at certain concentrations. There was some inhibition by CREAPlv, though this generally wasn't statistically significant, and the results for CREAPlv and CREAPsv were very similar. These results then suggest that the RS region of CREAP is not important for CRE regulation.

When CREB and CREAPlv, -sv or PCI neo were transfected into the same placental cells, the cAMP-treated, CREB-induced stimulation was significantly decreased by CREAP and PCI, while vehicle-treated levels were unaffected. The inhibitory effect by the expression vector backbone appeared peculiar to cAMP treatment and wasn't as apparent under basal conditions. There have been no reports of this inhibitory effect of the PCI neo vector and it is marketed as a mammalian expression vector by the source company. Given this observation it is possible that the lack of stimulation of the CRH promoter by CREAP is due to a negative effect from the vector backbone. Even if this is the case, CREAP is not capable of the same level of stimulation mediated by CREB.

In further consideration of the inhibitory effects of the PCI vector, both the PCI and CREB vector contain a human cytomegalovirus (CMV) promoter so it is unclear what is responsible for the difference in cAMP response. Table 5.1 shows comparison between the PCI and CREB vectors.

Feature	CMV-CREB	PCI Neo
CMV immediate early enhancer/promoter	√	√
SV40 early mRNA polyadenylation signal	√	
SV40 origin replication	√	
SV40 late polyadenylation signal		√
T7 RNA polymerase promoter		√
Phage f1 origin	√	√
β-lactamase (Amp) coding region		√
Kanamycin/neomycin resistance	√	
HSV-TK polyadenylation signal	√	
pUC plasmid replication origin	√	
ColE1-derived origin of replication		√
Chimeric intron		√

Table 5.1. Comparison of the features of CREB and PCI-neo Plasmids.

There are some common features between the two vectors and some differences but a major difference is the presence of a chimeric intron in PCI neo. The chimeric intron in PCI is composed of the 5'-donor site from the first intron of the human β-globin gene and the branch and 3'-acceptor site from the intron that is between the leader and the body of an immunoglobulin gene heavy chain variable region. Transfection studies have shown that the presence of an intron flanking the cDNA insert can increase the level of gene expression, though the level of the increase is cDNA insert-dependent

(Buchman and Berg 1988; Evans and Scarpulla 1989; Huang and Gorman 1990). So whether the inhibitory effect is due to the presence of this chimeric intron or another element of PCI neo is unclear. There is at least one study that has co-transfected PCI neo vector with a luciferase reporter and they didn't detect any inhibition in HaCat cells (Mendoza et al. 2006). However the data presented in this Chapter does show that the inhibitory effect of PCI neo seems to be cell line dependent (see below), cAMP-treatment-dependent and possibly luciferase reporter-dependent.

5.5.2 *AtT-20 Cells and Transfected CRH Regulation*

5.5.2.1 *AtT-20, CRH and CREAP*

The mouse corticotroph cell line, AtT-20, is often used as a model for hypothalamic CRH regulation (Dorin et al. 1989; Van et al. 1990; Dorin et al. 1993; Guardiola-Diaz et al. 1996; Malkoski et al. 1997; Malkoski and Dorin 1999; King et al. 2002). Glucocorticoids stimulate CRH in the placenta but inhibit such activation in the hypothalamus, most likely due to tissue-specific differences in transcription factors (King et al. 2002). It has been found that cAMP is able to stimulate the CRH promoter at two sites in AtT-20 cells, the consensus CRE (-228 to -221bps) and a caudal-type homeobox response element, CDXA (-125 to -118bps) (King et al. 2002).

Previously, transfected AtT-20 cell treatment with 3 mM cAMP resulted in a 14-fold increase in CRH-663 promoter activity over basal levels (King et al. 2002). In this study, 0.5 mM cAMP treatment was used and only resulted in a 1.7-fold increase, however when co-transfected with 5 µg CREB, there was over a 13-fold increase in

CRH-663 promoter activity. All transfections with CREB gave significant increases in CRH-663 promoter activity in AtT-20 cells. Unlike the placental cells, there was no significant inhibition by CREAPlv, -sv or PCI neo in vehicle-treated cells, and even some significant induction in both vehicle and cAMP-treated cells by CREAP and PCI neo. The difference in CREAP and PCI activities between placenta and AtT-20 cells suggests that tissue or species-specific effects may be responsible, such a difference between the brain and placenta, or a difference between mouse and human. However, these results lend confidence to the conclusion that CREAP was unable to drive CRH-mediated promoter activity in placental cells.

In further experiments, CREB and either CREAPlv, -sv or PCI neo were transfected into the same cells to see the effect on CREB-mediated stimulation of CRH in AtT-20 cells. CREB caused marked stimulation while CREAPlv and -sv and PCI were not significantly different to the vector alone. When CREB was co-transfected with CREAP or PCI neo there was a significant 17% inhibition by CREAPlv but not -sv or PCI in cAMP-treated cells. This is far less than that observed in placental cells (even if that was deemed insignificant). Most notable in these results is the fact that PCI neo does not seem to inhibit promoter activity in AtT-20 cells the way it did in placental cells. This is not surprising as contrasting results have already been observed for the hypothalamus and placenta, such as the opposite effect of glucocorticoids (Robinson et al. 1988; Guardiola-Diaz et al. 1996; Malkoski et al. 1997; Cheng et al. 2000b). Therefore the relative lack of stimulation of CRH promoter activity by CREAPlv or CREAPsv is more likely to be a true effect of the CREAP protein and not an effect from

the vector backbone. So even though CREAP contains leucine zipper motifs, it doesn't appear to stimulate CRH promoter activity like other bZIP proteins, such as CREB.

5.5.3 *JEG-3 and Transfected CRH and CRE Promoters.*

5.5.3.1 *JEG-3 Transfection Studies*

The regulation of CRH in the choriocarcinoma cell line, JEG-3 has not been extensively characterised. There are a few studies that have utilised JEG-3 in investigating CRH, but the results are not conclusive (Scatena and Adler 1996; Scatena and Adler 1998; Ni et al. 2002). JEG-3 are used as a model for the placenta but they do not express endogenous CRH, however they do readily express transfected CRH promoter (Scatena and Adler 1996; Scatena and Adler 1998; Ni et al. 2002).

Similarly, expression of the two transfected CREAP proteins in JEG-3 cells was confirmed with western blotting (data not shown) and protein bands of the appropriate sizes were detected with anti-CREAP sera. The JEG-3 cells were then transfected with CRH-663 and mCRE luciferase reporters and then co-transfected with CREB, CREAP_{lv}, -sv or PCI neo. Reporter activity was then measured by Dual Luciferase Assay following treatment with vehicle, cAMP, dexamethasone or PMA.

5.5.3.2 *Vehicle Treatment Results*

5.5.3.2.1 *CRH-663 and mCRE*

In vehicle-treated cells there was very little difference between the CRH-663 and mCRE reporters. There was a significant induction of promoter activity by CREB, but negligible effects observed due to CREAP or PCI. The similar activities of the CRH-663 and mCRE promoters under basal conditions is not surprising as it is only the CRE that is mutated in mCRE while all other regulatory elements are still present and functional so are able to respond to basal cellular stimuli. It was somewhat unexpected that CREB would significantly stimulate the mCRE promoter as the CRE is mutated but it could be acting through another element such as the cAMP responsive CDXA or a protein-protein interaction. However, under cAMP stimulation, CRH-663 activity was greater than that observed for mCRE, demonstrating a specific response to the CRE.

5.5.3.2.2 CRE-CRH-99 and CRH-99

Two other reporter constructs were also tested in JEG-3. The CRH-99 and CRE-CRH-99 luciferase reporters provide a more specific analysis of CRE mediated promoter activity. The presence of the CRE gave a dramatic increase in luciferase activity under basal conditions over that observed with CRH-99. There was however significant stimulation by CREB co-transfection even though CRH-99 doesn't contain any regulatory elements except a TATA box. It is possible that CREB could be interacting with proteins bound to the TATA box, either directly or through an adaptor such CBP (CREB binding protein) (Quinn 1994). CBP has been found in complexes with the TBP so in this way, CREB could still stimulate transcription without the presence of a CRE (Swope et al. 1996; Dallas et al. 1997; Schaufele et al. 2001). Neither CREAP or PCI had a significant effect on basal CRH-99 or CRE-CRH-99 promoter activities.

5.5.3.3 JEG-3 cAMP Treated Transfections

5.5.3.3.1 CRH-663 and mCRE

cAMP treatment stimulated CRH-663 and to a lesser extent mCRE activity, most likely through the cAMP responsive CDXA element (-125 to -118bps). King *et al.* found a 14-fold induction in CRH-663 activity in AtT-20 cells when treated with cAMP. The mCRE resulted in a 3.47-fold stimulation, while mutant CDXA had a 5.53-fold induction over basal levels, indicating that the CDXA is able to mediate cAMP stimulation and acts synergistically with the CRE (King et al. 2002). Scatena and Adler also found a region from -200 to -99bp of the CRH promoter that was responsive to cAMP in choriocarcinoma cells and they found a 58 kDa human specific factor that bound to this region (Scatena and Adler 1998). However, although CREAPlv would approximate this molecular mass, the functional results of this Chapter, coupled to the fact that this protein is not expressed in HeLa, while CREAP mRNA has been detected in HeLa cells (unpublished data) suggests that CREAP is not this human specific factor.

CREB co-transfection only caused a significant stimulation in CRH-663 cells while CREAPlv, sv and PCI neo were inhibitory to both promoters under cAMP-treated conditions, similar to the results from primary placental cells. However the level of stimulation by cAMP and CREB wasn't as great in JEG-3 cells as that observed in placenta and AtT-20 cells suggesting a cell specific difference in CRH regulation between placenta and JEG-3. The CREB activity in cAMP-treated mCRE transfected cells was similar to that observed under other treatment conditions, indicating that both cAMP treatment and a functional CRE are needed to induce a high level of promoter activity.

5.5.3.3.2 CRE-CRH-99 and CRH-99

The cAMP-treated CRH-99 results indicate that this reporter was slightly responsive to cAMP (though not significantly) which is enhanced by CREB co-transfection. Once again there was no significant effect of CREAPlv, -sv and PCI neo on either reporter. cAMP treatment produced an induction of CRE-CRH-99 promoter activity which was increased by CREB co-transfection indicating that the CRE is responsive to cAMP and CREB in JEG-3 cells, though the level of induction wasn't as great as that observed in placental cells. The induction of CRH-99 by CREB was most likely due to the previously mentioned protein-protein interaction with the TATA box.

5.5.3.4 *JEG-3-Dexamethasone-Treated Transfections*

5.5.3.4.1 CRH-663 and mCRE

To assess if glucocorticoids or phorbol ester affected CREAP, the CRH-663 and mCRE/CREAP co-transfected cells were treated with dexamethasone or PMA. Glucocorticoids inhibit CRH in the hypothalamus but stimulate in the placenta. Cheng *et al.* showed that dexamethasone treatment resulted in a two-fold stimulation of CRH-663 in placental cells and the stimulation is mediated by the CRE (Cheng et al. 2000b), but the response in JEG-3 cells has not been well characterised. In this study, dexamethasone treatment didn't induce stimulation in CRH-663 or mCRE transfected cells compared to the vehicle control, suggesting that JEG-3 may have a different response to glucocorticoids than placental cells. CREAP and PCI neo co-transfection gave similar promoter activity to the reporter alone, while CREB gave a two-fold

induction, which was significant compared to the dexamethasone vector control but not compared to the CREB transfected vehicle-treated cells. There was very little difference between CRH-663 and mCRE luciferase activity, indicating that the CRE is not important in the glucocorticoid response of JEG-3.

5.5.3.4.2 CRE-CRH-99 and CRH-99

Similar to the results with CRH-663 and mCRE there didn't seem to be any response to dexamethasone with the CRE-CRH-99 promoter in JEG-3. There was a significant stimulatory effect by CREB with both reporters but no significant difference with CREAP and PCI neo when treated with dexamethasone. These results are consistent with those observed with the CRH-663 and mCRE promoters, supporting the theory that glucocorticoid regulation of CRH or CRE containing promoters is not important in JEG-3. However, the lack of response to glucocorticoids in JEG-3 could be due to a low level of GR. Chen *et al.* found that cortisol and dexamethasone were able to stimulate transfected human gonadotrophin releasing hormone in JEG-3 cells but that the stimulation was dependent on the co-transfection of a GR cDNA expression vector (Chen et al. 1998).

5.5.3.5 JEG-3 Phorbol Ester-Treated Transfections

Phorbol ester (PMA, also known as TPA, 12-O-tetradecanoylphorbol 13-acetate) is a stimulator of the PKC pathway. Phorbol ester has been shown to stimulate expression of CRH. The CRH promoter has several partial TPA response elements (TRE's) (Vamvakopoulos et al. 1990). TPA has been shown to increase CRH gene expression

and peptide secretion in dissociated hypothalamic cultures (Emanuel et al. 1990; Parkes et al. 1993) but not in dissociated amygdala cultures (Kasckow et al. 1997). TPA also increases CRH expression in NPLC-KC, BE(2)-M17 and BE(2)-C cells (Parkes et al. 1993; Kasckow et al. 1995). The TRE and CRE sequences differ by only 1 base pair.

PKC isoforms alpha, epsilon and zeta have been detected in human placenta and activation of PMA-responsive PKCs (PKC alpha or PKC epsilon) appears to enhance ligand-stimulated cAMP production in trophoblasts (Karl and Divald 1996). This may be a physiologically important example of 'cross-talk' between various signalling pathways in human placental trophoblasts. JEG-3 cells have been used to show that both PKA and PKC are involved in the transcriptional activation of the TRE and that both kinases together have a synergistic effect, though it was found that TPA didn't stimulate CRE-directed transcription, but both cAMP and TPA stimulate the TRE (Hoeffler et al. 1989). It was also found that a common factor(s) from JEG-3 cells is capable of binding the CRE and TRE.

5.5.3.5.1 CRH-663 and mCRE

The CRE seems to be important in phorbol ester stimulation of CRH as deletion of the CRE from a CRH luciferase construct abolished the stimulatory effect of TPA on reporter expression in chicken macrophages (Van 1993). PMA was stimulatory to CRH-663 compared to vehicle-treated CRH-663-transfections. CREB co-transfection produced a significant induction in CRH-663-transfected cells but not mCRE cells. Transfection with CREAP or PCI had no significant effect on either promoter except an inhibition by PCI neo in CRH-663-transfected cells. The results for the PMA

treatments are similar to the cAMP-treated transfection results with a CRE seeming to be important for PMA-mediated CRH promoter stimulation in JEG-3 cells, as observed in other cell types.

5.5.3.5.2 CRE-CRH-99 and CRH-99

PMA treatment was stimulatory to CREB co-transfections of CRE-CRH-99 and CRH-99, while CREAP and PCI had no significant effect on either promoter. PMA-induced CRE-CRH-99 promoter activity but co-transfection with CREB led to less activity in PMA-treated cells than vehicle cells indicating that PMA doesn't enhance CREB activity on an isolated CRE. CREB is phosphorylated by PKA so a PKC stimulator is unlikely to augment CREBs activity, though PKC has been found to phosphorylate CREB in cultured rat striatal neurons (Mao et al. 2007), though this effect could be tissue specific. The PMA results for CRH-663 were more stimulatory in JEG-3 cells than with the CRE-CRH-99 promoter, indicating that regulatory elements on the CRH promoter in addition to the CRE may be important in PMA-mediated CRH stimulation.

5.5.4 Overview

There were differences in reporter activity across the cell types and treatment methods. Scatena and Adler determined that it is trans-acting factors that dictate the species-specific expression of placental CRH (Scatena and Adler 1996). King and colleagues also found that different transcription factors bind to the CRE from the CRH gene in AtT-20 cells versus placental cells. In the placenta CREB and cJun were found to bind while CREB and Fos bound the CRE in AtT-20 cells (King et al. 2002).

Treatment with cAMP and CREB co-transfection increased CRH- and CRE-containing promoter activities across all cell types though the level of induction varied. There did seem to be a distinct difference between AtT-20 and the placenta and JEG-3 cells, where the PCI neo vector had a tendency to be inhibitory, especially when treated with cAMP, in the placental type cells but not so in the AtT-20 cells. Therefore, there could be a difference in trans-acting factors between the cell types that alters the effect of the PCI neo vector in the presence of cAMP. However, even under conditions where the PCI neo vector is not inhibitory there is negligible effect of either CREAP_{lv} or CREAP_{sv} on the CRH promoter or CRE-containing reporters in any of the cell types investigated, indicating that it is unlikely that CREAP has any stimulatory effect on CRH. There was a slight tendency towards inhibition by CREAP in placenta and JEG-3, but stimulation in AtT-20 cells, though even when these effects reached statistical significance they were quite modest. Treatment with dexamethasone and phorbol ester also yielded little effect by CREAP on CRH or CRE promoters.

It is more probable that CREAP has no effect on CRH, or that it perhaps has a negative modulatory role as co-transfection of CREAP with CREB reduces CREB induced stimulation, although the reduction was not statistically significant. Whether this effect is due to competition for nuclear factors, DNA binding sites or by an inhibitory dimerisation with CREB is unknown. There are bZIP transcription factors which have been found to be antagonists of cAMP-induced transcription. Two isoforms of the CRE modulator (CREM) bZIP protein have been identified as repressors that act either by binding as non-functional homodimers to the CRE or by forming non-activating

heterodimers with other bZIP proteins (Foulkes et al. 1991). There is also an inducible repressor known as inducible cAMP early repressor (ICER) which is a truncated product of the CREM gene (Molina et al. 1993; Stehle et al. 1993). Overall, it seems that CREAP certainly doesn't behave in the same stimulatory manner on CRH- or CRE-containing reporter promoters as CREB, regardless of cAMP, dexamethasone or phorbol ester treatment. There also seems to be no real difference in transcriptional activity between the full length, CREAP_{lv} and the truncated CREAP_{sv}, which is missing its RS domain. Supershift EMSA experiments were also conducted to determine if CREAP is part of the placental protein complex that binds to the CRE, but these experiments were not successful (data not shown). It was unclear if supershift was not detected with CREAP antisera due to CREAP not being present in the complex with CREB and Jun, or if the rabbit polyclonal antibody was just not suitable for EMSA applications. Overall, CREAP is unlikely to be a major regulator of CRH expression, even if it has CRE-binding activity, but is possibly more involved in splicing as indicated by its localisation to splicing speckles and SR domains and an interaction with splicing factors as discussed in Chapter 4, or is involved in transcription in another way, or with a different gene.

Chapter 6. Analysis of CREAP Binding to Transcription Factors

6.1 TranSignal TF Array

6.1.1 Rationale

From the sequence analysis conducted in Chapter 3, it is clear that CREAP contains a unique set of modular domains which suggest roles in both transcription and splicing. A demonstrated link to splicing was found in Chapter 4 with CREAP being shown to localise to intranuclear splicing speckles and to co-localise and co-immunoprecipitate with the SR protein splicing factor, SC35. The transcriptional ability of CREAP was investigated in Chapter 5 by testing CREAP's ability to regulate the CRH promoter and CRE promoters. However, CREAP had negligible effect on CRH gene expression. To further investigate whether CREAP has any transcription factor characteristics, we looked to determine if CREAP was able to interact with other transcription factors, especially bZIP proteins, given that CREAP contains leucine zipper-like motifs which is a common dimerisation and protein-protein interaction domain.

6.1.2 The Transcription Factor Array

As a strategy to determine which transcription factors (TFs) interact with a CREAP, a commercial TranSignal™ TF Protein Array IV was employed. There are four arrays available with different combinations of TFs. We chose array IV as it contained a variety of bZIP proteins as well as some other factors with known roles in CRH regulation. This array enables you to determine how a particular protein interacts with

multiple other proteins (42 TFs on Array IV) in a single detection experiment. The array membrane is spotted with transcription factor proteins, which are expressed from full length TF cDNAs, with an N-terminal His Tag. A protein of interest is used as probe to search for interactions with the immobilized proteins. Interactions can be assessed either by using an antibody to the protein of interest, or with reagents to detect tags such as HA, GST or biotin. The signal is visualized via chemiluminescent detection with the signal strength corresponding to the strength of the interaction (see Figure 6.1).

In the case of the CREAP screen, purified, recombinant, cleaved CREAP protein and CREAP polyclonal antibody described in Chapter 4 were chosen to screen the array. However, firstly to determine if the antibody alone exhibited non-specific binding to the TFs, the array was pre-screened with CREAP antibody alone. As can be seen in Figure 6.2 (A), there was some non-specific binding of the antibody alone to some of the factors on the blot; therefore in further densitometric analysis the antibody alone densitometry values were subtracted from the CREAP protein results to ensure bona fide interactions were obtained.

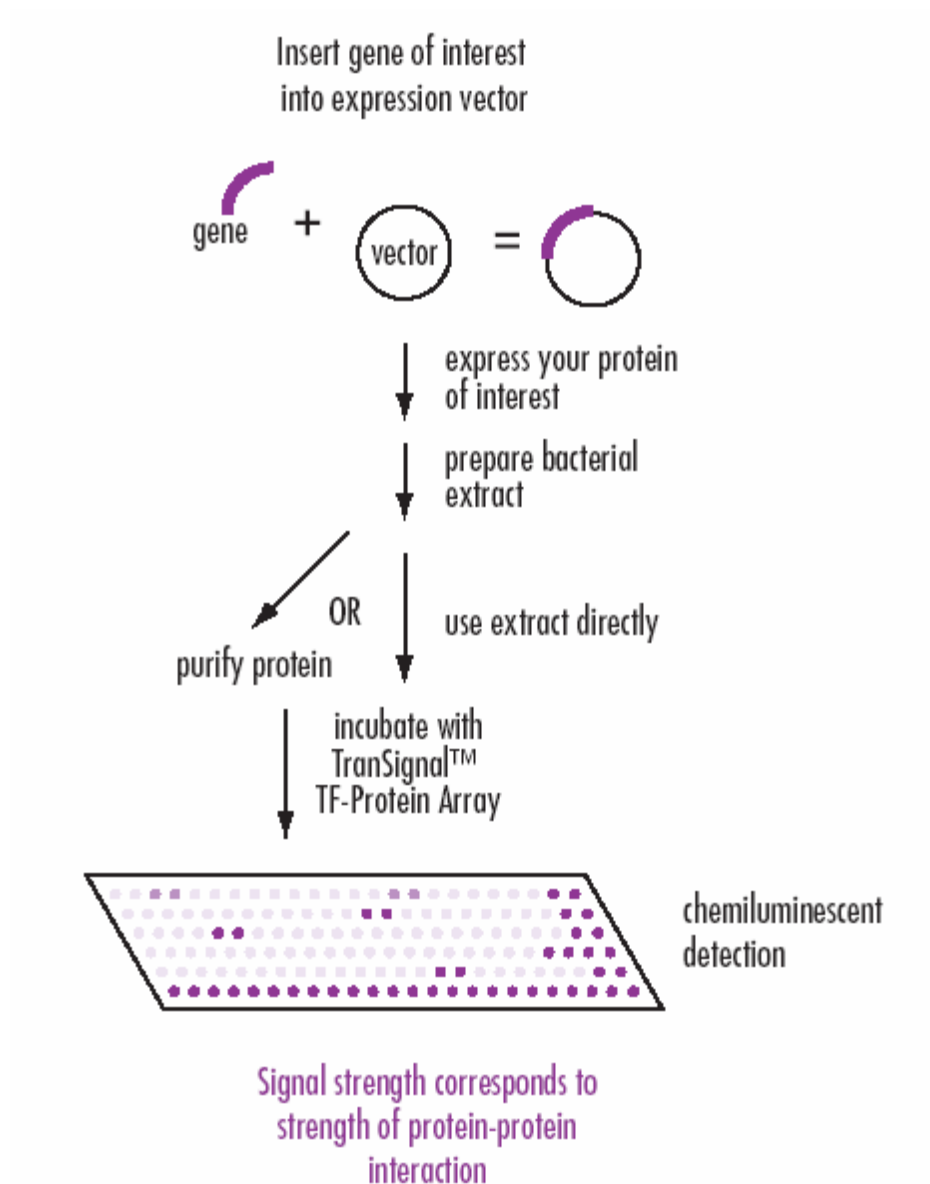
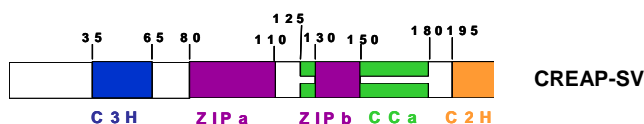


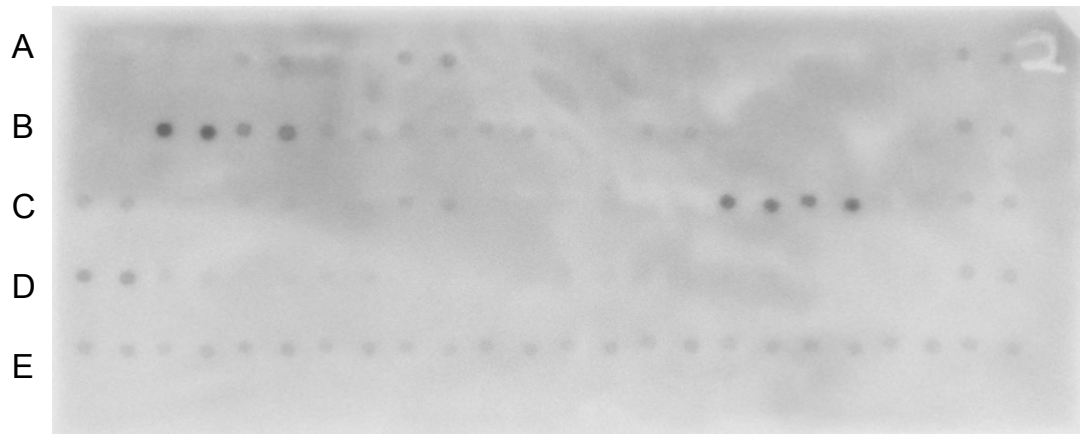
Figure 6.1. Flowchart of the TranSignal TF Array Method.

Highly purified CREAP protein prepared according to Section 2.5 was incubated with the TF Array according to the manufacturer's recommended conditions (see Section 2.15) and binding detected using the CREAP polyclonal antibody described in Section 2.7 of the Materials and Methods. Panel B of Figure 6.2 shows the results from screening the array with purified CREAPsv protein. This protein lacks the RS region, meaning that any interactions detected will be due to the transcriptionally related domains of CREAP including the leucine zipper-like and zinc finger-like domains. Comparing the antibody alone screen to that probed with CREAPsv, strong signals were observed with a range of TFs and CREAP. Those with the strongest 'signal to noise' ratio have been highlighted with red boxes. There was some overlap of the factors that interacted with the antibody alone and CREAPsv, such as EGR4 and PPAR α , but others that had interacted with the antibody didn't interact strongly with CREAP, such as ETS1 and PBX1. This indicates that the strong interactions with CREAP were likely to be genuine as the interaction displays specificity. Many of the strong interactors are members of the bZIP transcription factor family which supports the hypothesis that CREAP may be a novel member of the bZIP family or at least display bZIP-like activity.

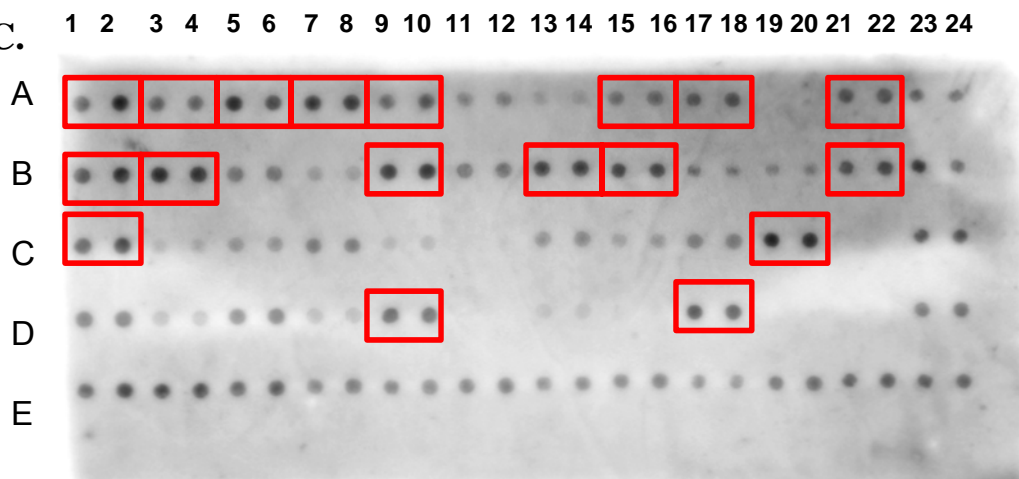
A.



B.



C.



ARRAY IV:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	AP2a		ATF1		ATF2		ATF3		ATF4		C/EBPa		CDX2		CREB1		E2F3		E2F4		EGR1		POS	
B	EGR2		EGR4		ETS1		ETS2		FOS		FOSB		GATA1		GR		HNF4G		IRF1		JUN		POS	
C	JUNB		MAX		MEF2A		MEF2B		NFE2		NFkBp50		NFkBp65		PAX6		PBX1		PPARa		PPARb		POS	
D	PPARg1		PPARg2		PXR-2		RXR-A		SP1		SP4		STAT1		v-Myc		YY1						POS	
E	POS		POS		POS		POS		POS		POS		POS		POS		POS		POS		POS		POS	

The TF Array IV was probed with CREAP antibody alone and also with purified CREAP protein. Panel A shows the schematic of the CREAPsv protein and the results from the antibody alone screen. Panel B shows the results from the antibody alone screen. Panel C shows the results from the CREAP screen and the location of each transcription factor on the array. Strong interactors are boxed in red.

To analyse these results in a non-biased fashion, densitometry was conducted on the arrays and the values from the antibody-alone array subtracted from the CREAP array results. The normalised values were then graphed in Figure 6.3 by rank. This confirmed that even some factors that interacted with the antibody still had strong interactions with CREAP while others didn't react strongly with CREAP even though they interacted with the antibody, suggesting that the strong interactions on the CREAP array are likely to be due to TF-CREAP interactions and not due to non-specific binding of the antibody.

The transcription factors were arbitrarily divided into strong, medium, low and no interactors based on visual assessment and densitometry of the interaction with CREAP. Those with a strong, medium, low or no interaction are listed in Tables 6.1 and 6.2. The majority of the strong interactors are from the bZIP and zinc finger transcription factor families.

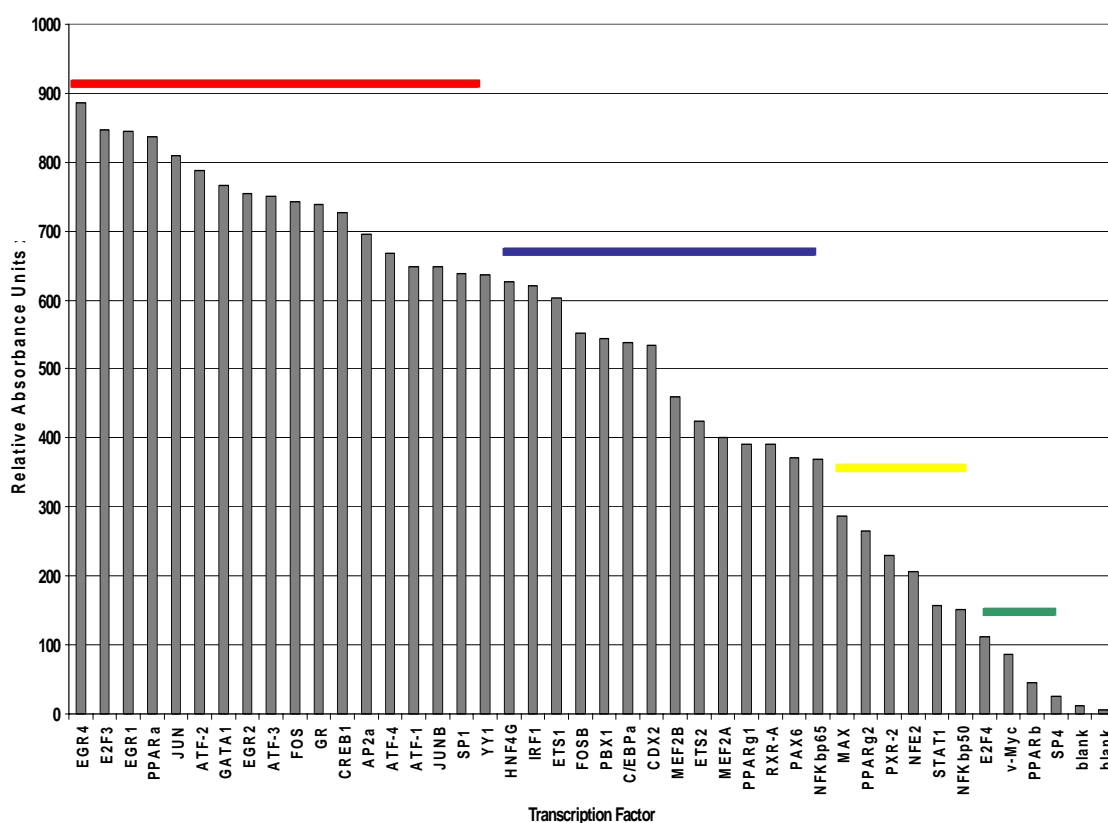


Figure 6.3. Normalised Densitometry Values for the CREAP-TF Array.

The densitometric values from the antibody alone and CREAP arrays were analysed using MultiGauge. The antibody alone values were subtracted from the CREAP values to account for any non-specific binding of the antibody. The relative absorbance units were plotted against the transcription factor. Red bars are strong interactors, blue bars are medium interactors, yellow bars are low interactors and green bars are no interactions.

<u>FACTOR</u>	<u>FAMILY</u>	<u>FUNCTION</u>
AP2α	bZIP and HLH like	Regulates CRH, hPL, hCG. Trophoblast differentiation
ATF1	bZIP	Transcriptional response to cAMP, maintain cell viability
ATF2	bZIP	Control of stress response genes, increase proliferation
ATF3	bZIP	Control of stress response genes
ATF4	bZIP	Repression of transcriptional response to cAMP
CREB1	bZIP	Modulates transcriptional response to cAMP. CRH regulation
E2F3	E2F, HLH	Regulates transcription during the cell cycle
EGR1	Zinc fingers	Neuronal plasticity, LH regulation
EGR2	Zinc fingers	Neuronal plasticity, Schwann cell development, peripheral nerve myelination
EGR4	Zinc fingers	Neuronal plasticity, male fertility
FOS	bZIP, AP1	CRH regulation. Differentiation, cell cycle, apoptosis
GATA1	Zinc fingers	Essential for normal erythropoiesis, and development of hematopoietic cells.
GR	Nuclear hormone receptor	CRH regulation, modulation of immune and inflammatory response, gluconeogenesis
JUN	bZIP, AP1	CRH regulation. Differentiation, cell cycle, apoptosis
JUNB	bZIP, AP1	Negative regulator of cell cycle
PPARα	Nuclear hormone receptor	Energy homeostasis, lipid catabolism,
SP1	C2H2 zinc fingers	Can act as activator of gene expression, important in growth and development. Regulation of steroid response in endometrium.
YY1	C2H2 zinc fingers	Multifunctional TF, can act as activator, repressor or initiator of transcription. May be an inhibitor of CRH

Table 6.1. Transcription Factors with a Strong Interaction with CREAP.

The transcription factors with a strong interaction with CREAP are listed in the table with their family and function.

<u>MEDIUM</u> <u>INTERACTION</u>	<u>LOW</u> <u>INTERACTION</u>	<u>NO</u> <u>INTERACTION</u>
C/EBPa	CDX2	E2F4
ETS1	ETS2	NFkBp50
ETS2	MAX	PPARb
FOSB	NFE2	SP4
HNF4G	PPARg2	vMyc
IRF1	PXR2	
MEF2A	STAT1	
MEF2B		
NFkBp65		
PAX6		
PBX1		
PPARg1		
RXRA		

Table 6.2. Transcription Factors with Medium, Low and No Interaction with CREAP.

The interaction of CREAP with the remaining transcription factors from the array were grouped by the strength of the interaction according to densitometry and visual examination.

6.2 Discussion

6.2.1 Overview

To determine if CREAP interacts with other transcription factors, a TranSignal TF Array IV was probed with CREAP_{sv} protein. With CREAP containing leucine zipper-like domains, protein dimerisation or interactions were predicted to be likely. The array chosen contained a variety of bZIP proteins and other factors known to be involved in CRH regulation. In this way the possibility of a role in CRH regulation could be investigated after the result of Chapter 5, with CREAP having negligible effect on CRH promoter activity. The array screen yielded the striking result that CREAP was able to interact with a relatively large number of TFs. These results will be discussed in regard to the available evidence to identify if these interactions may be relevant *in vivo* and indicate a biological role of these CREAP-TF interactions.

6.2.2 Roles of the Transcription Factors Identified to Interact with CREAP

6.2.2.1 AP2 α

AP2 α is a member of the AP2 (Activating Protein 2) family which has three isoforms, α , β and γ (Williams and Tjian 1991a). AP2 α is a 46 kDa protein which functions as a dimer which recognises the palindromic AP2 site (Williams and Tjian 1991b). It consists of a C-terminal DNA binding domain and an N-terminal proline-rich trans-activation domain. The C-terminal domain is responsible for DNA binding and dimerisation and is organised like a bZIP and helix-loop-helix (HLH) region with a basic region adjacent to an α -helical structure (Williams and Tjian 1991a; Williams and

Tjian 1991b). There are however many AP2 α -dependent genes that do not have a consensus AP2 site (Pena et al. 1999), suggesting that AP2 α may also function by interacting with other transcription factors. For example, AP2 α mediates the expression of CYP11A1 through its interaction with Sp1 (Pena et al. 1999) and the GR forms complexes with AP2 α in some cells (Ebert et al. 1998).

AP2 α induces the expression of hPL, hCG and other syncytiotrophoblast marker genes (Johnson et al. 1997; LiCalsi et al. 2000; Richardson et al. 2000). AP2 α also induces CRH promoter activity and mRNA levels but the induction is not due to AP2 α binding to the CRH promoter but due to its interaction with CREB (Cheng and Handwerger 2002).

6.2.2.2 *ATF1 , 2, 3 and 4*

Activating Transcription Factors (ATFs) are bZIP transcription factors that recognise the CRE and are related to CREB (Montminy et al. 1986). They are members of the bZIP transcription factor family. They can form homodimers or heterodimers with CREB and the AP1 proteins, Fos and Jun (Hai and Curran 1991). ATF1 modulates the transcriptional response to cAMP (De Cesare and Sassone-Corsi 2000), while ATF4 acts as negative regulator of CRE-dependent transcription (Hai and Hartman 2001). ATF2 and 3 are involved in the transcriptional control of stress response genes (Hai et al. 1999).

6.2.2.3 *CREB*

CRE-binding Protein (CREB) is a member of the bZIP transcription factor family. It binds to the CRE of target gene promoters as a dimer (Yamamoto et al. 1988). CREB must be phosphorylated by PKA at Ser133 to activate transcription (Montminy and Bilezikjian 1987; Gonzalez and Montminy 1989; Gonzalez et al. 1989). It is a major regulator of cAMP transcriptional effects. CREB is part of the protein complex that regulates CRH hormone in both the pituitary and placenta (King et al. 2002). The results of Chapter 5 suggested that CREAP had a slightly inhibitory effect on CREB-mediated CRH promoter activity. It is possible that CREAP may interact with CREB to form an inactive heterodimer, which reduces the number of active CREB homodimers available for gene induction.

6.2.2.4 *E2F3*

Transcription factor E2F denotes a family of helix-loop-helix proteins (Jordan et al. 1994) with eight members (Attwooll et al. 2004; Dimova and Dyson 2005). They have been shown to be essential for regulation of cell proliferation (Dyson 1998; Nevins 1998). E2F1-3 seem to function as transcriptional activators, E2F4 and 5 appear to function as retinoblastoma (Rb)-dependent transcriptional repressors while E2F6-8 may function as Rb-independent repressors (Dimova and Dyson 2005). They bind a defined DNA motif as a heterodimer together with the DP proteins (Huber et al. 1993; Wu et al. 1995). E2F3 is essential for cells to enter the S phase of the cell cycle (Kong et al. 2007).

There is some sequence similarity between E2F1-3 in the N-terminal domain, but E2F4 and -5 almost completely lack this region (Karlseder et al. 1996). E2F1, 2 and 3 have been shown to bind Sp1 *in vitro* through this region while E2F4 and 5 do not (Karlseder et al. 1996). This result may explain why E2F3 but not E2F4 interacted with CREAP on the array.

6.2.2.5 *EGR1, 2 and 4*

The Early Growth Response (EGR) family gene expression is induced by growth factors. The family has four members, EGR1, 2, 3 and 4. They have a highly conserved DNA-binding domain consisting of three C2H2 zinc-finger motifs which binds to a GC-rich consensus element (Gashler and Sukhatme 1995). EGR1 is the best characterised member of the family and is induced in various cell types under a range of stimuli such as growth factors, UV light, radiation and differentiation induction (Gashler and Sukhatme 1995). EGR1 has been shown to act as both a positive and negative regulator of transcription (Cao et al. 1993). Overlapping EGR1 and Sp1 binding sites have been identified in several gene promoters (Skerka et al. 1995).

6.2.2.6 *Fos, Jun and JunB*

The Activating Protein 1 (AP-1) transcription factor is mainly composed of Jun and Fos protein dimers. They bind to a consensus AP-1 site (TGAG/CTCA) (Angel and Karin 1991). The Jun family is composed of c-Jun (Jun), JunB and JunD while the Fos family is composed of c-Fos (Fos), FosB, Fra-1 and-2 (Hess et al. 2004). Each of these proteins is differentially expressed and regulated so that every cell type contains a

complex mixture of AP-1 dimers with different functions (Wagner 2001). The common motif of these proteins is the bZIP domain which is used for DNA binding and dimerisation. Jun and Fos are considered strong transactivators while JunB is considered a weak one (Hess et al. 2004). AP-1 has been linked to proliferation, differentiation, apoptosis, stress and neoplastic transformation (Angel and Karin 1991).

Jun and Fos have been found to form complexes with CREB on the CRE of the CRH promoter (King et al. 2002). CREB, Jun and Fos all have leucine zipper domains for protein dimerisation, as does CREAP, providing a means by which CREAP could be interacting with these factors.

6.2.2.7 *GATA-1*

GATA-1 is highly expressed in hematopoietic cells. Its primary function is to regulate the transcription of genes that are involved in the development of cells in the hematopoietic lineage (Morceau et al. 2004). The GATA protein contains two treble-clef zinc finger domains that are used in DNA binding and for protein-protein interactions (Lowry and Mackay 2006). GATA-1 has been shown to interact with a variety of proteins including itself, CREB-binding protein/P300 and Sp1 (Lowry and Mackay 2006). The CRH promoter contains four consensus GATA response regions suggesting a role for GATA and CREAP in CRH regulation.

6.2.2.8 GR

The Glucocorticoid Receptor (GR) is a member of the nuclear hormone receptor superfamily. The GR possesses a modular structure consisting of an N-terminal domain, DNA binding domain and ligand binding domain. When the GR binds its ligand, glucocorticoid, the activated receptor enters the nucleus and interacts with regulatory sites on the target gene to either enhance or repress transcription (Kumar et al. 2004). GR's recognise a specific glucocorticoid response element (GRE), however GRs also modulate expression of genes that do not contain a consensus GRE (Cheng et al. 2000b). It has been suggested that all the important functions of GR may be reliant on protein-protein interactions (Reichardt et al. 1998). Glucocorticoids modulate CRH expression in both the hypothalamus and placenta and this is more likely to be through protein-protein interactions as the CRH promoter lacks a full consensus GRE (Van et al. 1990; Guardiola-Diaz et al. 1996; Malkoski et al. 1997; Malkoski and Dorin 1999; Cheng et al. 2000b; King et al. 2002; van der Laan et al. 2007).

6.2.2.9 PPAR α

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that are involved in energy homeostasis (Escher and Wahli 2000). Upon ligand binding and activation, PPARs stimulate gene expression by binding to specific regions of the promoter of target genes. There are three isotypes of the family; PPAR- α , - β and - γ (Escher and Wahli 2000). PPAR α is well expressed in human liver, heart, kidney, skeletal muscle, intestine and pancreas and is detectable in lung, placenta, ovary, testis and adipose tissue (Auboeuf et al. 1997; Mukherjee et al. 1997; Froment et al. 2006).

PPAR α is involved in lipid oxidation, mitochondrial fatty acid metabolism, and the general mechanism maintaining lipid homeostasis (Escher and Wahli 2000).

6.2.2.10 *SP1*

Sp1 is a member of the Sp family of transcription factors that contain three conserved C2H2 zinc fingers that form the DNA-binding domain. They can bind and act through GC boxes to regulate the expression of multiple genes (Li et al. 2004). Sp1 may act as a positive or negative regulator of gene expression (Shou et al. 1998; Suske 1999; Barth et al. 2002; Bouwman and Philipsen 2002). Sp1 also regulates gene expression by interacting with other proteins. Protein-binding sites have been identified throughout Sp1 for transcription-associated proteins, DNA binding proteins, transcriptional regulators and chromatin remodelling factors, including YY1 and E2F (Li et al. 2004). It has been found that YY1 and Sp1 can form a physical complex and that domains within each protein mediate the interaction (Lee et al. 1993).

6.2.2.11 *Ying Yang 1 (YY1)*

YY1 is a multifunctional protein that can act as a transcriptional activator or repressor, or an initiator element binding protein that directs and initiates transcription (Shi et al. 1997). It contains four C2H2 type zinc fingers at its C-terminus which are responsible for sequence-specific DNA binding (Hariharan et al. 1991) as well as YY1's repression function (Lee et al. 1994; Bushmeyer et al. 1995; Lee et al. 1995a; Lee et al. 1995b).

YY1 has been shown to negatively regulate the transcriptional activity of CREB, most likely through a physical interaction between the zinc fingers of YY1 and the bZIP region of CREB (Zhou and Engel 1995; Zhou et al. 1995; Shi et al. 1997). A similar repression of Sp1 may also exist (Shi et al. 1997).

The CRH promoter contains a YYI response element only 7bp upstream of the CRE suggesting that these response elements and the proteins that bind them could interact to modulate CRH expression.

6.2.3 Validating the CREAP-TF Interactions

To try and confirm *in vivo*, some of these TF interactions with CREAP, immunoprecipitation experiments were performed on JEG-3 cells. Antibodies from Santa Cruz Biotechnology against CREB (sc-186 and sc-58), GR (sc-1003), JunB (sc-46) and Fos (sc-253) were used to immunoprecipitate (IP) nuclear and whole cell lysates, and the samples were western blotted. The protein lysates were incubated with antibodies against CREAP, CREB, GR, JunB or Fos and Protein A agarose (Santa Cruz). The IPs were washed to remove unbound proteins and the samples run on a NuPAGE gel, transferred to nitrocellulose for immunoblotting. CREAP does not appear to co-immunoprecipitate with any of these factors (no specific bands detected so data is not shown), however whether this is due to there truly being no interaction or a technical problem is unknown. Perhaps the antibodies were not the optimal antibodies to use for IP, the lysis method may not have been suitable to isolate interacting proteins, the interaction may be transient and difficult to capture, or the number of interacting molecules is too low to detect. Given that the majority of CREAP appears to localise to

nuclear speckles and this isn't a common site for transcription factors then it is possible that CREAP is capable of interaction with these factor but that only a very small number of protein complexes are formed at any one time and therefore can not be detected at the western blotting sensitivity level.

6.2.4 *Summary of Array Results*

The majority of transcription factors that interacted strongly with CREAP in the array are members of the bZIP and zinc finger families. Interaction with the bZIP family was predicted as CREAP contains leucine zipper like domains and these zippers mediate dimerisation of member proteins. CREAP also contains zinc finger motifs and these have been shown to be involved in protein-protein interactions in other factors. Many of these transcription factors have already been shown to interact with each other so it is possible that CREAP may be another component of these protein complexes. Many of the interacting proteins have roles in CRH regulation, further supporting the hypothesis that CREAP is involved in some aspect of CRH regulation.

Chapter 7. siRNA and Microarray Analysis of CREAP

7.1 Rationale

From the previous chapters it was found that CREAP contains domains that are implicated in both transcription and splicing. CREAP localises to the nucleus and to splicing speckles and was shown to interact with the splicing protein, SC35, suggesting a role in splicing. Investigation into whether overexpression of CREAP could regulate the CRH promoter or CRE-containing promoters indicated that CREAP is most likely not involved in CRH regulation. To determine if CREAP could interact with other transcription factors (TF), a TF array was probed with purified CREAP protein and several TF proteins, mostly from the bZIP protein family were found to have a strong interaction. Therefore, to try and further elucidate CREAPs role in transcription, siRNA mediated gene repression was conducted in conjunction with a genome wide expression analysis using microarray technology.

7.2 siRNA-Mediated Gene Knockdown of CREAP

7.2.1 Background

Gene ‘knockout’ by homologous recombination has previously been the common method to determine gene function in mammals, but it is costly, time consuming and not applicable to many organisms. The discovery of RNA interference (RNAi) gene ‘knockdown’ ignited a revolution in cell genetics (Dykxhoorn et al. 2003). RNAi was discovered when Fire and colleagues injected double-stranded (ds)RNA into *Caenorhabditis elegans* and found that it led to efficient sequence-specific gene

silencing (Fire et al. 1998). It was found that in *Drosophila*, long dsRNA molecules could be cleaved into short interfering dsRNA species (siRNAs) (Zamore et al. 2000). siRNAs are double-stranded ~22nt RNA species with two-nucleotide overhangs at each 3' end (Elbashir et al. 2001c). The introduction of chemically synthesised 21 and 22nt siRNAs to *Drosophila* embryo extracts resulted in the same type of degradation of homologous RNA as the cleaved dsRNA (Elbashir et al. 2001b).

There is a family of RNase III enzymes known as Dicer that mediate dsRNA cleavage (Bernstein et al. 2001; Billy et al. 2001; Ketting et al. 2001). A Dicer-R2D2 heterodimer cleaves the long dsRNA into siRNAs which are then incorporated into a multi-protein RNA-induced silencing complex (RISC). The siRNA is unwound leaving the antisense strand to guide RISC to its homologous target mRNA resulting in endonucleocytic cleavage of the target mRNA (Dykxhoorn et al. 2003) (Figure 7.1).

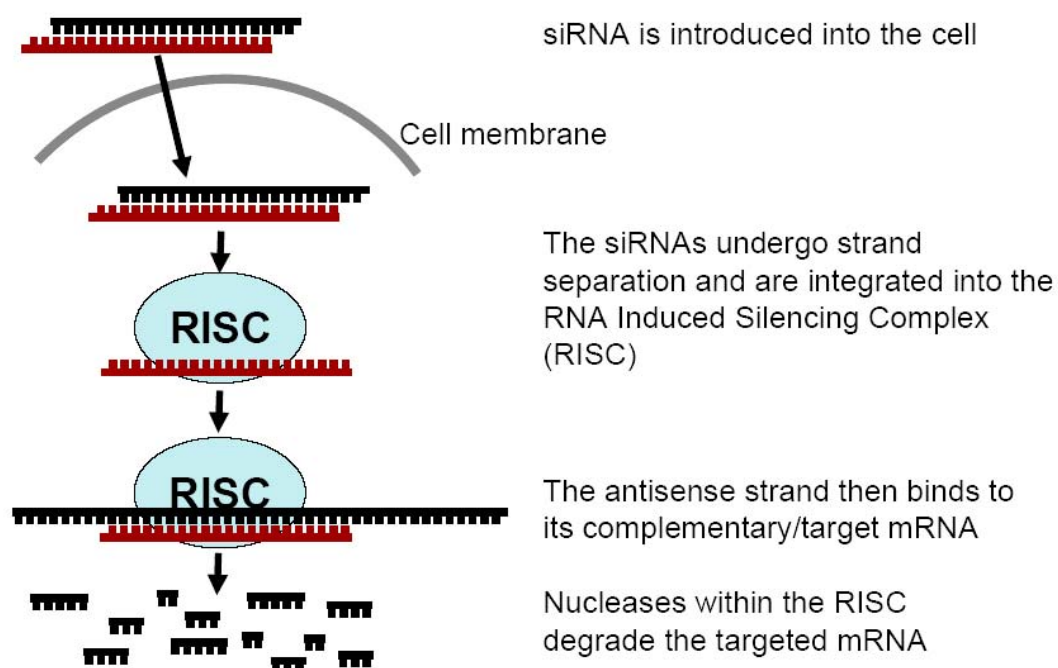


Figure 7.1. Mechanism of siRNA.

This diagram shows the mechanism of siRNA-mediated gene silencing. When siRNA is introduced into the cell it induces the RNA-Induced Silencing Complex (RISC) which directs the siRNA to the target mRNA sequences causing mRNA degradation and thus gene silencing. Adapted from Ambion, www.ambion.com.

Long dsRNA has been used to investigate gene function in various organisms. The dsRNA is cleaved into many siRNAs coding for different regions of the target sequence allowing efficient silencing, but the introduction of dsRNA longer than 30nt into mammalian cells induces a non-specific interferon response (Elbashir et al. 2001a). Interferon triggers degradation of mRNA and causes global inhibition of translation (Stark et al. 1998). Therefore synthetic siRNAs of less than 30nt must be used in mammalian cells so that the interferon response is not initiated (Holen et al. 2002; Dykxhoorn et al. 2003). siRNAs that target different regions of the same gene vary

markedly in their silencing effect (Holen et al. 2002; Miyagishi and Taira 2002). It is not only the base composition of the siRNA but also the secondary structure of the target mRNA and the location of any RNA binding proteins that determines the silencing efficiency of siRNA (Dykxhoorn et al. 2003). The design of synthetic siRNAs tends to be an empirical process with no firm guidelines in place. Many commercial companies are using patented design algorithms to predict the most effective siRNA sequences.

There are several disadvantages of siRNA. The transfection is only transient and the silencing effect is eventually lost. For stable knockdown, plasmid vector or virus vector mediated RNAi must be used. Also, the siRNA must be synthesised and cannot be replicated like a plasmid or virus vector, which increases the cost. However, it still remains a relatively affordable process and can tend to give more reliable and efficient results than relying on vector expression and correct post-transcriptional processing to generate functional siRNAs in the cell. Several plasmid-based RNAi systems were assessed for this project without success however siRNA transfection worked well to give knockdown of CREAP.

7.2.2 Optimisation in JEG-3 Cells

The first step in the siRNA pathway was to determine whether the JEG-3 cells were able to take up the siRNA. Qiagen provides a specific transfection reagent, HiPerFect, for siRNA. The ratio of HiPerFect Transfection Reagent to siRNA needs to be optimized for every new cell type and siRNA combination used. As a starting point for

optimization, 75ng (10 nM) Alexa Fluor 488 labelled siRNA and 3 or 4.5 μ l HiPerFect were used in 24 well plates. The cells were assessed for fluorescence as a measure of transfection efficiency, 24 h after transfection using a fluorescent microscope. The results are shown in Figure 7.2. The 3 μ l HiPerFect transfected cells appear to have more green transfected cells, however transfected cells are also present in the 4.5 μ l-treated cells. This shows that the JEG-3 cells were readily capable of being transfected with siRNA and 3 μ l HiPerFect is better than 4.5 μ l.

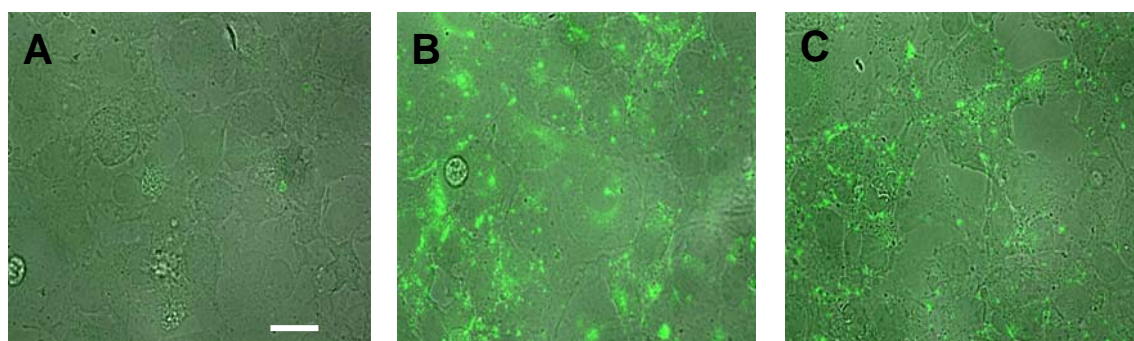


Figure 7.2. Transfection Efficiency of siRNA into JEG-3.

Alexa Fluor-488 labelled scrambled (negative control) siRNA was transfected into JEG-3 cells and fluorescent microscopy used to detect green transfected cells 24 h later. The merged image of the light and fluorescent images is shown. **A-** negative control untransfected cells. **B-** 75ng siRNA and 3 μ l Hiperfect transfection reagent. **C-** 75ng siRNA and 4.5 μ l Hiperfect transfection reagent. Scale bar represents 200 μ m.

7.2.2.1 *HP Guaranteed siRNAs*

With the amount of transfection reagent optimised, the concentration of CREAP-specific siRNA required for efficient knockdown of protein expression was determined.

Qiagen's HP Guaranteed siRNA consists of four custom designed siRNA's directed against the target gene. It is guaranteed that at least two of the siRNAs would produce knockdown.

Since CREAP siRNA was to be employed in a functional assay it was important to examine protein knockdown, rather than just mRNA knockdown, and quantitative western blotting was used. Different amounts of siRNA (scrambled negative control, MAPK positive control and the four CREAP siRNAs) with different time points were tested for CREAP and the positive siRNA control, MAPK, protein expression. The scrambled siRNA acts as a negative control by providing a non-silencing sequence, but subjects the cells to the process of RNA transfection. The results of these optimisation experiments are shown in Figure 7.3.

The optimal conditions were determined to be 40 nM siRNA and 96 h incubation, resulting in CREAP protein expression level knock down by approximately 80%. All four of the CREAP siRNA sequences gave significant knockdown of expression under these conditions. Interestingly, there did seem to be a slight knockdown of CREAP expression by the MAPK siRNA but whether this is an off-target effect or whether CREAP and the MAPK pathway may interact in some way is unknown.

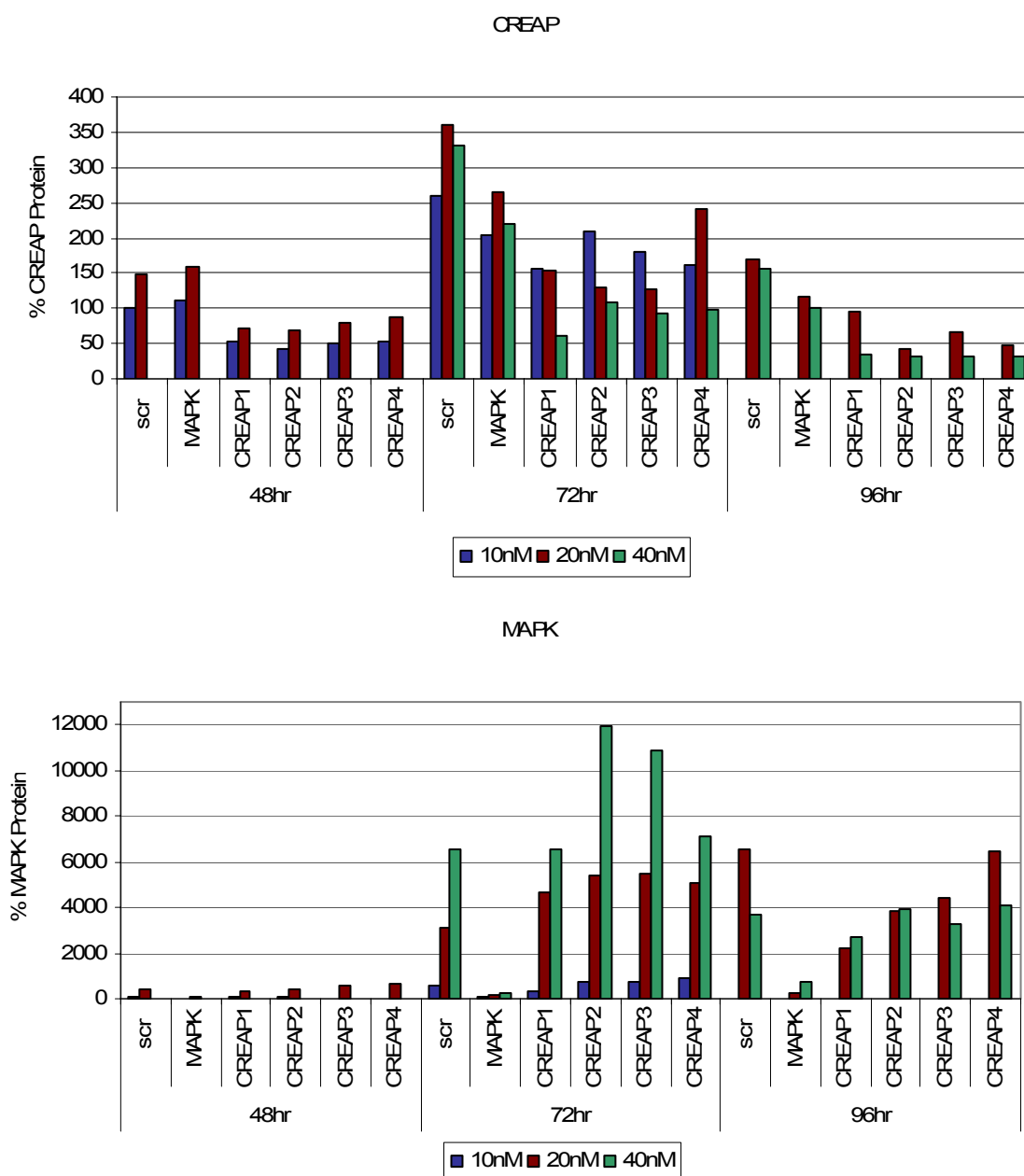


Figure 7.3. siRNA Optimisation Results on CREAP and MAPK Protein Expression.

Incubation times of 48, 72 and 96 h as well as siRNA concentrations of 10, 20 and 40 nM were tested on JEG-3 cells transfected using 3 μ l HiPerFect transfection reagent. Cells were lysed in LDS sample buffer and run on 10% Bis-Tris/MOPS gels and western blotted. Blots were probed with CREAP anti-sera, MAPK polyclonal antibody and normalised to actin. The percentage of remaining protein relative to 10 nM, 48 h scrambled control is shown.

Figure 7.4 shows an example of a typical western blot of the siRNA samples. As can be seen, the scrambled and MAPK siRNA transfected cells still show expression of 58 kDa CREAP while the CREAP siRNA cells have reduced expression. The MAPK antibody (Santa Cruz Biotechnology) picks up two isoforms of MAPK, a 44 and 42 kDa form. The 42 kDa MAPK1 isoform is targeted by the siRNA and shows dramatically reduced expression in treated cells.

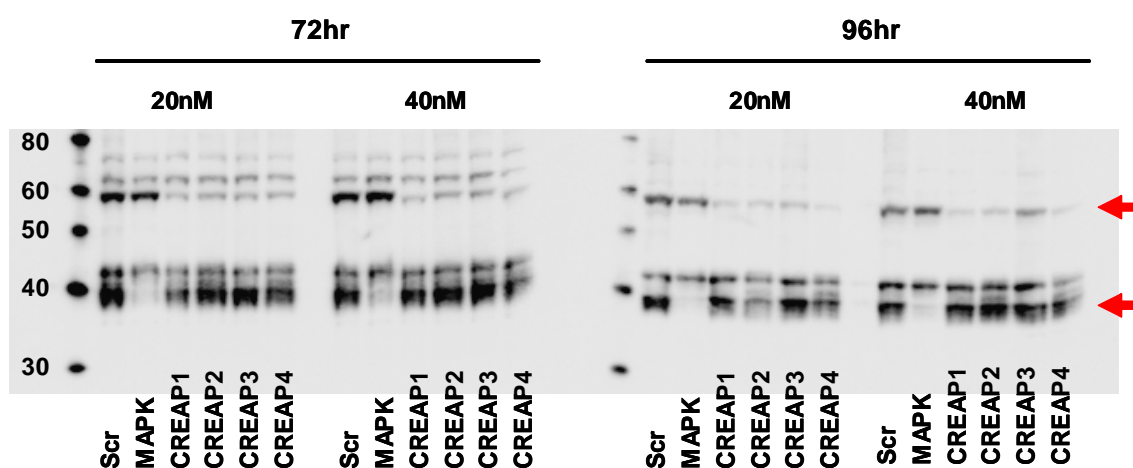


Figure 7.4. Typical Western Blot of siRNA Transfected JEG-3 Cells.

JEG-3 cells were transfected with scrambled, MAPK or CREAP siRNAs and the cells lysed in 2D buffer and western blotted with CREAP and MAPK antibodies. The red arrows indicate the 58 kDa CREAP and 42 kDa MAPK1 bands.

The MAPK siRNA was transfected as a validated positive control to ensure the protocol was working. The MAPK siRNA resulted in up to 99% knockdown of MAPK1 protein expression which confirmed the technique was working efficiently. The expression of MAPK1 in CREAP siRNA-treated cells was also analysed. Under the conditions of

optimal CREAP knockdown (40 nM, 96 h), there was some slight inhibition of MAPK1 by CREAP 1 and 3 sequences, but none by CREAP 2 and 4. Therefore, given that CREAP4 gave the best knockdown of CREAP and had no effect on MAPK1 expression, it was chosen for use in the microarray experiments.

7.2.3 Fibroblast Cell siRNA Transfection

The siRNA-mediated knockdown of CREAP was optimised as described above in JEG-3 cells. However to perform the microarray analysis, normal human fibroblasts were selected, based on the expert advice of Professor RJ Scott, University of Newcastle. A non-transformed, non-cancerous cell makes genetic analysis easier as the results would not be confounded by genetic mutations related to JEG-3 cells being derived from a choriocarcinoma.

To determine if the same siRNA transfection conditions as had been optimised for the JEG-3 cells were applicable to the fibroblast cells, transfection and western blotting analysis was conducted. Figure 7.5, shows a typical western blot of fibroblasts transfected with 40 nM siRNA, 3 μ l HiPerFect and incubated for 72 h or 96 h. The 96 h lysates show good knockdown of CREAP by all four CREAP siRNAs while the scrambled and MAPK transfected cells still show CREAP expression. This indicates that the conditions optimised in the JEG-3 cells are appropriate for use with the fibroblasts.

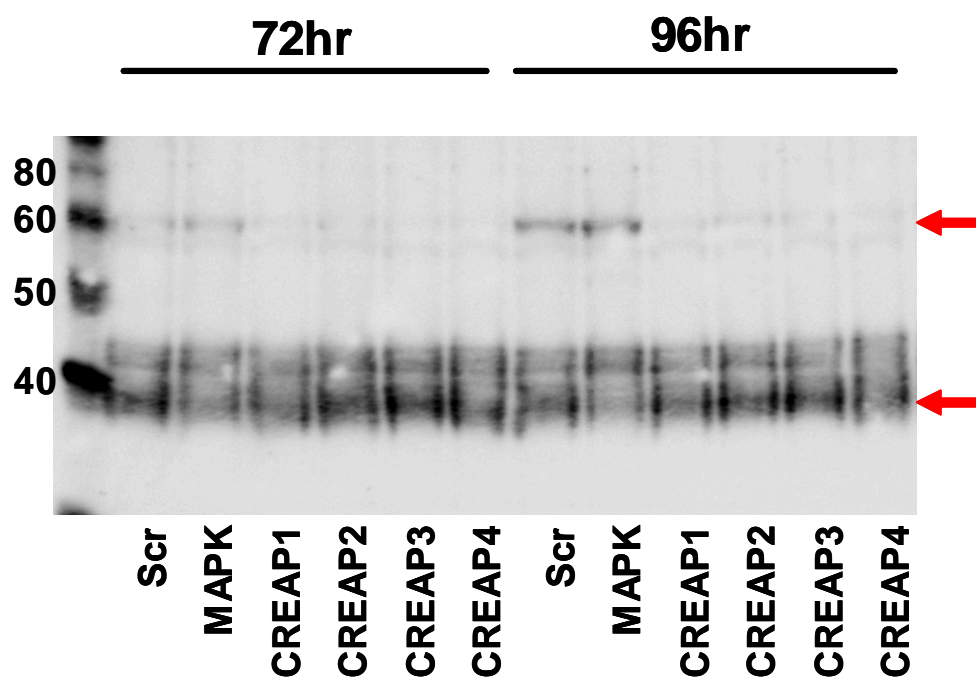


Figure 7.5. Typical Fibroblast siRNA Western Blot.

Fibroblast cells were transfected with 3 μ l HiPerFect and 40 nM siRNA. Cells were lysed at 72 or 96 h and samples western blotted and probed with CREAP and MAPK antibodies. The arrows indicate the CREAP and MAPK1 protein bands.

With efficient siRNA-mediated knockdown of CREAP protein levels confirmed in both the fibroblasts and JEG-3 cells, a robust and efficient knockdown protocol had been established. The CREAP4 siRNA seemed to give the highest levels of knockdown consistently and therefore it was used for all further experiments. The next step was to scale up the experiments in the fibroblast cells to obtain enough quality RNA for microarray analysis.

7.3 Gene Expression Profiling of CREAP Knockdown in Fibroblast Cells.

7.3.1 Illumina Microarray Technology

Microarray experiments allow high-throughput genome-wide expression profiling to investigate the effect of specific treatments or conditions. mRNA from the target cells or tissues is hybridised against an array of oligonucleotide probes for specific gene transcripts. In this way, up- or down-regulation of gene expression between samples can be determined and the data analysed using ontology annotation programs to determine if the altered genes fit into biological pathways, functions or processes. In this instance the effect of knocking down the expression of the CREAP gene was investigated to further elucidate what role CREAP is playing in the cell; whether it be transcriptional, splicing or something else entirely. With Illumina's multi-sample BeadChip format, up to eight samples can be profiled in parallel on single BeadChip, dramatically increasing throughput while decreasing experimental variability. The 100% hybridization-based QC on every probe ensures the best available performance

and reproducibility. The HumanRef-8 BeadChip used here covers over 24,000 transcripts with 18,631 unique, curated genes sourced from the RefSeq database.

7.3.2 *Sample Preparation*

The fibroblast siRNA transfections were conducted in three independent experiments in 6 well plates with duplicates of each treatment; untreated fibroblasts, scrambled siRNA to act as a control for the effect of transfection, and CREAP4 siRNA. RNA was extracted from all wells and identical treatments pooled, to give three experiments done in duplicate, giving a total of three RNA pools per siRNA transfection and two for untreated cells. Each pool of RNA was run as one sample on the HumanRef-8 v2 BeadChip so that there were three scrambled, three CREAP siRNA and two untreated control RNA samples on the chip. The results from biological replicates were pooled for analysis.

7.3.2.1 *Heat Map and Dendogram*

The gene expression profiles of the untreated control, scrambled negative control and CREAP siRNA were compared with a heat map. The up-regulated genes are shown in red, down-regulated genes in blue, and unchanged genes in yellow (see Figure 7.6). A dendogram is used to represent the relationships between the three groups. The untreated and scrambled control are grouped together on one branch and the CREAP siRNA on the other indicating that there is a significant difference in the gene expression profile of the knockdown sample compared to the controls. This also indicates that the untreated and scrambled controls have a very similar profile and that

the siRNA transfection procedure has not significantly altered the fibroblast's normal expression profile. 1440 genes were found to be significantly expressed ($p < 0.05$) between the CREAP siRNA and scrambled control using the statistical analysis described in Section 2.18.2.

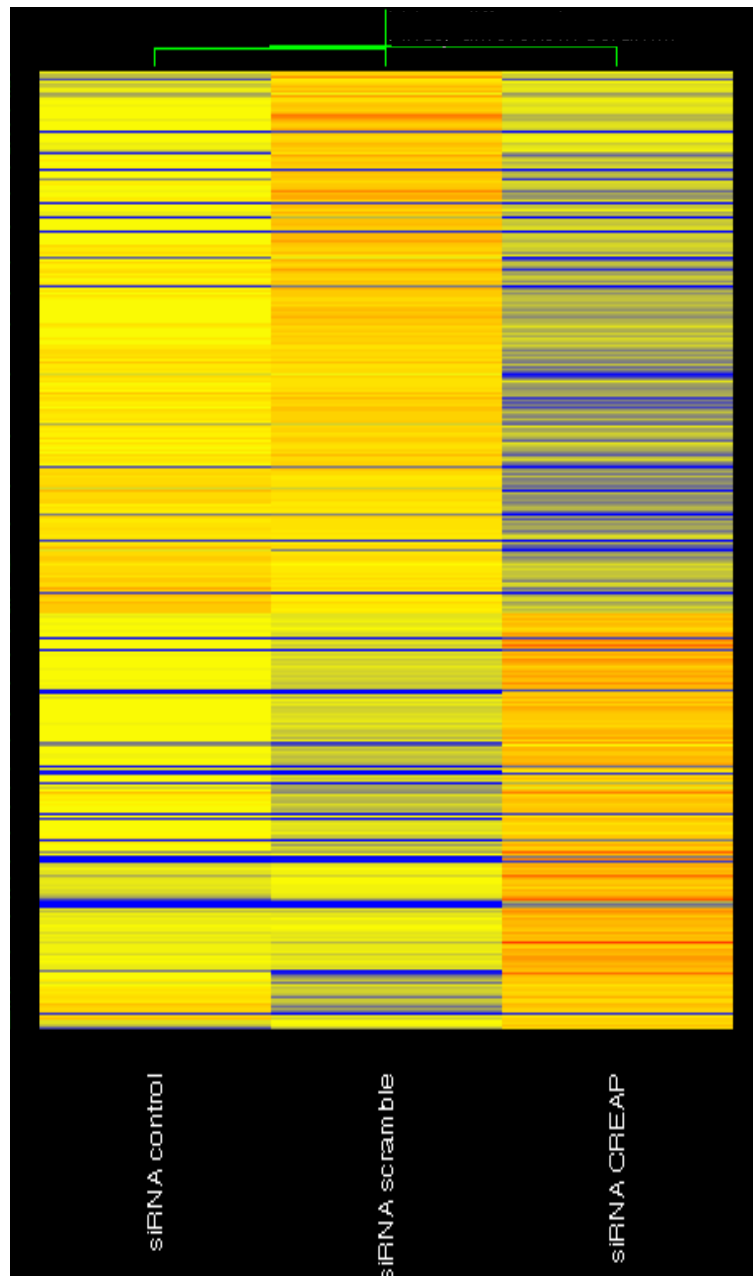


Figure 7.6. Comparison of Gene Expression Profiles.

The gene expression profiles for the untreated control, scrambled siRNA negative control and the CREAP siRNA samples is shown. Down-regulation is represented as blue, up-regulation as red and normal expression as yellow. The dendrogram on top represents relationship between and within groups, with the control and scrambled profiles being grouped together, indicating their profiles are similar, and the CREAP siRNA being on a different branch indicating its expression profile is different to the controls.

7.3.2.2 *Confirmation of CREAP mRNA Knockdown*

The HumanRef-8 v3.0 Expression Beadchip contained two variants of the CREAP identical protein, CROP. The CROP gene encodes the same protein as CREAP. To determine if CREAP/CROP mRNA was truly knocked down in the siRNA-treated sample, the expression of the CROP variants was examined. The values of expression intensity have been normalised by log transformation. CROP variant 2 lacks an internal region in the 3' UTR, as compared to variant 1, but encodes an identical protein. There was a difference in the expression of the two variants in siRNA-treated cells but both showed significant reduction in expression. Variant 1 was reduced 13.5-fold (p value=0.007) while variant 2 was reduced 30.8-fold (p value=0.0324) (Figure 7.7). The reason for this difference is unclear as the only variation in the isoforms is in the untranslated region, and the siRNA target sequence is located in the coding region so would be present in both variants. Nevertheless, the CREAP/CROP mRNA was significantly reduced in the knockdown samples indicating the siRNA has caused an efficient reduction in CREAP mRNA expression, consistent with the observed knockdown of protein levels demonstrated by western blotting (Figure 7.5).

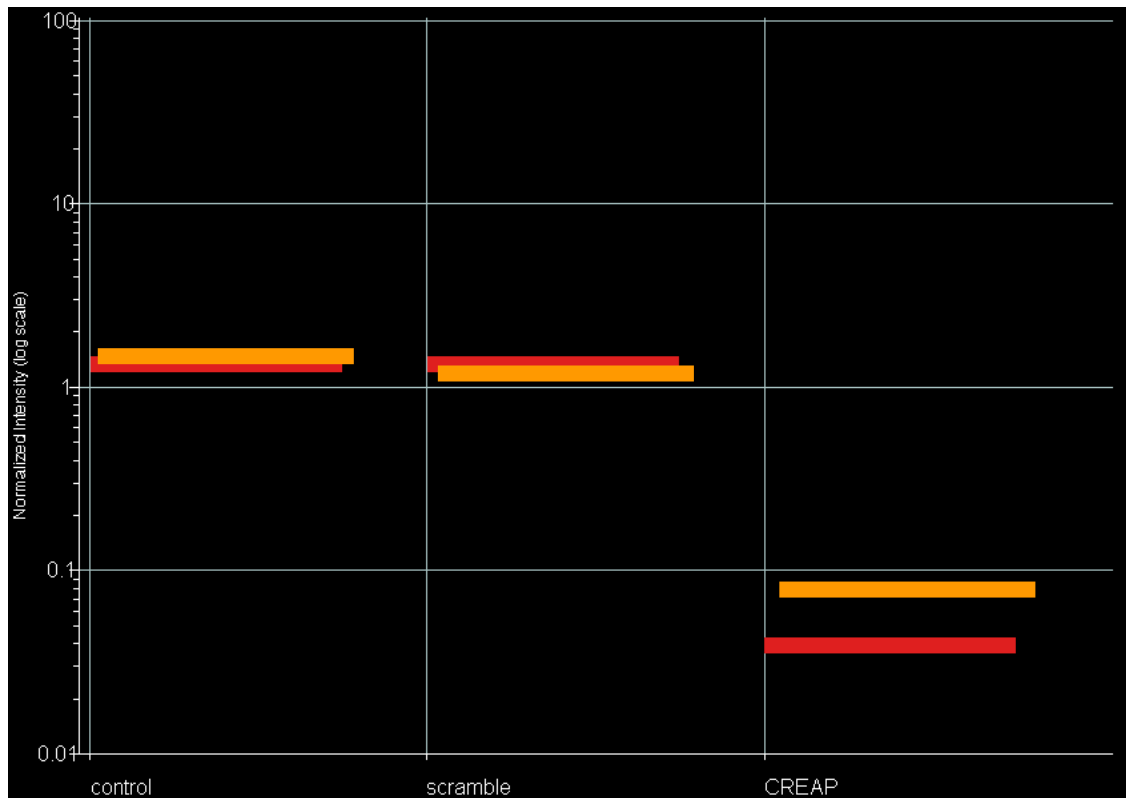


Figure 7.7. Comparison of the Expression of CROP/CREAP mRNA in siRNA Treated Fibroblasts vs Untreated and Negative Scrambled siRNA Controls.

The normalised expression intensities of the two CROP/CREAP variants present on the Human Ref-8 BeadChip from untreated control, scrambled negative control and CREAP siRNA-treated samples is graphically shown. The CROP variant 1 is represented by orange bars and the CROP variant 2 by red bars. CROP mRNA is significantly reduced in the CREAP siRNA samples, ($p=0.007$ and $p=0.032$ respectively).

7.3.2.3 Volcano Plot

To further analyse the significantly altered genes between scrambled and CREAP siRNA-treated cells, the data set was subjected to a volcano plot analysis. This divides the data set according to threshold values to generate a list of the most significantly

altered genes. Genes that are altered at least 2-fold and have a p value <0.05 are selected and a gene list and graph generated (see Figure 7.8). From this analysis, 617 genes were found to have fold changes greater than 2 and p values less than 0.05.

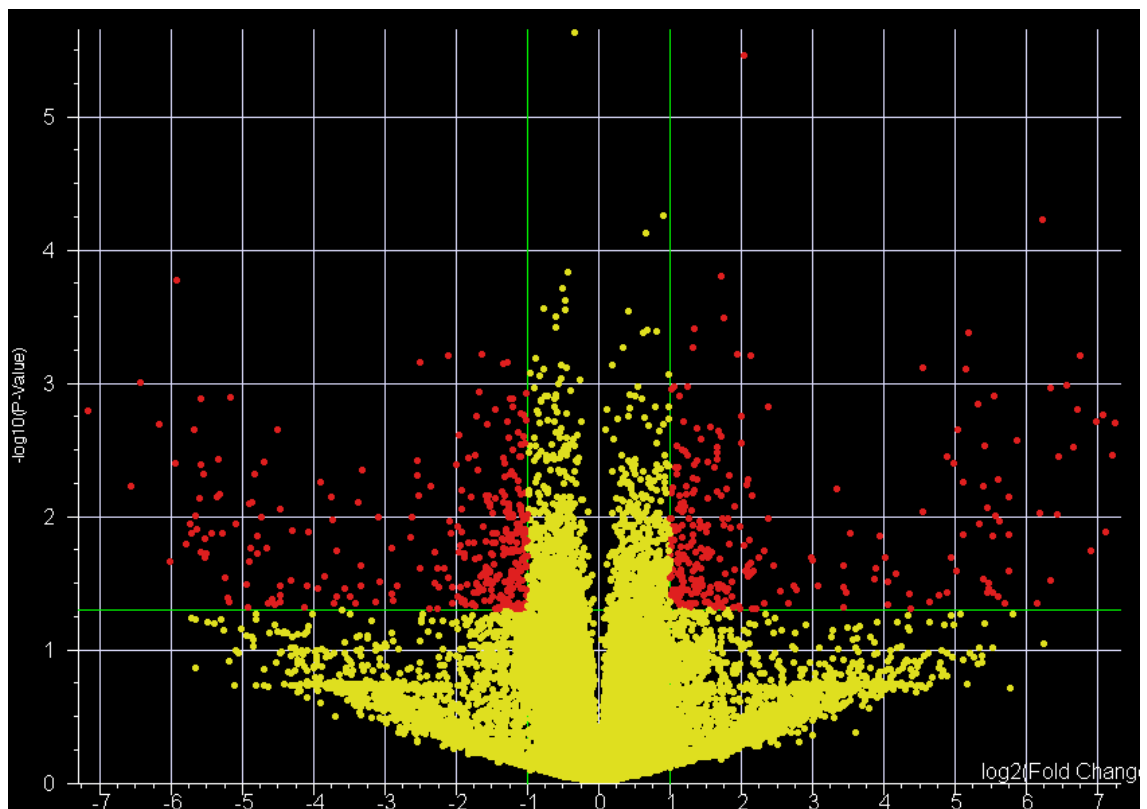


Figure 7.8. Volcano Plot of the Most Significantly Altered Genes.

To determine the genes that are the most significantly altered between scrambled and CREAP siRNA, the data was subjected to Volcano Plot analysis where the log of the p value is plotted against the log₂ of the fold change. A threshold value of $p > 0.05$ and a fold change greater than 2 (green lines), generated a set of genes that are very significantly altered (red dots) compared to the other altered genes (yellow dots).

The microarray data analysis had yielded a list of 1440 genes that have significantly altered gene expression in CREAP siRNA-treated cells, 617 of which have more than a two-fold difference. To determine if these genes fit into particular biological pathways, functions or processes, two programs were used to analyse the data. Both the 1440 and 617 genes lists were analysed using GATHER and PANTHER to determine significant functional relationships between the altered genes.

7.3.3 GATHER: Gene Annotation Tool to Help Explain Relationships

The GATHER program allows entry of a list of genes from a microarray experiment, or similar high-throughput genomic assay, and can analyse the gene expression signature against a series of data sources including Gene Ontology (Chang and Nevins 2006). Both the 1440 and 617 significant genes lists were analysed. There was a greater level of significance found with the 1440 genes with lower p values and higher Bayes factors. There was some overlap of the gene ontology annotations. See Table 7.1 and Table 7.2.

A Bayes factor is a measure of the strength of the evidence supporting an association of an annotation with your gene list. Higher Bayes factors indicate stronger evidence that the annotation is relevant to your genes. A positive Bayes factor indicates that the evidence supports the hypothesis that the annotation is more related to your list of genes than other genes in the genome. A negative Bayes factor indicates that the evidence suggests that the annotation is more strongly associated with other genes in the genome. A Bayes factor cutoff of 6 is recommended as that cutoff appears to balance false positives with false negatives (Chang and Nevins 2006).

Annotation	# Genes With Annotation	# Genes in Genome With Annotation	Bayes factor	p value
GO:0007186: G-protein coupled receptor protein signaling pathway	28	966	16.96	<0.0001
GO:0008104: protein localization	77	415	16.04	<0.0001
GO:0045184: establishment of protein localization	75	404	15.5	<0.0001
GO:0015031: protein transport	74	403	14.81	<0.0001
GO:0007166: cell surface receptor linked signal transduction	55	1366	13.47	<0.0001
GO:0006886: intracellular protein transport	48	266	7.43	<0.0001
GO:0050875: cellular physiological process	733	8362	7.4	<0.0001
GO:0046907: intracellular transport	67	438	7.05	<0.0001
GO:0007606: sensory perception of chemical stimulus	5	286	4.95	0.0002
GO:0007608: perception of smell	4	260	4.76	0.0002
GO:0008152: metabolism	573	6392	4.4	0.0003
GO:0050877: neurophysiological process	33	775	4.1	0.0004
GO:0044237: cellular metabolism	541	6024	3.67	0.0008
GO:0050874: organismal physiological process	99	1745	3.62	0.0008
GO:0007154: cell communication	211	3264	3.42	0.001
GO:0044238: primary metabolism	518	5768	3.07	0.002
GO:0007275: development	97	1690	2.94	0.002
GO:0043170: macromolecule metabolism	275	2871	1.91	0.005
GO:0044260: cellular macromolecule metabolism	259	2727	1.06	0.01
GO:0006811: ion transport	32	672	1.02	0.01
GO:0007582: physiological process	807	9655	0.9	0.01
GO:0007600: sensory perception	24	540	0.77	0.02
GO:0009605: response to external stimulus	69	1194	0.51	0.02

Table 7.1. Biological Processes found Significant by GATHER for the 1440 Genes.

The 1440 genes significantly altered by CREAP knockdown were analysed by GATHER to determine which biological processes were significantly altered. The gene ontology (GO) annotation description is listed with the number of genes from the 1440 with that annotation as well as the number of genes in the genome with the annotation. The Bayes factor and p value shows the significance of the association with the annotation. Bayes factors >6 and p values <0.05 are likely to be more valid.

Annotation	# Genes With Annotation	# Genes in Genome With Annotation	Bayes factor	p value
GO:0007186: G-protein coupled receptor protein signaling pathway	14	980	3.31	0.001
GO:0007166: cell surface receptor linked signal transduction	28	1393	1.15	0.01
GO:0009613: response to pest, pathogen or parasite	27	444	0.66	0.02
GO:0043118: negative regulation of physiological process	4	415	0.53	0.02
GO:0009156: ribonucleoside monophosphate biosynthesis	4	13	0.45	0.02
GO:0009161: ribonucleoside monophosphate metabolism	4	13	0.45	0.02
GO:0051179: localization	83	1902	0.34	0.03
GO:0009123: nucleoside monophosphate metabolism	4	14	0.23	0.03
GO:0009124: nucleoside monophosphate biosynthesis	4	14	0.23	0.03

Table 7.2. Biological Processes found Significant by GATHER for the 617 Genes.

The 617 genes significantly altered by CREAP knockdown, by greater than two-fold, were analysed by GATHER to determine which biological processes were significantly altered. The gene ontology (GO) annotation description is listed with the number of genes from the 617 genes with that annotation as well as the number of genes in the genome with the annotation. The Bayes factor and p value show how significant the association with the annotation is. Bayes factors >6 and p values <0.05 are likely to be more valid. The grey highlighted annotations overlap with the 1440 gene list processes.

7.3.4 PANTHER: Protein Analysis Through Evolutionary Relationships

PANTHER is a large collection of protein families that have been subdivided into functionally related subfamilies, using human expertise. These subfamilies model the divergence of specific functions within protein families, allowing more accurate association with function (ontology terms and pathways). The PANTHER ontology is similar to the Gene Ontology™ (GO), but greatly abbreviated and simplified to facilitate high-throughput analyses.

Both the 1440 and 617 CREAP siRNA lists were analysed using PANTHER (see Table 7.3). Several biological processes were deemed significant for each list though there was no overlap of significant processes except for the unclassified genes. The intracellular protein transport gene ontology was also identified by GATHER.

PANTHER Classification	NCBI: H. sapiens genes (REF)	617 Most Significant Genes				1400 Significant Genes				Gene Ontology Equivalent
Biological Process	# genes in classification	# genes detected	# genes expected	+/-	P value	# genes detected	# genes expected	+/-	P value	
Biological process unclassified	11321	215	268.43	-	0.00019	491	629.91	-	1.13E-12	biological_process unknown (GO:0000004)
Intracellular protein traffic	1008	33	23.9	+	1	100	56.09	+	1.21E-06	intracellular protein transport (GO:0006886)
Nucleoside, nucleotide and nucleic acid metabolism	3343	93	79.27	+	1	251	186.01	+	0.0000176	nucleobase, nucleoside, nucleotide and nucleic acid metabolism (GO:0006139)
General vesicle transport	251	12	5.95	+	1	31	13.97	+	0.00751	vesicle-mediated transport (GO:0016192)
Intracellular signaling cascade	871	32	20.65	+	1	74	48.46	+	0.044	intracellular signalling cascade (GO:0007242)
Cell proliferation and differentiation	1028	45	24.38	+	0.00249	77	57.2	+	0.192	cell proliferation;GO:0030154 cell differentiation (GO:0008283)
Regulated exocytosis	48	7	1.14	+	0.0353	9	2.67	+	0.348	regulation of exocytosis (GO:0017157)
Signal transduction	3406	108	80.76	+	0.032	202	189.51	+	1	signal transduction (GO:0007165)
Transport	1306	52	30.97	+	0.00723	87	72.67	+	1	transport (GO:0006810)

Table 7.3. Significant Biological Processes Identified by PANTHER.

The 1440 and 617 genes lists were subjected to analysis by PANTHER. The significant processes identified from both lists are tabulated with the number of genes associated with the process in the genome, the number of genes from each list, the number of genes expected, whether there are more or less genes altered than expected +/-, the p value and the associated gene ontology classification. Significant p values are highlighted in grey.

7.3.4.1 Gene Ontology Lineage Linkage

After collating the data from PANTHER and GATHER, a gene ontology lineage diagram was constructed. Considering that hundreds of genes were significantly altered in response to CREAP siRNA-mediated gene silencing, looking at the genes as a linked processes diagram more clearly shows the broader areas that are affected. The data from the 1440 gene lists was used to construct a gene ontology lineage diagram which shows the way individual annotations are linked (Figure 7.9). From the diagram it appears that the major areas of gene expression perturbation are protein transport, signal transduction and metabolism.

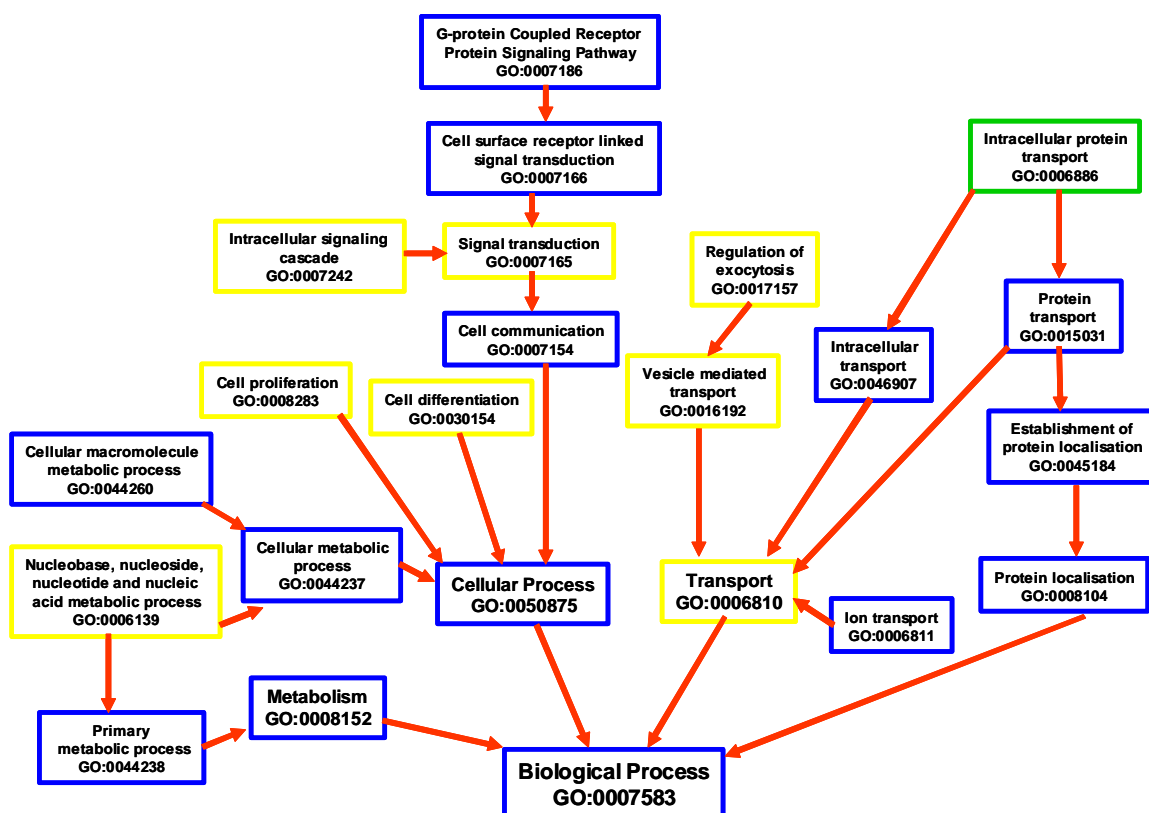


Figure 7.9. Graphical View of the Gene Ontology Lineage Linking Significant Biological Processes.

Gene ontology classifications that were significantly altered in CREAP knockdown cells are graphically represented, showing the links between biological processes. The processes identified by the GATHER program are boxed in blue, while those identified by PANTHER are boxed in yellow. The hierarchical clustering is organised according to a parent-child arrangement with red arrows showing this relationship.

7.3.4.2 Fold-Difference Analysis

The PANTHER program also has the ability to analyse a gene list with its corresponding fold-difference data. When the 1440 gene list, with the fold-difference expression data was analysed, three molecular functions were found to be significant (see Table 7.4). These three functions appear to be related, in that KRAB Box transcription factors are a type of zinc finger, and zinc finger transcription factors are a part of transcription factors in general. So when the magnitude of the differences in gene expression from CREAP knockdown cells were analysed rather than just a list of gene names, transcription became significant.

Molecular Function	# Genes	+/-	P value	Description
Zinc finger transcription factor	67	+	1.93E-03	A transcription factor containing zinc finger domain(s), which is composed of conserved cysteines and histidines co-ordinate with zinc ion(s). Examples of zinc finger domains are C2H2 zinc finger, C3HC4 zinc finger, CHC2 zinc finger, etc.
KRAB box transcription factor	48	+	6.14E-03	A transcription factor containing a C2H2 zinc finger domain and a KRAB (Kruppel-associated box) domain
Transcription factor	134	+	3.76E-02	A protein required for the regulation of RNA polymerase by specific regulatory sequences in or near a gene.

Table 7.4. PANTHER Fold difference Analysis of 1440 CREAP siRNA Gene List.

When-fold difference data was analysed using PANTHER, three transcription factor-related molecular functions were identified as significant. The table lists the molecular function, the number genes from the list associated with the function, whether there are more or less genes than expected +/-, the p value and a description of the function.

7.3.4.3 *Are TF Array Factors Affected by CREAP Knockdown*

With transcription being shown to be affected by CREAP siRNA mediated knockdown, the expression levels of the transcription factors identified as interacting with CREAP on the TF Array in Chapter 6 were investigated. The list of microarray genes was interrogated for specific transcription factors from the array. However, most factors from the array were not significantly altered. The comparison of the strong interacting factors is shown in Table 7.5. Only ATF2, CREB1 and EGR2 were significantly altered and only EGR2 had a greater than 2-fold difference. This is not unexpected as unless CREAP is regulating the expression of these particular transcription factors, the lack of an interacting partner is unlikely to affect their expression. There were, however, some related factors that were also significantly altered such as upregulation of CREB binding protein, ATF6, JunD and down regulation of ATF2-like basic leucine zipper transcription factor.

Transcription Factor	Fold Difference	p value
ATF1	1.26	-
ATF2	1.20	0.0241
ATF3	1.05	-
ATF4	-1.09	-
CREB1	1.49	0.026
E2F3	-1.17	-
EGR1	-1.64	-
EGR2	-2.82	0.0156
EGR4	1.19	-
FOS	2.25	-
GATA1	1.00	-
GR	1.14	-
JUN	-1.04	-
JUNB	-2.60	-
PPARa	-1.22	-
SP1	1.04	-
YY1	-1.04	-

Table 7.5. TF Array Factors Gene Expression Data from CREAP Knockdown Cells.

The strong interacting factors from the TF Array were assessed for gene expression changes in CREAP knockdown cells. The-fold difference and the p value of significantly altered genes is shown.

7.3.4.4 CRH and CREAP Knockdown

In Chapter 4, the effect of CREAP transfection on CRH promoters was tested and it seemed that CREAP had negligible effects on CRH promoter activity. To assess if

CREAP knockdown has any effect on CRH, the gene expression levels from CREAP siRNA-treated cells was examined. There was a 2.15-fold reduction in CRH expression in CREAP knockdown cells though this was not statistically significant. This further supports the evidence that CREAP does not regulate CRH expression either positively or negatively.

7.4 Discussion

7.4.1 *CREAP siRNA*

In investigating gene silencing technologies for CREAP knockdown, siRNA was found to be an efficient and reproducible technique. CREAP protein levels were considerably reduced by all the siRNA sequences tested with the optimal conditions resulting in around an 80% reduction of CREAP. This technique was applicable to both the choriocarcinoma cell line, JEG-3 and normal human fibroblasts.

The yeast CREAP homologue, Luc7p, is essential for yeast viability (Fortes et al. 1999a) but in higher animals it would seem that there is redundancy in splicing functions. CREAP does not appear to be an essential factor in human cells as substantial knockdown was not lethal to cells and didn't result in an observable change in phenotype, at least not in the short term (data not shown). This would suggest that CREAP is not involved in the splicing or transcriptional regulation of any essential cell factors.

7.4.2 *Microarray Analysis*

Several gene expression studies have been conducted using Illumina's BeadChip microarrays and due to the reliability and accuracy of the platform, RT-PCR confirmation is usually unnecessary (Lian et al. 2007; Myers et al. 2007; Doring et al. 2008; Ehlers et al. 2008; Idaghdour et al. 2008). There were 1440 differentially expressed genes in siRNA-mediated CREAP knockdown cells, and 617 of those had fold-differences greater than 2. Analysis by GATHER and PANTHER gave slightly different results when it came to annotating the differentially expressed genes, due to variation in their analysis algorithms. Importantly, there was some overlap of results and similar ontology classifications identified by both programs. There was an over-representation of differentially expressed genes from CREAP siRNA-treated cells in the significant PANTHER biological processes. When the combined gene ontology lineage diagram was plotted there were connections between most of the biological process and three broad classifications appeared to exist.

With 1440 genes significantly altered in expression in CREAP knockout cells, approximately 6% of the genes tested were differentially expressed. Other studies on the microarray analysis of gene knockdown yield a range of values for the number of genes significantly altered in expression. An Affymetrix microarray study on human endometrial stromal cells showed that treatment with estradiol, medroxyprogesterone acetate and dibutyryl cAMP (H + cAMP) to induce decidualization resulted in 2361 (~5%) significantly altered genes, while this treatment coupled with siRNA-mediated knockdown of the homeobox protein HOXA10, resulted in 494 genes (~1%) altered by knockdown (Lu et al. 2008). Knockdown of the transcription factor TCERG1 in

HEK293T cells, resulted in 1039 (4.7%) of the genes screened being differentially expressed (Pearson et al. 2008). When the transcription factor E2F1 was knocked down in HeLa cells (Goto et al. 2006), 79 genes (0.2%) were altered by more than three-fold. A three-fold difference threshold applied to CREAP, still results in 216 differentially expressed genes. It seems that the number of genes affected by the knockout of a particular gene varies considerably, however CREAP siRNA knockdown does seem to have affected a large number of genes.

The affected processes from both GATHER and PANTHER seem to centre around protein transport, signal transduction and metabolism. With tens to hundreds of individual genes associated with each ontology, detailed analysis of individual genes was beyond the scope of this study. In a general sense the possible role that CREAP could be playing in these processes is more likely to be coupled to transcription as splicing factors tend to be redundant. As a possible transcription factor, CREAP could be involved in regulating proteins involved in metabolism and protein transport and localisation. There were several processes that related to signal transduction, including G-protein coupled signalling, cell surface receptor-linked signalling and intracellular signalling cascades. Specific targets of interest to the CRH field are discussed below.

7.4.2.1 Signal Transduction

Several components of the protein kinase A (PKA) pathway are affected by knockdown of CREAP. The catalytic subunit of PKA is upregulated 119-fold, while CREB1 is slightly upregulated by 1.5-fold. The increase in CREB expression could be tied in with the results from Chapter 5, where CREAP seemed to have a slightly inhibitory effect on

CREB and cAMP-mediated promoter induction. The bZIP proteins ATF2, ATF6 and JunD were also upregulated by CREAP knockdown. The mechanism by which CREAP knockdown is affecting the expression of components of the PKA pathway and other signalling cascades is unclear.

The PKC pathway also appears to be affected by CREAP knockdown with the protein kinase C isozyme, PKC γ , down-regulated by 26.5-fold and phospholipase C (PLC δ 4) down-regulated by 2.5-fold. The MAPK1 mRNA levels were also significantly reduced by 1.6-fold, which is consistent with the results of the western blotting, where there was a reduction in MAPK1 protein levels in CREAP siRNA-treated cells. Whether this is an off-target effect, that CREAP regulates MAPK in some way or if there is cross-talk with the cAMP and MAPK pathways is unclear.

7.4.2.2 *Transcription*

When the data was analysed by fold difference, three transcriptional functions became clear- KRAB box transcription factors, zinc finger transcription factors and transcription factors. At least fifty zinc finger proteins showed differential expression in response to CREAP knockdown. CREAP itself contains two zinc finger-like domains but how this would relate to the expression of other zinc finger proteins is unknown. The finding that transcription factor functions became significant when the quantitative expression data was analysed did substantiate the initial hypothesis that CREAP has transcriptional activity. The results suggest that CREAP may be regulating other transcription factors, or its knockdown may force the upregulation of other factors as a compensatory mechanism.

7.4.3 Splicing and CREAP Knockdown

There were no splicing or splicing-related annotations associated with CREAP knockdown. Given CREAPs (and CROP/hLuc7A's) association with splicing speckles and splicing proteins such as SC35, it would be expected that knockdown might influence these pathways and processes. However, redundancy is a demonstrated feature of SR proteins. In cellular extracts lacking SR proteins, individual SR proteins can each complement the extract and splicing can be restored (Mayeda and Krainer 1992; Mayeda et al. 1992; Zahler et al. 1992). In most cases SR proteins are functionally redundant, but they can exhibit specific splicing functions of specific genes, so that if a SR protein is depleted, most splicing will continue as normal, but there may be a few specific genes which are affected and may not be noticed unless they are essential proteins (Hastings and Krainer 2001). RNAi knockouts of SR proteins in *C.elegans* showed that SF2/ASF was essential for viability but no other SR protein knockouts produced any observable phenotype unless more than one protein was targeted at the same time (Kawano et al. 2000; Longman et al. 2000). Therefore, it's not surprising that splicing-related functions were not affected by CREAP knockdown as any function CREAP has in splicing is likely to be redundant. It is unknown however, whether any of the genes affected in the other pathways are altered due to a splicing defect that is mediated by CREAP, or by a direct effect of CREAP.

7.4.4 *CREAP Knockdown and CRH*

siRNA-mediated knockdown of CREAP did not significantly affect the expression of CRH. There was a 2.15-fold reduction in CRH levels compared to the controls, but it wasn't statistically significant. Given the results from Chapter 5, where over-expression following transfection of CREAP had negligible effect on CRH promoter activity, the effect of knocking down CREAP was of interest. However, CREAP knockdown did not have an effect, once again suggesting that even though CREAP has CRE-binding capabilities, it is not an important regulator of CRH gene expression.

7.4.5 *Summary*

Efficient knockdown of CREAP mRNA and protein levels were achieved using siRNA-mediated gene silencing. Up to 80% knockdown of protein levels was achieved in both fibroblast and JEG-3 cells. To assess the effect of CREAP knockdown on whole genome expression, an Illumina Ref-8 v2 BeadChip microarray was utilised. 1440 genes were differentially expressed in CREAP siRNA-treated fibroblasts, which does appear to be quite a large number, especially given the redundancy of splicing factors. Gene analysis revealed that the altered genes fitted into several biological process including protein transport, metabolism, signal transduction and transcription. Supporting our previous findings, CREAP knockdown had no significant effect on the expression of CRH. The mechanism by which CREAP is able to modulate the expression of so many genes remains unknown- is CREAP affecting splicing, transcription or something else entirely. While it was hoped that microarray analysis would shed greater light on the function of CREAP in the cells, especially if it has

transcriptional activity, further work needs to be done to elucidate its role and investigate the significance of siRNA microarray data set.

Chapter 8. Conclusions

8.1 Final Summary

CRH expression increases exponentially over gestation and peaks at labour. It is hypothesised that a placental clock exists which controls the timing of birth and CRH is an important factor in this process. The regulation of CRH gene expression in the placenta and hypothalamus is an important research focus of this laboratory, as understanding placental CRH regulation could lead to a better understanding and thereby, prevention of premature birth, which is still the leading cause of perinatal mortality and morbidity.

CREAP was discovered as a novel protein capable of binding to the CRE. It was isolated from a placental cDNA library and hypothesised to bind the CRE of the CRH promoter, and be a key regulator of expression. Sequence analysis revealed that CREAP comprises a unique set of protein domains consisting of two-leucine zipper-like regions, two coiled-coils, a C3H and a C2H2 type zinc finger and an RS domain. The presence of the leucine zippers fitted well with the CRE-binding capability, as bZIP transcription factors can bind to CREs. The C2H2 zinc finger is a DNA binding motif and the C3H zinc finger has been shown to be able to bind to DNA and RNA. The coiled-coil motif is known to be important in protein-protein interactions while the RS domain is a characteristic of the SR family of splicing factors. Thus, CREAP contains domains related to both DNA binding and transcription, RNA binding, protein interactions and splicing. CREAP is also a member of a new protein family with at

least three members and at least one major alternative splice variant. Its expression appears to be quite ubiquitous.

Beginning with this knowledge, a range of reagents were produced to allow further study of CREAP. These included mammalian expression vectors and a GST fusion protein. The fusion protein was used as an antigen to raise polyclonal antibodies and as a probe for protein interactions. The expression vectors were used in a Dual Luciferase Assay to assess promoter activity. The polyclonal antibodies were also used for localisation studies, interaction studies and western blotting.

Consistent with it containing multiple NLSs, CREAP was shown to have a nuclear localisation. This consisted of both diffuse nucleoplasmic distribution and concentration within intranuclear splicing speckles. This result was confirmed with both western blotting and indirect immunofluorescent staining. CREAP co-localised and co-immunoprecipitated with the SR protein, splicing factor, SC35. Other researchers have also identified that the CREAP identical protein CROP/hLuc7A also interacts with the splicing factors SF2/ASF and the U1-70K component of U1 snRNP (Umehara et al. 2003; Puig et al. 2007). The yeast homologue Luc7p is also an essential splicing factor in yeast. Therefore a role for CREAP in splicing seems very likely.

To investigate whether CREAP was capable of regulating CRH gene expression, luciferase reporter assays with CRH and CRE promoters were transfected into primary placental, AtT-20 and JEG-3 cells, and exposed to various treatment conditions. CREAP co-transfection, however, had negligible effect on CRH promoter activity,

indicating that even though it can bind to a CRE, it is not regulating gene expression in these experimental systems.

To further investigate if CREAP has potential transcriptional activity, CREAP protein was used to screen a transcription factor protein array. Interestingly, CREAP strongly interacted with a large list of different proteins, especially those factors from the bZIP and zinc finger families. Several of the interacting factors have a demonstrated role in CRH regulation, suggesting that CREAP may still be involved in some aspect of CRH regulation. These interactions were detected *in vitro* but how relevant they are *in vivo* remains unknown.

With the possibility that CREAP may still have a transcriptional role, siRNA was conducted to see if CREAP knockdown might illuminate the pathways and processes that CREAP is involved in. CREAP protein levels were reduced by 80% in siRNA-treated cells. The effect of knockdown was studied using Illumina's Ref-8v2 BeadChip microarray. 1440 genes were differentially expressed in knockdown cells compared to controls, with 617 genes showing greater than a two-fold change. Gene ontology programs were used to classify the genes into functions and processes. The altered genes seemed to cluster into three main processes; protein transport, metabolism and signal transduction, with components of the PKA pathway being notably affected. There was no significant effect on CRH gene expression in CREAP siRNA-treated cells, in agreement with the findings from the promoter study. When the genes were also analysed by expression level, then transcription factor functions, especially those related to zinc finger transcription factors became significant. One possible reason for

this could be an up-regulation of transcription factors to compensate for the loss of CREAP.

8.2 Conclusions

Initially CREAP was identified as a novel CRE-binding protein from placenta. With an ability to bind to the CRE and a structure similar to the bZIP protein family, it was hypothesised that it may be a regulator of CRH expression, similar to the bZIP proteins CREB, Fos and Jun. However, the findings of this thesis, coupled with the small but growing literature on the CREAP identical protein CROP/hLuc7A, seem to suggest that CREAPs main function is in splicing. It co-localises and interacts with other SR proteins and splicing factors within speckles. Nevertheless, the leucine zipper and zinc finger domains, together with its ability to bind the CRE, interact with other transcription factors and the differential expression of genes related to transcription, signal transduction, transport and metabolism implicate a still undiscovered role in transcription and gene regulation.

A hypothetical model for CREAP function is shown in Figure 8.1. The possible roles in transcription and splicing are united at the periphery of splicing speckles, where RNA transcription and processing are coupled to lead to the most efficient gene expression.

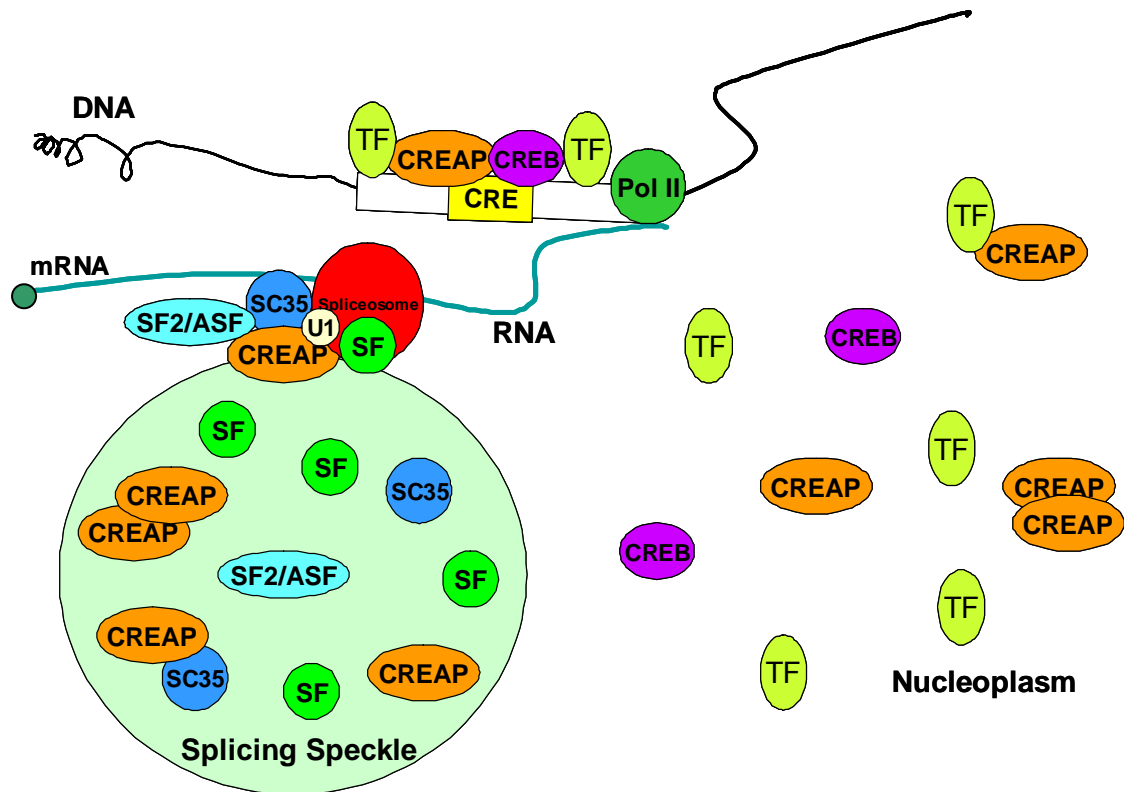


Figure 8.1. Hypothetical Model of CREAP Function in the Cell Nucleus.

CREAP is localised to the nucleoplasm and splicing speckles. Transcription and splicing takes place at the periphery of and within speckles. CREAP binds to the CRE of gene promoters, and has been shown to bind to other transcription factors (TFs) including CREB. CREAP therefore may be regulating transcription by direct binding to the CRE or by interacting with CREB and other TFs. CREAP is also located within speckles and has been shown to interact with splicing factor such as SC35, SF2/ASF and U1 snRNP, suggesting that CREAP may form part of the spliceosome to affect splicing. It is also likely that CREAP forms a homodimer. TF-transcription factors, SF-splicing factors.

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