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The Nuclear Response to cAMP: Role of Transcription Factor CREM [and Discussion]

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The nuclear response to cAMP: role of transcription factor CREM

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SUMMARY

In eukaryotes, transcriptional regulation upon stimulation of the adenylate cyclase signalling pathway is mediated by a family of cAMP-responsive nuclear factors. This family consists of a large number of members which may act as activators or repressors. These factors contain the basic domain/leucine zipper motifs and bind as dimers to cAMP-response elements (CRE). The function of CRE-binding proteins is modulated by phosphorylation by several kinases. The ICER (inducible cAMP early repressor) protein is the only inducible member of this family. The induction of this powerful repressor is likely to be important for the transient nature of cAMP-induced gene expression. CRE-binding proteins have been found to play an important role in the physiology of the pituitary gland, in regulating spermatogenesis, in the response to circadian rhythms and in the molecular basis of memory.

1. INTRODUCTION

Transcription factors are elements able to integrate information from promoter sequences and signal transduction pathways to control the rate of gene expression. Several transcription factors have been characterized at both the structural and functional level. Their organisation 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 by duplication and divergence of existing genes, but also by a domain shuffling process to generate factors with novel properties (Harrison 1991).

An important step forward in the study of transcription factors has been the discovery that many constitute final targets of specific signal transduction pathways. The two major signal transduction systems are those including 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).

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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). The binding of a specific ligand to a receptor results in the activation or inhibition of the cAMP-dependent pathway. cAMP, in turn, binds cooperatively to two sites on the regulatory subunit of protein kinase-A (PKA), releasing the active catalytic subunit (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. GENES RESPONSIVE TO cAMP

The analysis of promoter sequences of several genes allowed the identification of promoter elements which mediate the transcriptional response to changes in the levels of intracellular cAMP. A number of sequences have been identified of which the best characterized is the CRE (Ziff 1990; Lalli & Sassone-Corsi 1994). A consensus CRE site is constituted by an 8 b.p. palindromic sequence (TGACGTCA) with a higher conservation in the 5′ half of the palindrome with respect to the 3′ sequence. Several genes which are regulated by a variety of endocrinological stimuli contain similar sequences in their promoter regions although at different positions (Borrelli *et al.* 1992).

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However, the CRE consensus sequence has also been found in the context of other distinct promoter elements where they apparently confer different transcriptional properties (Liou *et al.* 1988; Wagner & Green 1993). Another extensively studied example is the ATF (activating transcription factor) element which is present in the early promoters of adenovirus and mediates transcriptional activation by the viral oncogenic protein E1A (Sassone-Corsi 1988; Lee *et al.* 1989).

3. A MULTIGENE FAMILY OF TRANSCRIPTION FACTORS

The first CRE-binding factor to be characterized was CREB (CRE-binding protein; see Hoeffler *et al.* 1988) but subsequently at least ten additional CRE-binding factor cDNAs have been cloned (see figure 1). They were obtained by screening a variety of cDNA expression libraries, with CRE and ATF sites (Hai *et al.* 1989; Foulkes *et al.* 1991). Some key features of these transcription factors are listed as follows.

1. They all belong to the bZip transcription factor class (see figure 1). The number of leucines in the zipper heptad repeat varies from 4 to 6.

2. Outside of the bZip region, homology between these factors is relatively poor. Based upon regions of sequence similarity however, they can be divided into subfamilies.

3. The different factors are able to heterodimerize with each other 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 is likely to reside 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.

4. Alternative splicing of CRE-binding bZip factors seems common. The CREM gene generates a large family of alternatively spliced transcripts although unlike CREB, the function and physiological role of many of these isoforms has been determined (Foulkes *et al.* 1992; Foulkes & Sassone-Corsi 1992; Laoide *et al.* 1993). Alternative polyadenylation and translation initiation have also been observed in the CREM gene (Foulkes *et al.* 1993; Delmas *et al.* 1993).

5. CRE-binding proteins act as both activators and repressors of transcription. Some alternatively spliced CREM isoforms act as antagonists of cAMP-induced transcription. The cAMP-inducible ICER product deserves special mention since it is generated from an alternative promoter of the CREM gene (Molina *et al.* 1993; Stehle *et al.* 1993), and is responsible for its early

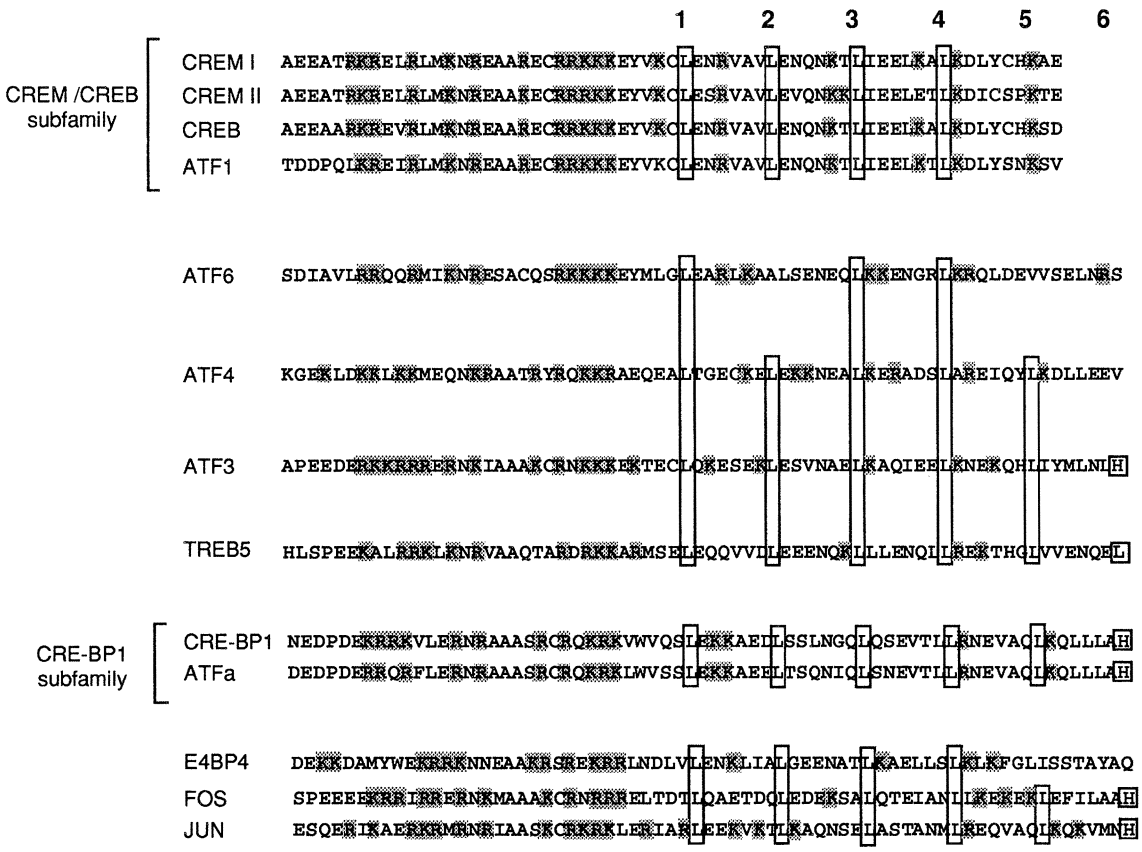

Figure 1. Sequence comparison of the bZip domains of all CRE-binding proteins described to date. The sequences of c-Fos and c-Jun are included for comparison. The boxed histidine residues at the C-terminus of some of the sequences may substitute for leucine to extend the zipper structure At position 2 in the leucine zipper of ATF6, an alanine substitutes for a leucine. Related sequences constituting the CREM/CREB and CREBP1 subfamilies are indicated.

response inducibility which is unique amongst CRE-binding factors.

4. PHOSPHORYLATION-DIRECTED TRANSCRIPTIONAL ACTIVATION

Important steps in the understanding of the control of transcription factor function by phosphorylation have been made with the characterization of the transcriptional activators CREB and CREM (Gonzalez & Montminy 1989; Foulkes *et al.* 1992). Figure 2 shows the general structure of the activator proteins. Interestingly, the relative position of the different functional domains is well conserved among various other CRE-binding proteins such as ATF1 and ATF2 (Hai *et al.* 1989). The transcriptional activation domain contains two independent regions (Lalli & Sassone-Corsi 1994). The first, known as the phosphorylation box (P-box) or kinase inducible domain (KID), 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, divided in two parts, 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*). It is apparent that this domain represents the convergence point for phosphorylation events stimulated by several signal transduction pathways. Indeed, it has been shown that the residue Ser 117 in CREM is the target for phosphorylation by PKC, CamK, p34cdc2 as well as PKA *in vitro* (see figure 2) (de Groot *et al.* 1993*a*, *b*). These phosphorylation events are relevant *in vivo*, because treatment with forskolin, TPA or the Ca^{2+} ionophore A23187 all lead to the enhanced phosphorylation of Ser 117. In PC12 cells, increases in the levels of intracellular Ca^{2+} by membrane depolarization have been shown to increase the phosphorylation of serine 133 in CREB and a concomitant induction of c-*fos* gene expression mediated by a CRE in the c-*fos* 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, becasue PKA also seems to be necessary for c-*fos* induction mediated by Ca^{2+} influx in PC12 cells (Ginty *et al.* 1991). Although phosphorylation appears indispensable for activation by CREB, it is not sufficient for full activity. An acidic region just downstream of serine 133 (140-DLSSD) has been shown to be important for CREB function (Lee *et al.* 1990).

An interesting finding that reveals the complexity of the transcriptional response elicited by these factors

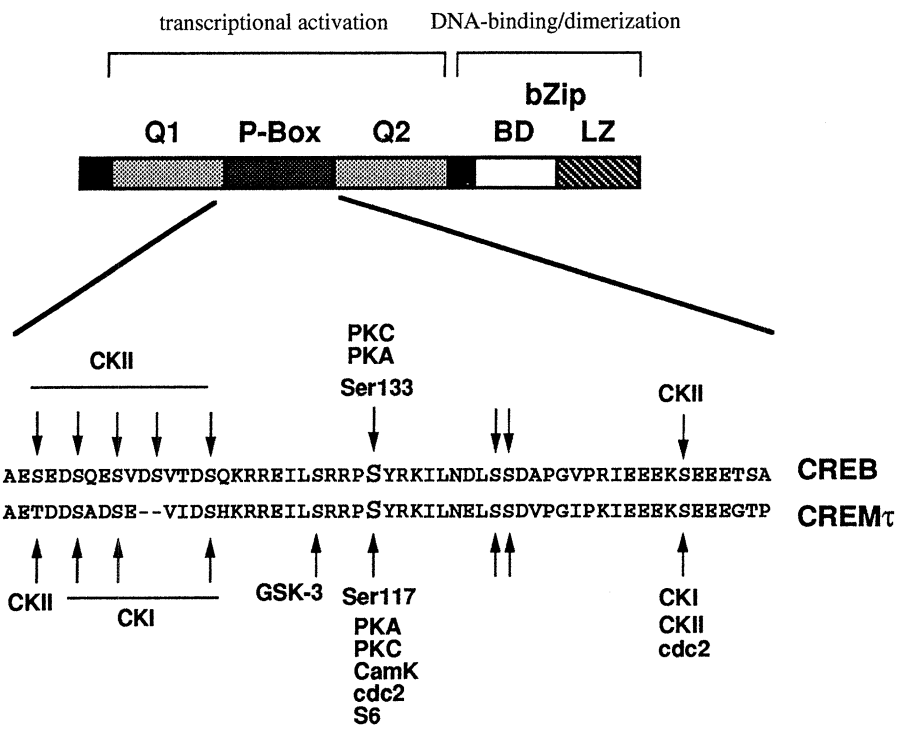

Figure 2. Structure of a CRE-binding protein activator. The two glutamine-rich domains (Q1 and Q2) and the bZip region (BD and LZ) are indicated in addition to the P-Box. This schematic representation is valid for the transcriptional activators CREB and CREM. The detailed amino acid sequence of the CREM and CREB P-box domains. Arrows indicate the serine and threonine residues in CREB and CREM which have been demonstrated *in vivo* or *in vitro* to be phosphorylated by the indicated kinases.

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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).

5. THE GLUTAMINE-RICH ACTIVATION DOMAIN

The two domains flanking the P-box contain about three-fold 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. The Q2 domain appears to make a more significant contribution to the transactivation function than Q1 (Laoide *et al.* 1993). Furthermore, ATF1 lacks a counterpart of the Q1 domain, and still functions as an efficient transcription activator (Rehfuss *et al.* 1991). Thus the P-box region and the Q2 domain are sufficient to mediate cAMP-induced transcription.

It is apparent that the activation domain is inherently a modular structure. Indeed the Q2 domain when fused to a heterologous DNA binding domain, still retains a non-inducible activation function, while the P-box is able to confer PKA inducibility on a heterologous acidic activation domain (for example, GAL4) (Laoide *et al.* 1993). Furthermore, the P-box is able to confer this inducibility in *trans* as well as in *cis*. Thus theoretically, the P-box could be involved in the regulation not only of the adjacent Q domains but also in controlling the activation function of other factors bound to separate promoter elements (Lalli & Sassone-Corsi 1994).

6. MECHANISMS OF REPRESSION

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). 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 decreased 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.

The discovery of the CREM gene opened a new dimension in the study of the transcriptional response to cAMP (Foulkes & Sassone-Corsi 1992). The dynamic and versatile pattern of CREM expression combined with its tissue- and developmental-specific pattern, contrasts with that of the remaining members of the CRE-binding factor family which seem to be constant and ubiquitous (Hai *et al* 1989; Borrelli *et al.* 1992). A striking feature of the CREM gene is the presence of two bZip domains used alternatively by differential splicing (DBDI and DBDII; see figure 3). These features offered the first clue that CREM occupied a priviliged position amongst this group of factors and pointed further to it fulfilling a pivotal role in the nuclear response to cAMP.

Various studies have established that differential transcript processing is central to the regulation of CREM expression. The importance of this mechanism is reinforced by the fact that all the CREM isoforms which incorporate the P-box exons (see figure 3) are generated from a GC-rich promoter (P1) which has been shown to behave as a housekeeping promoter directing a non-inducible pattern of expression (Molina *et al.* 1993; Stehle *et al.* 1993).

7. INDUCIBLE REPRESSOR ICER

An alternative promoter lying within an intron near the 3′ end of the CREM gene, directs the transcription of a truncated product, termed ICER (Inducible cAMP Early Repressor) (Molina *et al.* 1993; Stehle *et al.* 1993). The ICER open reading frame is constituted by the C-terminal segment of CREM (see figure 3). The predicted open reading frame encodes a small protein of 120 amino acids with an expected 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 provocative 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.

The expression of ICER was first described in the pineal gland where it is the subject of a dramatic circadian pattern of expression (Stehle *et al.* 1993). However, recent data implicates dynamic ICER expression as a general feature of neuroendocrine

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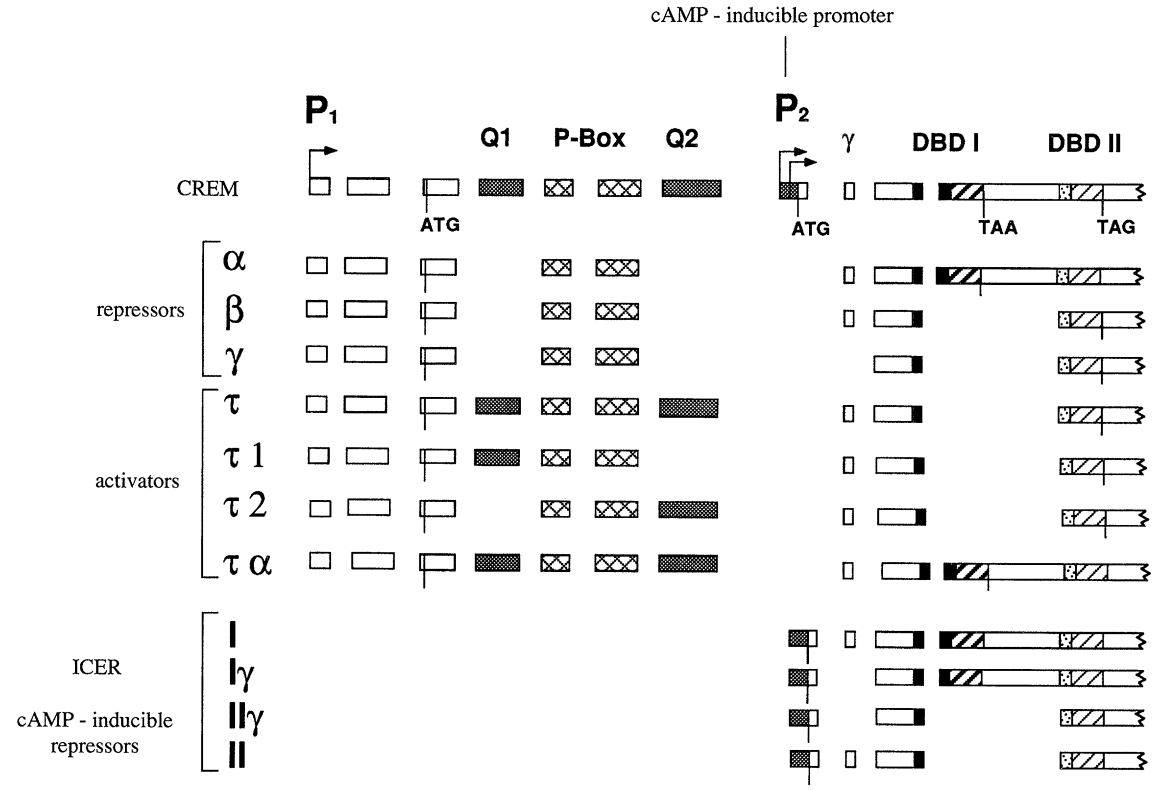

Figure 3. Activators and repressors from the same gene. Schematic representation of the CREM gene. The various activator and repressor CREM isoforms are indicated. The P1 promoter is GC-rich and directs a non-inducible pattern of expression, while the P2 promoter is strongly inducible by activation of the cAMP-dependent signalling pathway.

systems (E. Lalli, N. S. Foulkes & L. Monaco, unpublished data). An important feature about ICER is its inducibility. This makes ICER the only CRE-binding protein whose function is physiologically regulated by altering its cellular concentration.

8. 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 is that 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 since the expression of no other CRE-binding factor has been shown to be inducible to date. For example, the recently characterized CREB promoter is GC-rich and reminiscent of the promoters of constitutively expressed, housekeeping genes (Meyer *et al.* 1993). Similarly, the promoter which directs expression of the other CREM isoforms (P1) is not cAMP inducible (Molina *et al.* 1993).

Clues that the CREM gene was cAMP inducible first came from the demonstration 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 below the threshold of detectability. 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 implicates 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 (Molina *et al.* 1993).

The 5′ end of the ICER clones corresponds to an alternative transcription start site. The start of transcription, which identifies the so-called P2 promoter, is within the 10 kb intron which is C-terminal to the Q2 glutamine-rich domain exon. In contrast to the promoter which generates all the previously characterized CREM isoforms (P1), 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. These features make P2 unique amongst cAMP-regulated promoters and are suggestive of cooperative interactions among the factors binding to these sites.

9. A NEGATIVE AUTOREGULATORY LOOP

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

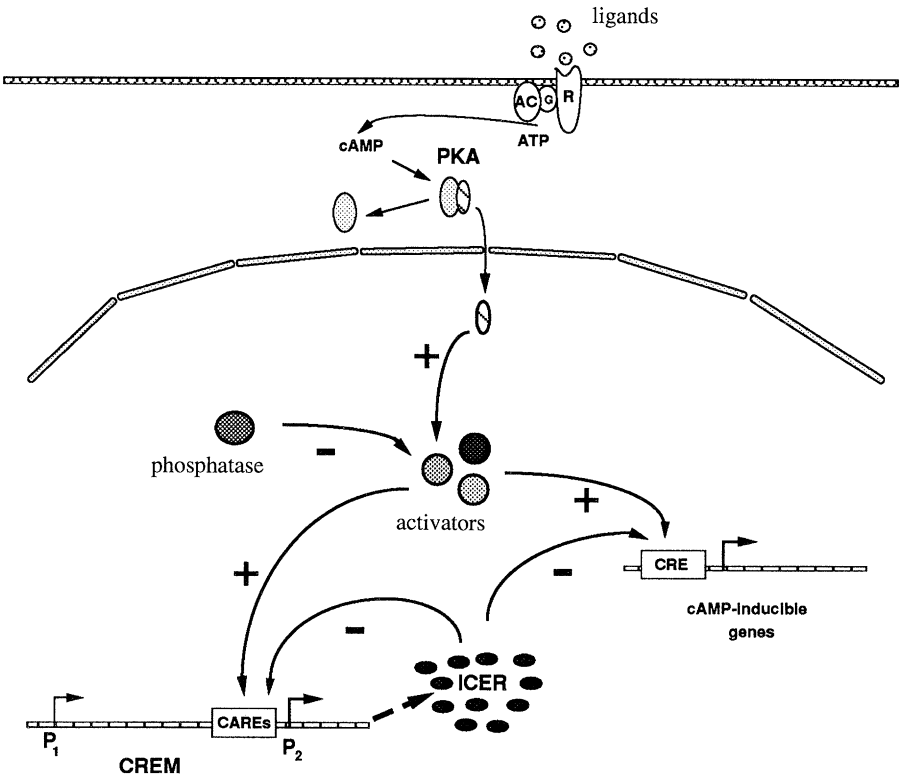

Figure 4. The cAMP signal transduction pathway. Schematic representation of the route whereby ligands at the cell surface interact with and thereby membrane receptors (R) and result in altered gene expression. Ligand binding activates coupled G-proteins (G) which in turn stimulates the activity of the membrane-associated adenylyl cyclase (AC). This converts ATP to cAMP which causes the dissociation of the inactive tetrameric protein kinase A (PKA) complex into the active catalytic subunits and the regulatory subunits. Catalytic subunits migrate into the nucleus where they phosphorylate and thereby activate transcriptional activators. Attenuation of the activators may occur via a nuclear phosphatase. Activators interact with the cAMP response enhancer element (CRE) found in the promoters of cAMP-responsive genes to activate transcription. Phosphorylated factors activate also transcription from the CREM P2 promoter via the CARE elements and ultimately lead to a rapid increase in ICER protein levels. ICER represses cAMP-induced transcription, including that from its own promoter. The consequent fall in ICER protein levels eventually leads to a release of repression and permits a new cycle of transcriptional activation.

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. 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 (see figure 4). The CREM feedback loop predicts the presence of a refractory inducibility period in the gene's transcription (Sassone-Corsi 1994). Recent results are consistent with this hypothesis (M. Lamas, unpublished data).

10. ROLE OF CREM IN 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 the abundant CREM transcript encodes exclusively the activator form, whereas in prepubertal testis only the repressor forms were detected at low levels. Thus the developmental switch of CREM expression also constitutes 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.

CREM activator protein is detected in mature germ cells, such as round spermatids, which have undergone meiosis (Delmas *et al.* 1993). Thus CREM transactivator function must be restricted to the late phase of transcription before the compaction of the DNA. Interestingly, several genes have been identified which are transcribed at the time of appearance of the CREM protein and which include CRE-like sequences in their promoter regions. Several lines of evidence demonstrate that CREM constitutes the first step of a transcriptional cascade which is responsible for the activation of several germ specific genes. 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. Importantly, the dramatic increase in the levels of CREM protein correlates with its concomitant phosphorylation at serine 117 by a cAMP-stimulated PKA activity in round spermatid extracts (Delmas *et al.* 1993). Thus CREM appears to participate in the testis-specific promoter activation of numerous haploid-expressed genes.

A remarkable aspect of the CREM developmental switch in germ cells is constituted by its exquisite hormonal regulation. The spermatogenic differentiation program 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. 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. Injections of FSH lead to a rapid and significant induction of the CREM transcript. The hormonal induction of CREM by FSH is not transcriptional, as predicted by 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 as germ cells do not have FSH receptors. Recent results show that another hormonal message originating from the Sertoli cells upon FSH stimulation is mediating CREM activation in germ cells (L. Monaco, unpublished data).

11. ROLE OF CREM IN CIRCADIAN RHYTHMS

Crucial elements for the synchronization of biological rhythms in mammals are the pineal gland (Tamarkin *et al.* 1985) and the suprachiasmatic nucleus (SCN) (Moore 1983). Environmental lighting conditions are transduced by the pineal gland from a neuronal to an endocrine message, the rhythmic secretion of melatonin (Tamarkin *et al.* 1985). This hormone synthesis is controlled by the SCN, being elevated at night and low during the day (Moore 1983). The cAMP-dependent signal transduction pathway serves as a relay to stimulate melatonin synthesis. Thus, from neuronal pathways which include the retina and the SCN, the pineal gland acts as a temporal regulator for the function of the hypothalamic-pituitary-gonadal axis (Tamarkin *et al.* 1985).

The study of CREM expression in the rat brain indicated a specific pattern of expression (Mellström *et al.* 1993). Analysis of CREM expression in the pineal gland has revealed a dramatic day–night regulation, with peak during the night. The CREM isoform in the pineal gland corresponds to ICER, the early response repressor known to be cAMP-inducible in endocrine cells (Stehle *et al.* 1993). The transcript shows very characteristic and reproducible kinetics of expression. It appears likely that the autoregulatory loop shown to control ICER transient inducibility (Molina *et al.* 1993) would also play a role in the day–night cyclic expression in the pineal gland (N. S. Foulkes, unpublished data).

By a series of physiological experiments, the mechanism controlling this pattern of ICER expression was determined and found to require clock-distal elements. Indeed, it is known that at night, postganglionic fibers originating from the superior cervical ganglia (SCG) release norepinephrine which in turn regulates melatonin synthesis via adrenergic receptors. These analyses have shown that signals from the SCN direct the induction of CREM expression (Stehle *et al.* 1993).

The question of possible targets for downregulation by ICER in the gland is of particular interest. It has been proposed that a reasonable target could be the enzyme which catalyses the rate-limiting step of melatonin synthesis, namely N-acetyl transferase or factors which regulate its activity (J. H. Stehle & N. S. Foulkes, unpublished data).

Another important finding concerning the role of CRE-binding factors in circadian rhythms concerns the cyclic phosphorylation of CREB in the suprachiamatic nucleus (Ginty *et al.* 1993). During the night, upon light stimuli which phase-shift the clock, CREB appears to be efficiently phosphorylated by an SCN-endogenous kinase at the serine 133 residue. Phosphorylation at this site turns CREB into an activator and may be mediated by a number of kinases (Lalli & Sassone-Corsi 1994). Although the nature of the SCN-endogenous kinase has not been established, it seems likely that it could be PKA (Ginty *et al.* 1993). This result would suggest a key role for this kinase or of a counteracting phosphatase in the regulation of the clock function. The target genes for the activated CREB in the SCN have yet to be established.

12. CONCLUSIONS AND PERSPECTIVES

To date, much of the research in transcription factor biology has been devoted to understanding the structure and function relation of these factors. Progress has been extremely rapid and now the basic principles of transcription factor function are close to being elucidated. As a result of this work, new questions have been raised and so it is clear we still have a long way to go before we understand completely how the promoters and enhancers of genes execute transcriptional control. However a much greater challenge lies ahead and that is to relate transcriptional control mechanisms to the physiology and biology of the organism. The use of homologous recombination to inactivate specific gene products offers a powerful tool

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to address such questions. Paradoxically however, in some cases it has complicated our understanding because it is clear that many important factors operate in the context of networks where there is considerable overlap of function. Thus it is possible that the phenotype obtained by loss of a single factor could reflect more the compensatory adjustments made by other factors in its network rather than the function of the target factor itself. CRE-binding proteins appear to play a central role in the physiology of the neuroendocrine system. Further studies of their molecular and functional characteristics will therefore represent another major step forward in our understanding of hormonal regulation and metabolism.

REFERENCES

Borrelli, E., Montmayeur, J. P., Foulkes, N. S. & Sassone-Corsi, P. 1992 Signal transduction and gene control: the cAMP pathway. *Crit. Rev. Oncogenesis* **3**, 321–338.

Cambier, J. C., Newell, N. K., Justement, J. B., McGuire, J. C., Leach, K. L. & Chen, K.K. 1987 Iα binding ligands and cAMP stimulate nuclear translocation of PKC in β lymphocytes. *Nature, Lond.* **327**, 629–632.

Courey, A. J. & Tjian, R. 1989 Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine activation motif. *Cell.* **55**, 887–898.

Dash, P. K., Karl, K. A., Colicos, M. A., Prywes, R. & Kandel, E. R. 1991 cAMP response element-binding protein is activated by Ca^{2+}/calmodulin- as well as cAMP-dependent protein kinase. *Proc. natn Acad. Sci. U.S.A.* **88**, 5061–5065.

de Groot, R. P., Ballou, L. M. & Sassone-Corsi, P. 1994 Positive Regulation of the cAMP-responsive Activator CREM by the p70 S6 Kinase: An Alternative Route to Mitogen-induced Gene expression. *Cell* **79**, 81–91.

de Groot, R. P., den Hertog, J., Vandenheede, J. R., Goris, J. & Sassone-Corsi, P. 1993*a* Multiple and cooperative phosphorylation events regulate the CREM activator function. *EMBO J.* **12**, 3903–3911.

de Groot, R. P., Derua, R., Goris, J. & Sassone-Corsi, P. 1993*b* Phosphorylation and negative regulation of the transcriptional activator CREM by p34cdc2. *Molec. Endocrinol.* **7**, 1495–1501.

Delmas, V., Laoide, B. M., Masquilier, D., de Groot, R. P., Foulkes, N. S. & Sassone-Corsi, P. 1992 Alternative usage of initiation codons in mRNA encoding the cAMP-responsive-element modulator (CREM) generates regulators with opposite functions. *Proc. natn Acad. Sci. U.S.A.* **89**, 4226–4230.

Delmas, V., van der Hoorn, F., Mellström, B., Jégou, B. & Sassone-Corsi, P. 1993 Induction of CREM activator proteins in spermatids: downstream targets and implications for haploid germ cell differentiation. *Molec. Endocrinol.* **7**, 1502–1514.

Foulkes, N. S., Borrelli, E. & Sassone-Corsi, P. 1991 CREM gene: Use of alternative DNA binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* **64**, 739–749.

Foulkes, N. S., Mellström, B., Benusiglio, E. & Sassone-Corsi, P. 1992 Developmental switch of CREM function during spermatogenesis: from antagonist to transcriptional activator. *Nature, Lond.* **355**, 80–84.

Foulkes, N. S. & Sassone-Corsi, P. 1992 More is better: Activators and Repressors from the Same Gene. *Cell* **68**, 411–414.

Foulkes, N. S., Schlotter, F., Pévet, P. & Sassone-Corsi, P. 1993 Pituitary hormone FSH directs the CREM functional switch during spermatogenesis. *Nature, Lond.* **362**, 264–267.

Ginty, D. D., Kornhauser, J. M., Thompson, M., Bading, H., Mayo, K. E., Takahashi, J. S. & Greenberg, M. 1993 Regulation of CREB Phosphorylation in the suprachiasmatic Nucleus by Light and a circadian clock. *Science, Wash.* **260**, 238–241.

Ginty, D. D., Glowacka, D., Bader, D. S., Hidaka, H. & Wagner, J. A. 1991 Induction of immediate early genes by Ca^{2+} influx requires cAMP-dependent protein kinase in PC12 cells. *J. biol. Chem.* **266**, 17454–17458.

Gonzalez, G. A. & Montminy, M. R. 1989 Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at Ser 133. *Cell* **59**, 675–680.

Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T. & Montminy, M. 1992 Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. *Cell* **70**, 105–113.

Hai, T. Y. & Curran, T. 1991 Cross-family dimerization of transcription factors Fos:Jun and ATF/CREB alters DNA binding specificity. *Proc. natn Acad. Sci. U.S.A.* **88**, 3720–3724.

Hai, T. Y., Liu, F., Coukos, W. J. & Green, M. R. 1989 Transcription factor ATF cDNA clones: An extensive family of leucine zipper proteins able to selectively form DNA binding heterodimers. *Genes Dev.* **3**, 2083–2090.

Harrison, S. C. 1991 A structural taxonomy of DNA-binding domains. *Nature, Lond.* **353**, 715–719.

Hoeffler, J. P., Meyer, T. E., Yun, Y., Jameson, J. L. & Habener, J. F. 1988 Cyclic AMP-responsive DNA-binding Protein: Structure Based on a Cloned Placental cDNA. *Science, Wash.* **242**, 1430–1433.

Jégou, B. 1993 The Sertoli-germ cell communication network in mammals. *Int. Rev. Cytol.* **147**, 25–96.

Kistler, M., Sassone-Corsi, P. & Kistler, S. W. 1994 Identification of a functional cAMP response element in the 5′-Flanking Region of the Gene for Transition Protein 1 (TP1), a basic Chromosomal Protein of Mammalian Spermatids. *Biol. Reprod.* **51**, 1322–1329.

Lalli, E. & Sassone-Corsi, P. 1994 Signal Transduction and Gene Regulation: The nuclear Response to cAMP. *J. biol. Chem.* **269**, 17359–17362.

Laoide, B. M., Foulkes, N. S., Schlotter, F. & Sassone-Corsi, P. 1993 The functional versatility of CREM is determined by its modular structure. *EMBO J.* **12**, 1179–1191.

Lee, C. Q., Yun, Y., Hoeffler, J. P. & Habener, J. F. 1990 Cyclic-AMP-responsive transcriptional activation involves interdependent phosphorylatedsubdomains. *EMBO J.* **9**, 4455–4465.

Lee, K. A. W., Fink, S. J., Goodman, R. H. & Green, M. R. 1989 Distinguishable promoter elements are involved in transcriptional activation by E1A and cyclic AMP. *Molec. Cell. Biol.* **9**, 4390–4397.

Liou, H. C., Boothby, M. R. & Glimcher, L. H. 1988 Distinct cloned class II MHC DNA-binding proteins recognize the X-box transcription element. *Science, Wash.* **242**, 69–71.

Masquilier, D. & Sassone-Corsi, P. 1992 Transcriptional cross-talk: nuclear factors CREM and CREB bind to AP-1 sites and inhibit activation by Jun. *J. biol. Chem.* **267**, 22460–22466.

McKnight, S. G., Clegg, C. H., Uhler, S. R., Chrivia, J. C., Cadd, G. G. & Correll, L. L. 1988 Analysis of the cAMP-dependent protein kinase system using molecular genetic approaches. *Rec. Progr. Horm. Res.* **44**, 307–335

Mellström, B., Naranjo, J. R., Foulkes, N. S., Lafarga, M. & Sassone-Corsi, P. 1993 Transcriptional response to cAMP

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in brain: specific distribution and induction of CREM antagonists. *Neuron* **10**, 655–65

Meyer, T. E., Waeber, G., Lin, J., Beckman, W. & Habener, J. 1993 The promoter of the gene encoding 3′ 5′-cyclic adenosine monophopsphate (cAMP) response element binding protein contains cAMP response elements: Evidence for positive autoregulation of gene transcription. *Endocrinology* **132**, 770–777

Molina, C. A., Foulkes, N. S., Lalli, E. & Sassone-Corsi, P. 1993 Inducibility and negative autoregulation of CREM: An alternative promoter directs the expression of ICER, an early response repressor. *Cell* **75**, 875–886.

Moore, R. Y. 1983 Organization and function of the central nervous system circadian oscillator; the suprachiasmatic hypothalamic nucleus. *Fed. Proc.* **42**, 2783–2789.

Nichols, M., Weih, F., Schmid, W., DeVack, C., Kowenz-Leutz, E., Luckow, B. & Schutz, G. 1992 Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. *EMBO J.* **11**, 3337–3346.

Nishizuka, Y. 1986 Studies and perspectives of protein kinase C. *Science*, *Wash.* **233**, 305–312.

Rehfuss, R. P., Walton, K. M., Loriaux, M. M. & Goodman, R. H. 1991 The cAMP-regulated enhancer-binding protein ATF-1 activates transcription in response to cAMP-dependent protein kinase A. *J. biol. Chem.* **266**, 18431–18434.

Roesler, W. J., Vanderbark, G. R. & Hanson, R. W. 1988 Cyclic AMP and the induction of eukaryotic gene expression. *J. biol. Chem.* **263**, 9063–9066.

Sassone-Corsi, P. 1988 Cyclic AMP induction of early adenovirus promoters involves sequences required for E1A-transactivation. *Proc. natn Acad. Sci. U.S.A.* **85**, 7192–7196.

Sassone-Corsi, P. 1994 Rhythmic Transcription and Autoregulatory Loops: Winding up the Biological Clock. *Cell* **78**, 361–364.

Sassone-Corsi, P., Ransone, L. J. & Verma, I. M. 1990 Cross-talk in signal transduction: TPA-inducible factor Jun/AP-1 activates cAMP responsive enhancer elements. *Oncogene* **5**, 427–431.

Sassone-Corsi, P., Visvader, J., Ferland, L., Mellon, P. L. & Verma, I. M. 1988 Induction of proto-oncogene *fos* transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. *Genes Dev.* **2**, 1529–1538.

Sheng, M., McFadden, G. & Greenberg, M. E. 1990 Membrane depolarization and calcium induce c-*fos* transcription via phosphorylation of transcription factor CREB. *Neuron* **4**, 571–582.

Stehle, J. H., Foulkes, N. S., Molina, C. A., Simonneaux, V., Pévet P. & Sassone-Corsi, P. 1993 Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. *Nature*, *Lond.* **365**, 314–320.

Sun, Z., Sassone-Corsi, P. & Means, A. 1995 Calspermin Gene Transcription is Regulated by two cyclic AMP response elements contained in an alternative Promoter in the Calmodulin Kinase IV gene. *Molec. Cell. Biol.* **15**, 561–571.

Tamarkin, L., Baird, C. J. & Almeida, O. F. X. 1985 Melatonin: A coordinating signal for mammalian reproduction? *Science*, *Wash.* **227**, 714–720.

Verma, I. M. & Sassone-Corsi, P. 1987 Proto-oncogene *fos*: complex but versatile regulation. *Cell* **51**, 513–514.

Wagner, S. & Green, M. R. 1993 HTLV-I *tax* protein stimulation of DNA-binding of bZip proteins by enhancing dimerization. *Science*, *Wash.* **262**, 395–399.

Williams, T., Admon, A., Luscher, B. & Tjian, R. 1988 Cloning and Expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes Dev.* **2**, 1557–1569.

Yoshimasa, T., Sibley, D. R., Bouvier, M., Lefkowitz, R. J. & Caron, M. G. 1987 Cross-talk between cellular signalling pathways suggested by phorbol ester adenylate cyclase phosphorylation. *Nature*, *Lond.* **327**, 67–70.

Ziff, E. B. 1990 Transcription factors: a new family gathers at the cAMP response site. *Trends Genet.* **6**, 69–72 .

Discussion

Question. Are any of the processes Dr Sassone-Corsi studied influenced by testosterone?

P. Sassone-Corsi. Testosterone had no effect in our experiments. It should be stressed that the effect of FSH is indirect because there are no FSH receptors on the germ cells; these are all on the Sertoli cells. What we are talking about here is communication between the Sertoli cells and germ cells. We now have evidence that Sertoli cells release a factor, maybe a peptide, in response to FSH which then communicates information onward to the germ cells.

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