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CREM: A Master-Switch in the Transcriptional Response to cAMP

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CREM: a master-switch in the transcriptional response to cAMP

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SUMMARY

The CREM gene encodes both repressors and activators of cAMP-dependent transcription in a tissue and developmentally regulated manner. In addition, multiple and cooperative phosphorylation events regulate the function of the CREM proteins. CREM plays a key physiological and developmental role within the hypothalamic-pituitary axis. There is a functional switch in CREM expression during the development of male germ cells which is directed by the pituitary hormone FSH. The CREM protein in germ cells is a powerful activator which appears to function as a master-switch in the regulation of post-meiotic genes.

CREM is inducible by activation of the cAMP signalling pathway with the kinetics of an early response gene. The induction is transient, cell-specific, does not involve increased transcript stability and does not require protein synthesis. The subsequent decline in CREM expression requires *de novo* protein synthesis. The induced transcript encodes ICER and is generated from an alternative, intronic promoter. ICER functions as a powerful repressor of cAMP-induced transcription, and represses the activity of its own promoter, thus constituting a negative autoregulatory loop.

1. INTRODUCTION

The structural organization of most transcription factors is intrinsically modular, in most cases including a DNA-binding domain and an activation domain. It has been shown that these domains can be interchanged between different factors and still retain their functional properties. This modularity suggests that, during evolution, increasing complexity of gene expression may have resulted not only from duplication and divergence of existing genes, but also from a domain-shuffling process which generated factors with novel properties (Harrison 1991).

An important step forward in the study of transcription factors has been the discovery that many of them constitute the final targets of specific signal transduction pathways. The two major signal transduction systems are those which include cAMP and diacylglycerol (DAG) as secondary messengers (Nishizuka 1986). Each pathway is also characterized by specific protein kinases (Protein Kinase A and Protein Kinase C, respectively) and its ultimate target DNA control element (cAMP-responsive element (CRE) and TPA-responsive element (TRE), respectively). Although initially characterized as distinct systems, accumulating evidence points towards extensive cross-talk between these two pathways (Cambier *et al.* 1987; Yoshimasa *et al.* 1987; Masquilier & Sassone-Corsi 1992).

Intracellular levels of cAMP are regulated primarily by adenylate cyclase. This enzyme is in turn modulated by various extracellular stimuli mediated by receptors and their interaction with G proteins (McKnight *et al.* 1988). cAMP binds cooperatively to two sites on the regulatory subunits of protein kinase-A (PKA), releasing the active catalytic subunits (Roesler *et al.* 1988; Lalli & Sassone-Corsi 1994). These are translocated from cytoplasmic and Golgi complex anchoring sites and phosphorylate a number of cytoplasmic and nuclear proteins on serines in the context X-Arg-Arg-X-*Ser*-X (Lalli & Sassone-Corsi 1994). In the nucleus, PKA-mediated phosphorylation ultimately influences the transcriptional regulation of various genes through distinct, cAMP-inducible promoter responsive sites (Ziff 1990; Borrelli *et al.* 1992).

2. INDUCTION OF GENE EXPRESSION BY ACTIVATION OF THE cAMP SIGNALLING PATHWAY

The consensus cAMP-responsive element (CRE) is constituted by an 8 base pair (b.p.) palindromic sequence (TGACGTCA) with a higher conservation in the 5′ half of the palindrome than the 3′ sequence.

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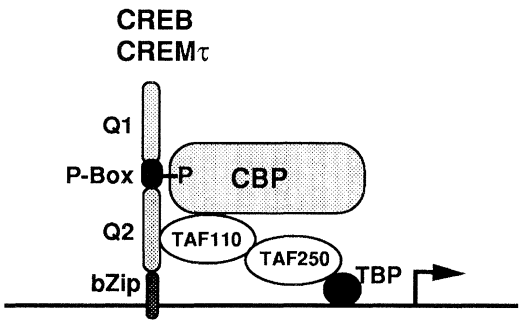

Figure 1. Scheme of the possible interaction between the transcriptional activators CREB and CREM with the coactivator CBP (Chrivia *et al.* 1993). Interaction is phosphorylation-dependent and allows the contact with other elements of the transcriptional machinery, such as TAF110, TAF250 and the TATA-binding protein TBP.

Several genes which are regulated by a variety of endocrine stimuli contain similar sequences in their promoter regions although at different positions (Sassone-Corsi 1988; Borrelli *et al.* 1992).

The first CRE-binding factor to be characterized was CREB (CRE-binding protein; Hoeffler *et al.* 1988) but subsequently at least ten additional CRE-binding factor cDNAs have been cloned. They were obtained by screening a variety of cDNA expression libraries, with CRE and ATF sites (Hai *et al.* 1989; Foulkes *et al.* 1991). These proteins belong to the bZip transcription factor class.

The different factors are able to heterodimerize with eachother but only in certain combinations. A 'dimerization code' exists which seems to be a property of the leucine zipper structure of each factor. Some ATF/CREB factors are able to heterodimerize with Fos and Jun, and this may change the specific affinity of binding to a CRE with respect to a Fos-Jun binding site (Hai & Curran 1991). This property resides in the similarity between the CRE (TGACGTCA) and TRE (TGACTCA) sequences (Sassone-Corsi *et al.* 1990; Masquilier & Sassone-Corsi 1992) and demonstrates the versatility of the transcriptional response to signal transduction. There are both activators and repressors of cAMP-responsive transcription. Some alternatively spliced CREM isoforms act as antagonists of cAMP-induced transcription. The cAMP-inducible ICER product deserves special mention as it is generated from an alternative promoter of the CREM gene and is responsible for its early response inducibility which is unique amongst CRE-binding factors (Molina *et al.* 1993; Stehle *et al.* 1993).

3. ACTIVATION BY PHOSPHORYLATION

The characterization of the transcriptional activators CREB and CREM (Gonzalez & Montminy 1989; Foulkes *et al.* 1992) has helped in the elucidation of the molecular mechanisms involved in transcriptional activation. These factors contain a transcriptional activation domain which is divided into two independent regions (Lalli & Sassone-Corsi 1994). The first, known as the phosphorylation box (P-box) contains several consensus phosphorylation sites for various kinases, such as PKA, PKC, p34cdc2, glycogen synthase kinase-3 and casein kinases (CK) I and II (Gonzalez & Montminy 1989; Lee *et al.* 1990; de Groot *et al.* 1993*a*, *b*). The second region flanks the P-box and is constituted by domains rich in glutamine residues (Lalli & Sassone-Corsi 1994).

Upon activation of the adenylyl cyclase pathway, a serine residue at position 133 of CREB and at position 117 of CREM is phosphorylated by PKA (Gonzalez & Montminy 1989; de Groot *et al.* 1993*a*). The major effect of phosphorylation is to convert CREB and CREM into powerful transcriptional activators. Within the P-box, serine 133/117 is located in a region of about 50 amino acids containing an abundance of phosphorylatable serines and acidic residues which was shown to be essential for transactivation by CREB and CREM (Lee *et al.* 1990; de Groot *et al.* 1993 *a*).

Interestingly, in PC12 cells, increases in the levels of intracellular Ca^{2+} caused by membrane depolarization have been shown to induce the phosphorylation of serine 133 in CREB and a concomitant activation of c-*fos* gene expression mediated by a CRE in the promoter (Sassone-Corsi *et al.* 1988; Sheng *et al.* 1990). Although Ca^{2+}-dependent CamK was shown to be able to phosphorylate serine 133 *in vitro* (Dash et al. 1991), *the in vivo* significance remains unclear, as PKA also seems to be necessary for c-*fos* induction mediated by Ca^{2+} influx in PC12 cells (Ginty *et al.* 1991).

An important finding that reveals the complexity of the transcriptional response elicited by these factors concerns the mitogen-induced p70 S6 kinase, which phosphorylates and activates CREM (de Groot *et al.* 1994). This finding implicates $p70^{s6k}$, a kinase generally considered cytoplasmic, in the mitogenic response also at the nuclear level. Interestingly, because CREM and other factors of the CREB/ATF family represent the final targets of the cAMP-pathway, these results show that they may also act as effectors of converging signalling systems and possibly as mediators of pathway cross-talk (de Groot *et al.* 1994).

4. INTERACTION WITH COACTIVATOR CBP

The two domains flanking the P-box contain about threefold more glutamine residues than in the remainder of the protein in both CREB and CREM. Glutamine-rich domains have been characterized in other factors, such as AP-2 and Sp1 (Williams *et al.* 1988; Courey & Tjian 1989) as transcriptional activation domains. The current notion is that they constitute surfaces of the protein which can interact with other components of the transcriptional machinery. Indeed, further steps towards an understanding of the mechanism of action of the P-box has come with the identification of a 265 K, 2441 amino acid protein, CBP (CREB-binding protein) that is able to interact specifically with the phosphorylated CREB P-box domain (Chrivia *et al.* 1993). The CBP sequence reveals two zinc finger domains, a glutamine-rich domain at its C-terminus and a single consensus PKA recognition site. Phosphorylation of Ser-133 promotes

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binding to CBP and consequently the interaction with TFIIB, a general transcription factor involved in RNA polymerase II activity (Kwok *et al.* 1994). Thus, CBP may act as a link between CREB and the transcription preinitiation complex. This interaction may need some RNA polymerase II cofactors, such as TAF110 (figure 1). Finally, the adenoviral E1A oncoprotein-associated p300, which is thought to play a role in preventing the cell cycle G0/G1 transition, is structurally very closely related to CBP (Arany *et al.* 1995). Both CBP and p300 appear to have intrinsic activating properties which are inhibited by the E1A protein (Arany *et al.* 1995). Thus, it is clear that studies of the transcriptional activation domain of CRE-binding bZip factors continues to provide important insights into the function of transcription factors in general.

5. CREM IS AN EARLY RESPONSE GENE

During studies of CREM expression within the neuroendocrine system, an unexpected new facet emerged: namely the transcription of the CREM gene is inducible by cAMP (Molina *et al.* 1993). Furthermore, the kinetics of this induction are those of an early response gene (Verma & Sassone-Corsi 1987). This important finding further reinforces the notion that CREM products play a fulcral role in the nuclear response to cAMP because the expression of no other CRE-binding factor has been demonstrated as inducible to date.

The demonstration that the CREM gene was cAMP inducible first came from the discovery that adrenergic signals direct CREM transcription in the pineal gland (Stehle *et al.* 1993). The inducibility phenomenon was then characterized in detail in the pituitary corticotroph cell line AtT20. In unstimulated cells the level of CREM transcript is undetectable. However, upon treatment with forskolin (or other cAMP analogues), within 30 mins there is a rapid increase in CREM transcript levels which peak after 2 h and then progressively decline to basal levels by 5 h. These characteristic kinetics classify CREM as an 'early response gene' and thus directly implicate the cAMP pathway in the cell's early response for the first time. CREM inducibility is specific for the cAMP pathway because it is not inducible by TPA or dexamethasone treatment. The inducible CREM transcript corresponds to a truncated product, termed ICER (inducible cAMP early repressor) (Molina *et al.* 1993; Stehle *et al.* 1993).

The 5′ end of the ICER clones correspond to an alternative transcription start site. The start of transcription, which identifies the P2 promoter, is within the 10 kb intron which is C-terminal to the Q2 glutamine-rich domain exon. In contrast to the promoter generating all the previously characterized CREM isoforms (P1) and which is GC-rich and not inducible by cAMP (N. S. Foulkes, unpublished data), the P2 promoter has a normal A-T and G-C content and is strongly inducible by cAMP. It contains two pairs of closely spaced CRE elements organized in tandem, where the separation between each pair is only three nucleotides (figure 2). These features make P2 unique amongst cAMP-regulated promoters and are suggestive of cooperative interactions among the factors binding to these sites.

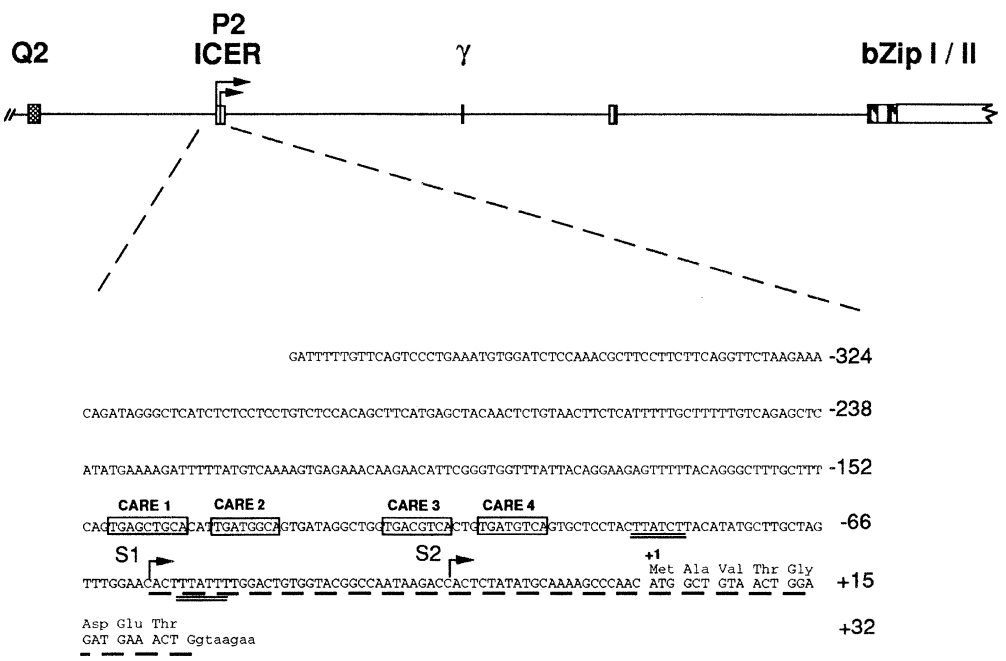

Figure 2. The ICER promoter. Schematic representation of the ICER 5′ flanking region. The position of the two starts of transcription (S1 and S2) and the Kozak ATG codon is indicated. A 400 b.p. genomic sequence including the ICER 5′ exon is shown. Dashed underlining delineates the ICER 5′ exon. Lower case sequence represents the beginning of the first intron of the ICER transcript. Putative TATA elements are indicated by double underlining whereas the four CRE-like elements (CAREs) are boxed and labeled. The position +1 corresponds to the A of the Kozak ATG initiation codon (Molina *et al.* 1993).

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The ICER open reading frame is constituted by the C-terminal segment of CREM. The predicted open reading frame encodes a small protein of 120 amino acids with a predicted molecular mass of 13.4 kDa. This protein, compared with the previously described CREM isoforms, essentially consists of only the DNA binding domain, which is constituted by the leucine zipper and basic region. The structure of ICER is suggestive of its function and makes it one of the smallest transcription factors ever described (Molina *et al.* 1993; Stehle *et al.* 1993).

The intact DNA binding domain directs specific ICER binding to a consensus CRE element. Importantly, ICER is able to heterodimerize with the other CREM proteins and with CREB. ICER functions as a powerful repressor of cAMP-induced transcription in transfection assays using an extensive range of reporter plasmids carrying individual CRE elements or cAMP-inducible promoter fragments (Molina *et al.* 1993). Interestingly, ICER-mediated repression is obtained at substoichiometric concentrations, similarly to the previously described CREM antagonists (Laoide *et al.* 1993). ICER escapes from PKA-dependent phosphorylation and thus constitutes a new category of CRE binding factor, for which the principle determinant of their activity is their intracellular concentration and not their degree of phosphorylation. Recent data implicates dynamic ICER expression as a more general feature of neuroendocrine systems (Lalli & Sassone-Corsi 1995; Monaco *et al.* 1995).

6. ATTENUATION AFTER INDUCTION

Dephosphorylation appears to represent a key mechanism in the negative regulation of CREB activation function. It has been proposed that a mechanism to explain the attenuation of CREB activity following induction by forskolin is dephosphorylation by specific phosphatases (Hagiwara *et al.* 1992; see figure 3). After the initial burst of phosphorylation in response to cAMP, CREB is dephosphorylated *in vivo* by protein phosphatase-1 (PP-1). However, the situation is more complex because it has been shown that both PP-1 and PP-2A can dephosphorylate CREB *in vitro* (Nichols *et al.* 1992) resulting in an apparent decrease in binding to low-affinity CRE sites *in vitro*. Therefore, the precise role of PP-1 and PP-2A in the dephosphorylation of CREB remains to be determined.

Upon cotreatment with cycloheximide, the kinetics of CREM gene induction by forskolin are altered in that there is a significant delay in the post-induction decrease in the transcript; elevated levels persist for as long as 12 h. This implicates a *de novo* synthesised factor which might downregulate CREM transcription (Molina *et al.* 1993). This observation combined with the presence of CRE elements in the P2 promoter, suggested that the transient nature of the inducibility could be due to ICER (figure 3). Consistently, the CRE elements in the P2 promoter have been shown to bind to the ICER proteins. Detailed studies have demonstrated that the ICER promoter is indeed a target for ICER negative regulation (Molina *et al.* 1993). Thus, there exists a negative autoregulatory mechanism controlling ICER expression. The CREM feedback loop predicts the presence of a refractory inducibility period in the gene's transcription (Sassone-Corsi 1994).

7. CREM AND SPERMATOGENESIS

CREM is a highly abundant transcript in adult testis whereas in prepubertal animals is expressed at very low levels. Thus in testis CREM is the subject of a developmental switch in expression (Foulkes *et al.* 1992). Further charaterization revealed that the abundant CREM transcript encodes exclusively the activator form, whereas in prepubertal testis only the

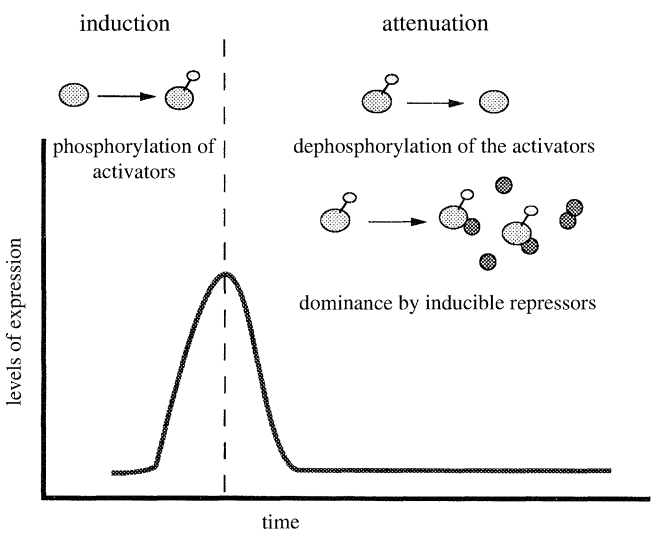

Figure 3. Kinetics of CREM inducibility. After the induction phase, due to the phosphorylation of the activators (i.e. CREB), expression is attenuated by at least two mechanisms: (*a*) dephosphorylation of the activators by some specific phosphatases; and (*b*) negative autoregulation by the *de novo* synthesized ICER repressor on the P2 promoter (see figure 2) (Molina *et al.* 1993; Sassone-Corsi 1994).

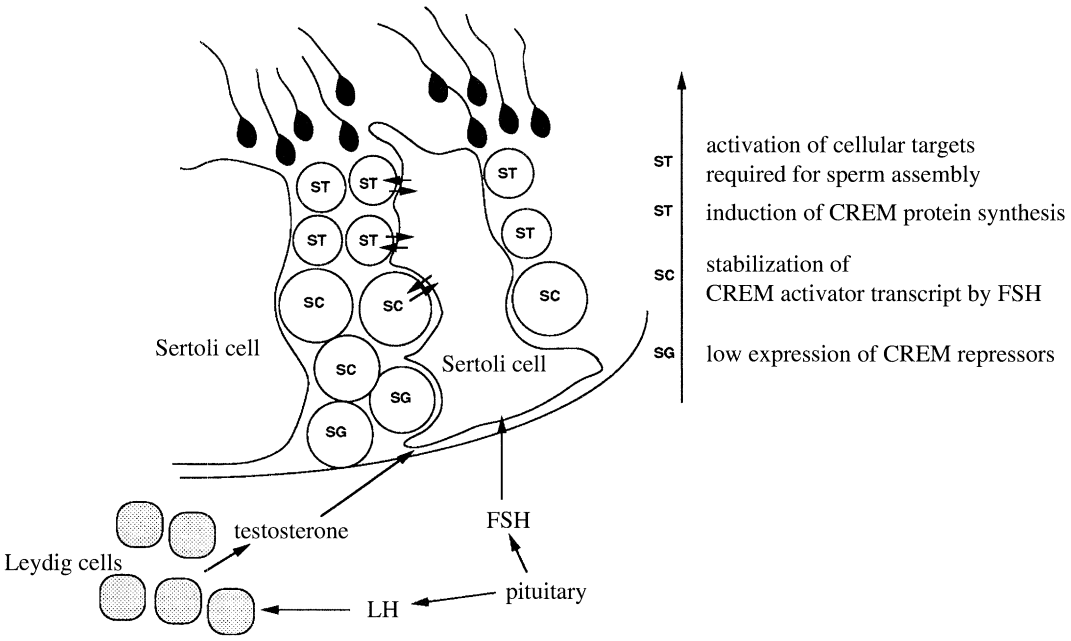

Figure 4. Schematic representation of a section of a seminiferous tubule where the CREM expression pattern is indicated. CREM expression is regulated at multiple levels during spermatogenesis. Premeiotic germ cells (spermatogonia, SG) express a low level of CREM repressor isoforms. During meiotic prophase, the pituitary Follicle Stimulating Hormone (FSH) is responsible for the stabilization of CREM activator transcripts in spermatocytes (SC); CREM protein, on the other hand, is detected only after meiosis in haploid spermatids (ST).

repressor forms were detected at low levels. Thus the developmental switch of CREM expression also constitiutes a reversal of function (Foulkes & Sassone-Corsi 1992).

Spermatogenesis is a process occurring in a precise and coordinated manner within the seminiferous tubules (Jégou 1993). During this entire developmental process the germ cells are maintained in intimate contact with the somatic Sertoli cells. As the spermatogonia mature, they move from the periphery towards the lumen of the tubule until the mature spermatozoa are conducted from the lumen to the collecting ducts.

A remarkable aspect of the CREM developmental switch in germ cells is constituted by its exquisite hormonal regulation. The spermatogenic differentiation programme is under the tight control of the hypothalamic-pituitary axis (Jégou 1993). The regulation of CREM function in testis seems to be intricately linked to FSH both at the level of the control of transcript processing and at the level of protein activity (figure 4). For example, surgical removal of the pituitary gland leads to the loss of CREM expression in the rat adult testis (Foulkes *et al.* 1993).

Furthermore, hypophysectomization in prepubertal animals, prevents the switch in CREM expression at the pachytene spermatocyte stage, thus implicating the pituitary directly in the maintenance of as well as the switch to high levels of CREM expression. Injection of FSH leads to a rapid and significant induction of the CREM transcript. The hormonal induction of CREM by FSH is not transcriptional; and this is consistent with the housekeeping nature of the P1 promoter. Instead, by a mechanism of alternative polyadenylation, AUUUA destabilizer elements present in the 3′ untranslated region of the gene are excluded, dramatically increasing the stability of the CREM message. CREM is the first example of a gene whose expression is modulated by a pituitary hormone during spermatogenesis (Foulkes *et al.* 1993). The implication of these findings is that hormones can regulate gene expression at the level of RNA processing and stability. Importantly the effect of FSH can not be direct because germ cells do not have FSH receptors. Recent data suggest that another hormonal message originating from the Sertoli cells upon FSH stimulation is mediating CREM activation in germ cells (L. Monaco, unpublished results).

8. CREM, A REGULATOR OF GENE EXPRESSION IN HAPLOID GERM CELLS

A first hint as to the role of CREM during spermatogenesis was indicated by its protein expression pattern. In the seminiferous epithelium, CREM transcripts accumulate in spermatocytes and spermatids, but CREM protein is detected only in haploid spermatids (Delmas *et al.* 1993). The absence of CREM protein in spermatocytes reflects a strict translational control and indicates multiple levels of regulation of gene expression in testis. It will be extremely important to analyse further the mechanism of this delay in translation and to define whether it is also hormonally dependent.

Phosphorylation by PKA activates CREM function allowing the relay of the hormonal signal from the cytoplasm to the nucleus (Lalli & Sassone-Corsi 1994). The CREM activator is efficiently phosphorylated by cAMP-dependent PKA activity endogenous to the

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spermatids, indicating that the CREM protein is a nuclear target for the cAMP pathway in haploid spermatogenic cells (Delmas *et al.* 1993).

The expression of CREM activator protein in spermatids coincides with the transcriptional activation of several genes containing a CRE motif in their promoter region. These genes encode mainly structural proteins required for spermatozoon assembly (transition protein, protamine, etc), suggesting a role for CREM in the activation of genes required for the late phase of spermatid differentiation (figure 4). This observation implies that the transcription of some key structural genes is directly linked to hormonal control and consequently to the level of cAMP present in seminiferous epithelium.

To date at least three genes, RT7 (Delmas *et al.* 1993), transition protein-1 (Kistler *et al.* 1994) and calspermin (Sun *et al.* 1995) have been shown to be targets of CREM-mediated transactivation in germ cells.

A demonstration of the role of CREM in the expression of one of these genes, RT7, was shown using *in vitro* transcription experiments. A CREM-specific antibody blocks RT7 *in vitro* transcription with nuclear extracts from seminiferous tubules but not with extracts from liver (Delmas *et al.* 1993). In conclusion, CREM might participate in testis- and developmental-specific regulation of genes containing a CRE in their promoter region, by expressing the repressor isoforms before meiosis, and high levels of the activator after meiosis.

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