An Intron Facilitates Activation of the Calspermin Gene by the Testis-specific Transcription Factor CREM<sup>*</sup>

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Calmodulin is a high affinity Ca<sup>2+</sup>/calmodulin binding protein that is found only in postmeiotic male germ cells. Our previous studies have shown that the calspermin transcript is produced by utilization of a testis-specific promoter located within an intron of the calmodulin kinase IV gene. This promoter contains two cAMP response element-like motifs that bind the testis-specific transcription factor CREM<sup>+</sup>. This interaction is required for transcriptional activation. Here we describe a novel regulatory element, the 111-base pair first intron of the calspermin gene, which is also required for enhancement of transcription by CREM<sup>+</sup> via the cAMP response element motifs. Deletion or inversion of this intron results in loss of CREM<sup>+</sup>-mediated stimulation of transcription. However, CREM<sup>+</sup> stimulates calspermin promoter activity when the intron is moved upstream of the promoter but only when inserted in the proper orientation. Footprint, linker scanning, and deletion analyses were used to identify regulatory elements in the intron. We suggest that the intron functions as an orientation-dependent but position-independent regulatory element to activate the calspermin promoter by facilitating the stimulatory effect of CREM<sup>+</sup> on transcription.

Calspermin is a high affinity Ca<sup>2+</sup>/calmodulin binding protein that is present exclusively in testicular germ cells (1–3). In situ hybridization experiments have shown that the calspermin transcript first appears in primary spermatocytes undergoing the final stages of meiosis and increases in abundance throughout the postmeiotic differentiation process known as spermiogenesis (2). During postnatal development of the rat testis, calspermin mRNA is first detected at day 24 and reaches the high levels characteristic of the adult at about day 35. Therefore, expression of the calspermin gene is both tissue-specific and developmentally regulated. The calspermin transcript is derived from a gene encoding the Ca<sup>2+</sup>/calmodulin-dependent protein kinase IV (CaM kinase IV)<sup>1</sup> (2, 4, 5). Calspermin represents the carboxyl-terminal 169 amino acids of CaM kinase IV and lacks the entire kinase catalytic domain. Primer extension assays have mapped the calspermin transcription initiation site within an intron of the CaM kinase IV gene, which is at least 35 kilobases downstream of the kinase transcription initiation site.<sup>2</sup> Our previous studies showed that the calspermin transcript is produced by utilization of a testis-specific promoter within the same intron of the CaM kinase IV gene that contains the initiation site of the calspermin transcript (6).

Multiple regulatory elements contribute to the testis-specific expression of the calspermin gene. Results from analysis of transgenic mice demonstrate that the −200 to +321 region of the calspermin promoter is sufficient to direct expression of a β-galactosidase reporter gene specifically to testis of postpubertal transgenic mice.<sup>3</sup> We suggested that this region contained at least three regulatory elements. First, there are negative regulatory elements between nucleotides −200 and −80 that may contribute to silencing the calspermin gene in somatic cells. This is based on the finding that whereas a transgene containing the −80 to +361 region of the gene is transcriptionally active in NIH3T3 cells, the −200 to +361 region is inactive (6). Second, there are two CRE-like motifs at −70 and −50 that bind the testis-specific transcription factor CREM<sup>+</sup>. Interaction of CREM<sup>+</sup> with the CRE sites is required for maximal transcriptional regulation of the calspermin gene in NIH3T3 cells (6). Ectopic expression of CREM<sup>+</sup> is sufficient to restore activity to an otherwise inactive calspermin promoter in NIH3T3 cells and to a calspermin promoter-LacZ chimeric transgene in skin fibroblasts prepared from transgenic mice, which normally express the transgene only in testis (6).<sup>2</sup> From these data, we suggested that CREM<sup>+</sup> is the transcription factor primarily responsible for activation of the calspermin gene in testis. Third, we postulated that the 111-bp intron from +130 to +241 might also serve a regulatory role since maximal promoter activity was obtained with constructs that included this region (6). In this paper, we show that this element is absolutely required for CREM<sup>+</sup> to activate transcription from the calspermin promoter.

We demonstrate that CREM<sup>+</sup> stimulates transcription from a wild-type calspermin promoter in NIH3T3 cells but not from the promoter when the intron is deleted or inverted. By placing the intron in different positions and different orientations, we show that the intron serves as a regulatory element in an orientation-dependent but position-independent manner. A region within the intron is protected from DNase I digestion by nuclear extract from NIH3T3 cells. 5′, 3′, and internal deletions of the intron sequence were made to identify regulatory elements. Based on these data we conclude that the entire 111-bp intron is required to facilitate the effect of CREM<sup>+</sup>. We suggest that multiple factors must bind to the intronic sequence to enable CREM<sup>+</sup> to interact with the CREs to regulate transcription of the calspermin gene.

**Experimental Procedures**

Linker Scan Mutagenesis—Linker scan mutagenesis of the first intron of the calspermin gene was performed using PCR. The DNA frag-

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1 The abbreviations used are: CaM kinase IV, Ca<sup>2+</sup>/calmodulin-dependent protein kinase IV; bp, base pair(s); PCR, polymerase chain reaction; CRE, cAMP response element; CAT, chloramphenicol acetyltransferase.

2 Z. Sun, R. L. Means, B. LeMagueresse, and A. R. Means, submitted for publication.
ment between −80 and +361 was divided into two segments from the site where the linker cassette was located. A novel restriction enzyme site was created in the linker cassette to replace the intron sequence. The restriction sites used were KpnI, BglII, and NotI to create 6-, 8-, and 11-nucleotide linker cassettes. Four oligonucleotides were synthesized previously (6).

Deletion Mutation—Different fragments of the calserpin promoter were amplified from the 6-kilobase genomic DNA fragment by PCR according to the conditions described by Boehringer Mannheim for its Taq DNA polymerase. In order to subclone the PCR fragments into the pCAT basic vector (Promega), the 5′ primers (synthesized using an Applied Biosystems 392 DNA/RNA synthesizer) were designed to contain a PstI site, and the 3′ primer was designed to contain a XbaI site. PCR products (amplified by a Perkin Elmer Cetus DNA thermal cycler), which were digested by PstI and XbaI, were then subcloned into the PstI and XbaI sites of the pCAT basic vector. In all cases, the PCR-derived fragments were sequenced to insure that no errors had been introduced. The oligonucleotide sequences used in the construction of the many different promoter fragments are available upon request. Transfection of DNA, CAT assays, preparation of NIH3T3 cell nuclear extract and DNase I footprinting were performed as described previously (6).

RESULTS

An Intron Is Essential for CREM to Stimulate Calserpin Promoter Activity—The calserpin coding region is contained within the CaM kinase IV gene as shown schematically in Fig. 1. Exon I is a calserpin-specific exon. Exons II and III are common to both CaM kinase IV and calserpin. Previous experiments have shown that a DNA fragment around the transcription initiation site (position +1) for the calserpin transcript has strong promoter activity in NIH3T3 cells (6). Optimal promoter activity was obtained from a −80 to +361 DNA fragment, which included the 111-bp intron sequence extending from +130 to +241. In order to investigate the role of this intron in calserpin gene transcription, a series of deletion mutants was made from nucleotide +361 extending to +50, and the mutated promoters were linked to a CAT reporter gene as shown on the left side of Fig. 2. The constructs were transfected into NIH3T3 cells, and CAT activity was assayed. The results are expressed in Fig. 2 (black bars) as a fold stimulation relative to the activity obtained from pCAT basic, a promoterless CAT construct. Deletion of sequences 3′ to the intron did not significantly affect expression of CAT (−80/+321 and −80/+300). However, CAT activity was markedly reduced by deletion of intron sequences (−80/+200, −80/+180). We postulated that since the acceptor sites for splicing in the −80/+200, −80/+180 constructs were deleted, the mRNA generated from both constructs might not be stable. Therefore, we made another four constructs containing no intron (−80/+50), an inverted intron (inversion), an internal deletion of the intron (deletion) or an internal deletion of 50 nucleotides of the intron (internal deletion). The inversion was designed so that the original donor and acceptor sites for splicing were kept intact. All four mutations resulted in markedly reduced CAT activity. The results suggested that the intron positively regulates calserpin promoter activity, but only when in the original orientation.

Previous studies have shown that CREM or CREB binding to the two CRE motifs greatly stimulates activity of the wild-type calserpin promoter but not of a promoter in which both CREs were either mutated or deleted (6). Since the CREs and the intron appear to be regulatory elements of the calserpin promoter, we studied the relationship between these elements. The constructs in Fig. 2 were cotransfected into NIH3T3 cells with or without expression vectors for CREM and a constitutively active fragment of CaM kinase IV (6). The black bars indicate the amount of CAT activity generated following trans-
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Fig. 3. Position and orientation effects of the intron. The intron was placed in different positions and orientations relative to the calspermin promoter. The intron is denoted by a rectangle containing an arrow, which indicates the orientation of the intron. The structure of each promoter construct is shown in the left side of the figure. CAT activity obtained from transfection with white bars or without expression vectors encoding CREM and CaM kinase IV (black bars) is shown. CAT activity is calculated as the ratio of the activity obtained for the construct to that obtained for pCAT basic, which is a promoterless CAT construct. The results are the mean values of at least three independent experiments ± S.E.

Fection of the promoter-CAT constructs alone, and the white bars indicate the amount of CAT activity resulting from cotransfection with expression vectors for CREM and CaM kinase IV. When the intron was intact, CREM-stimulated promoter activity (Fig. 2, constructs −80/+361, −80/+321, −80/+300, and −80/+242). By deleting part of the intron, CREM stimulation of promoter activity was abolished (Fig. 2 constructs −80/+200, −80/+180, and −80/+50). Deletion (−80/+50), inversion of the intron, internal deletion of the entire intron or of the central 50 nucleotides of the intron (Fig. 2, last two constructs) eliminated CREM stimulation of promoter activity. These results suggested that the intron was required for CREM activation of promoter activity.

The Intron Functions in a Position-independent but Orientation-dependent Manner—In order to study the requirements for regulation of the calspermin promoter by the intron, constructs in which the intron was inserted at different positions, orientations, or copy numbers were made as shown in Fig. 3. The intron is denoted by a rectangle containing an arrow, which indicates its orientation. Fig. 3 (black bars) shows the CAT activity from each of the constructs. The intron in the −80/+361 promoter was in the original position and orientation (+). Its presence resulted in a 4-fold increase in CAT activity relative to that produced by the −80/+50 promoter, which does not contain the intron (Fig. 3, compare constructs II and III), whereas the intron itself produced no promoter activity when placed upstream of CAT reporter gene (Fig. 3, construct I). If the intron was moved upstream of the −80/+50 promoter and placed in the plus orientation, activity was increased 3-fold relative to the intronless control (Fig. 3, construct IV). Switching the intron to the minus orientation abolished the −80/+50 promoter activity (Fig. 3, construct V). Two copies of the intron in the plus orientation upstream of the −80/+50 promoter had the same stimulatory effect as one copy (Fig. 3, construct VI), suggesting that the effect of the intron is not copy number-dependent. Constructs VII and VIII contained the −80 to +361 promoter region, which included an intron in the original position and the plus orientation. Both constructs also contained an additional copy of the intron placed upstream of the −80/+361 promoter. This upstream intron had no significant effect on CAT activity produced by the −80/+361 promoter regardless of its orientation. Constructs IX and X contained the −80/+361 promoter, which included a copy of the intron in the original position but in the minus orientation (acceptor and donor sites for splicing were kept intact) and one or two copies of the intron positioned upstream in the plus orientation, respectively. Neither of these latter constructs was transcriptionally active. Therefore, when the intron is in the proper position but in the minus orientation, transcription is strongly inhibited and cannot be overcome by additional properly oriented copies placed upstream of −80.

The constructs in Fig. 3 were also cotransfected into NIH3T3 cells with expression vectors for CREM and CaM kinase IV. In Fig. 3, the black bars indicate the amount of CAT activity generated following transfection of the promoter-CAT constructs alone, and the white bars indicate the amount of CAT activity resulting from cotransfection with expression vectors for CREM and CaM kinase IV. Constructs II and III served as positive and negative controls, respectively. CREM-stimulated CAT activity from the −80/+361 promoter, which contained a properly positioned and oriented intron (Fig. 3, construct III), but not from the −80/+50 promoter, which contained no intron (II). Placing an intron upstream of the −80/+50 promoter in the plus orientation rescued the stimulatory effect of CREM (Fig. 3, construct IV). Switching this upstream intron to the minus orientation resulted in a complete loss of the stimulatory effect (Fig. 3, construct V), suggesting an orientation-dependent requirement for the intron to facilitate the stimulatory effect of CREM. CREM-mediated enhancement of transcription was somewhat reduced when two copies rather than one copy of the intron were placed upstream of the −80/+50 promoter (Fig. 3, construct VI). An additional copy of the intron placed in either orientation upstream of the −80/+50 promoter containing an intron at the proper position and in the plus orientation had no effect on CREM stimulation of transcription (Fig. 3, constructs VII and VIII). If the intron in the −80/+361 promoter was in the correct position but in the minus orientation, CREM did not stimulate promoter activity even when one or two copies of the intron were positioned upstream of the promoter in the plus orientation (constructs IX and X). Therefore, one copy of the intron in the proper position was dominant and either stimulated or inhibited the ability of CREM to enhance activity from the calspermin promoter depending on its orientation.

Identification of a Protected Region in the Intron by DNase I Footprint Assay—In order to test for the presence of regulatory sequences in the intron, we performed DNase I footprint assays. A 32P-end-labeled DNA probe containing sequences from −200 to +361 was subjected to DNase I digestion after addition of nuclear extract from NIH3T3 cells. The results of this analysis are shown in Fig. 4. One region protected by CREM and CaM kinase IV digestion mapped between nucleotides +170 and +200 of the intron (Fig. 4, lane C). This region was protected by presence of NIH3T3 cell nuclear extract but not by the bovine serum albumin control (Fig. 4, lanes A and B). In order to examine the specificity of this protection, a competition assay was performed. An unlabeled, double-stranded oligonucleotide representing the +170 to +195 region of the intron was preincubated with nuclear extract for 20 min before adding the labeled
probe. Addition of this oligonucleotide to the nuclear extract effectively competed for binding of the putative factor (Fig. 4, lane D), whereas a random sequence oligonucleotide did not (Fig. 4, lane E). Internal deletion of 50 nucleotides containing the protected region of the intron resulted in disruption of the stimulatory effect of CREM (Fig. 2, last construct). Computer-assisted analysis of the calspermin promoter region identified a potential binding site for glucocorticoid receptor (similar to one in the murine mammary tumor virus promoter) in the region protected in the DNase I footprint assay. To determine whether the glucocorticoid receptor could stimulate calspermin promoter activity, an expression vector encoding the glucocorticoid receptor was cotransfected with the CAT construct containing the −80/+361 region of the calspermin promoter, and dexamethasone was added to cells to serve as a receptor ligand. There was no stimulation of promoter activity (data not shown), suggesting that the factor binding to the intron region is not glucocorticoid receptor.

Analysis of the Intron by Linker Scanning Mutagenesis—Since a region in the calspermin intron between +170 and +200 appears to bind transacting factors and acts as a regulatory element for transcription, and an internal deletion of 50 nucleotides from +150 to +200 of the intron, which contained the region protected from DNase I digestion, resulted in the loss of the CREM stimulatory effect (Fig. 2, last construct), we attempted to identify the regulatory sequences functionally by linker scanning mutagenesis as shown in Fig. 5. Sequences indicated by rectangles were replaced with linker cassettes, which scanned from +143 to +213. None of these constructs resulted in a significant change in promoter activity relative to the wild-type promoter, and their transcriptional activity was similarly stimulated by cotransfection with expression vectors encoding CREM and CaM kinase IV. Therefore, the regulatory element may not be confined in a small contiguous DNA fragment or the regulatory element may not be disrupted by linker scan mutagenesis.

Deletion Analysis of the Intron—The 3' deletions of the intron sequences in Fig. 1 that decreased transcription would interfere with the splicing machinery since the acceptor sites for splicing were deleted. Such results are difficult to interpret because it is possible that the intron would serve a structural role in transcript processing. To exclude this possibility, we made 5' deletions from the intron inserted upstream of the −80/+50 construct (Fig. 3, construct III). Deletions were made every 20 nucleotides as shown in the left side of Fig. 6. All of these deletions resulted in significantly reduced promoter activity relative to the parent construct (black bars), and their transcriptional activity was not stimulated by cotransfection with expression vectors encoding CREM and CaM kinase IV (white bars). Therefore, 5', 3', or internal deletions of the intron all resulted in disruption of the stimulatory effect of the intron in a position-independent manner. Collectively these results suggest that the shortest functional fragment that facilitates CREM to stimulate transcription is the entire 111 bp intron.

**DISCUSSION**

The calspermin transcript is derived from a gene encoding the CaM kinase IV gene in postmeiotic male germ cells (2, 4, 5). Our earlier studies have shown that the calspermin transcript is produced by utilization of a promoter located within an intron of the CaM kinase IV gene (6). About 15 CAT constructs were made to analyze promoter activity from different DNA

**Fig. 5. Analysis of the Intron by Linker Scanning Mutagenesis.** The sequences between +143 and +213 were subjected to linker scan analysis. The constructs contain a −80 to +361 fragment of the calspermin promoter, except the sequences in rectangles (linker cassettes) are replaced by a restriction enzyme site indicated on the top of rectangles. These constructs were cotransfected with or without expression vectors for CREM and CaM kinase IV. CAT activity obtained from transfection with (white bars) or without the expression vectors for CREM and CaM kinase IV (black bars) is shown. CAT activity is calculated as the ratio of the activity obtained for the construct to be tested to that obtained for pCAT basic. The results are the mean values of at least three independent experiments.
fragments of the calsperrmin promoter and, in each case, the intron was required for maximal promoter activity (6). This result has been repeated in several cell lines including NIH3T3, HeLa, and NS20 cells. The -200 to +361 fragment of the calsperrmin promoter, which contains the 111-bp intron, accurately initiates transcription in vitro in testis nuclear extracts. The same DNA fragment targets a lacZ reporter gene specifically to postmeiotic male germ cells of transgenic mice. Therefore, it seems likely that the intron is important for the function of the calsperrmin promoter in vitro as well as in vivo, although the latter has not been assessed experimentally. We demonstrate here that the intron is essential for stimulation of transcription from the calsperrmin promoter by the testis-specific transcription factor CREM$^\tau$.

Introns have been shown to regulate the expression of many genes (7–13). When a 200-bp DNA fragment contained in the first intron of the human DRA gene was linked to the 5′ or 3′ end of a CAT reporter gene driven by the thymidine kinase promoter, it stimulated thymidine kinase promoter activity. The effect was independent of position, orientation, or distance (14), which are the characteristics of the classically defined enhancer. This enhancer is actively involved in the regulation of DRA gene transcription, since several DNase I hypersensitive sites were mapped to the element, and high level expression of the DRA gene depends on its presence. Similarly, transcription of several other genes has been shown to be influenced by a regulatory element within an intron including those for the immunoglobulin heavy chain gene (15), the renin gene (16), and the type IV collagen gene (17).

The transcriptional activation function of CREM$^\tau$ is dependent on the presence of the entire 111-bp intron of the calsperrmin gene. Our previous studies have identified two CRE-like motifs at -70 and -50, which bind the testis-specific transcription factor CREM$^\tau$ and stimulate transcription from the calsperrmin promoter (6). We demonstrate here that both the CRE motifs and the intron are required for the stimulation by CREM$^\tau$, since CREM$^\tau$ no longer stimulates transcription from the calsperrmin promoter in which the intron has been deleted or placed in reverse orientation. When the intron is moved upstream of the promoter and correctly oriented, CREM$^\tau$ stimulates promoter activity. However, CREM$^\tau$ loses its effect when the orientation of the upstream intron sequence is reversed. In addition, 5′ deletions of even 20 nucleotides resulted in the loss of activity as did 3′ or internal deletions of the intron in the original or upstream (data not shown) position. Therefore, the effect of the intron on the function of CREM$^\tau$ is position-independent but orientation-dependent.

There are at least four theoretical ways that the intron could influence the stimulatory effect of CREM$^\tau$. First, the most obvious possibility is that the intron is necessary for stability of the primary transcript. Second, the intron could also contain CRE-like elements. Third, the intron could be required for the physical binding of CREM$^\tau$ to the upstream CRE motifs. Fourth, there could be interactions between CREM$^\tau$ binding at the CRE motifs and intron binding proteins that stabilize the transcription complex or are otherwise required for CREM$^\tau$ function. Since the intron is position-independent but orientation-dependent, we do not favor the stability theory. Inversion of the intron while carefully maintaining the integrity of the splicing junctions or deletion of the central 50 bp of the intron both result in the loss of activity. On the other hand, the 111-bp intron sequence can be moved upstream of the CREs and maintains its regulatory role in this position. We also excluded the second possibility that the intron contains CRE-like elements, because no region in the intron is protected by purified CREM$^\tau$ in a footprint analysis, whereas both CRE-like motifs are protected (6). The effect of the intron is orientation-dependent. The CRE is an orientation-independent response element, because CRE is an inverted repeat sequence element. In addition, CREM$^\tau$ does not stimulate transcription from the intron sequence when it is linked to an SV40 promoter (data not shown). We can excluded the third possibility, because our previous studies demonstrate that CREM$^\tau$ physically binds to the CRE motifs of the calsperrmin promoter in the absence of the intron. The CRE motifs are protected from DNase I digestion by purified CREM$^\tau$ or CREM$^\tau$ in testis nuclear extract, even though the DNA probe (from -500 to +50) contained no intron (6). Bandshift assays indicate that purified CREM$^\tau$ or the CREM$^\tau$ present in testis nuclear extract binds to double-stranded oligonucleotides containing the CREs but not the intron (6). Collectively these results indicate that CREM$^\tau$ binds to the CRE motifs, but in the absence of the intron it does not stimulate transcription from the calsperrmin promoter. Our results support the fourth possibility that there are interactions between CREM$^\tau$ and the intron binding proteins. Since the interactions require both CREs and the intron, mutation of either CREs or the intron should have the same negative effect on the calsperrmin promoter. In support of this idea, the levels of CAT activity produced from the calsperrmin promoter with an internal deletion of the intron is the same as that from the calsperrmin promoter with both CREs mutated or deleted.

Interactions between transcription factors are the basis for transcriptional regulation (18). Interactions between CRE binding factors and other transcription factors have been postulated. Imai et al. (19) revealed that optimal transcription of the phosphoenolpyruvate carboxykinase gene in response to glucocorticoids required not only a glucocorticoid response element but also a CRE in the promoter region. Since a monoclonal antibody to the glucocorticoid receptor coprecipitated CREB, a protein-protein interaction between glucocorticoid receptor and CREB was proposed to account for the role of the CRE in the response of the phosphoenolpyruvate carboxykinase gene to glucocorticoids. Unfortunately, this type of interaction does not explain the calsperrmin promoter. While a region in the intron that is protected from DNase I digestion in nuclear extract corresponds in sequence to a glucocorticoid response element-like element, activity of the calsperrmin promoter was not stimulated in response to glucocorticoids, and the intron failed to bind glucocorticoid receptor (data not shown). In another study, Delegeane et al. (20) identified an enhancer in the promoter region of the human glycoprotein hormone $\alpha$-subunit gene. This enhancer had no independent
activity but, in combination with the CRE found upstream of the enhancer, it significantly increased the tissue-specific activity of both the α-subunit promoter and a heterologous promoter. In this case, the interactions between the CRE binding proteins and other transcription factors facilitated tissue specific expression of the gene.

Whereas we cannot experimentally prove the reason for the orientation dependence of the intron, it might be explained in the following way. Interaction of the intron with the calspermin promoter likely involves several DNA-binding proteins. Productive interactions between a series of DNA-binding proteins that recognize elements in the 5′-flanking region of the calspermin promoter, such as the CRE motifs, and another series that binds the intron may require the correct spatial and directional orientation of these proteins. Reversal of the intron containing sequences crucial for these interactions might lead to disruption of the normal spatial geometry of the transcription complex. In other words, when the intron is in the plus orientation, the interactions between the intron and the promoter elements facilitate formation or stabilization of the transcription initiation complex. When the intron is in the minus orientation, the proteins bound to the intron may prevent the formation of the transcription initiation complex or at least render it sufficiently unstable to support transcription. Several CAT constructs that contain an inverted intron in its natural position have no transcriptional activity (Fig. 3). In these cases, the inverted intron is placed downstream of the transcription initiation site, thus the proteins bound to the inverted intron may not only prevent formation of the transcription initiation complex but also may physically block progression of RNA polymerase II. This may be why two additional plus orientation introns upstream of promoter containing the inverted intron do not rescue the promoter activity. Our studies suggest that one of the factors that binds to the 5′-flanking region of the promoter and interacts with the intron binding proteins is CREMt.

Three lines of evidence demonstrate a requirement for CREMt in the regulation of testis-specific expression of the calspermin gene. 1) CREMt restores activity to a normally inactive calspermin promoter in NIH3T3 cells (6). 2) Anti-CREMt antibody inhibits in vitro transcription activity in testis nuclear extract from the calspermin promoter. 3) CREMt restores transcription from a calspermin promoter-LaZ chimeric transgene in skin fibroblasts prepared from transgenic mice. The CREMt transcript is generated exclusively in testis from an alternative splicing event (21), and CREMt protein is accumulated to high levels in postmeiotic germ cells where the calspermin gene is activated (2, 22). Based on these results, we suggested that appearance of abundant CREMt in postmeiotic germ cells results in activation of the calspermin gene. The results reported here suggest that the intron may contribute to the regulation of testis-specific expression of the calspermin gene. We demonstrate that the intron serves as a position-independent but orientation-dependent regulatory element that may function to facilitate the stimulatory function of CREMt.

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REFERENCES

1. Means, A. R., and Cruzalegui, F. (1993) Rec. Prog. Hormone Res. 48, 79–97
2. Means, A. R., Cruzalegui, F., LeMagueresse, B., Needleman, D., Slaughter, G. S., and Ono, T. (1991) Mol. Cell. Biol. 11, 3960–3971
3. Ono, T., Slaughter, G. R., Cook, R. G., and Means, A. R. (1989) J. Biol. Chem. 264, 2083–2087
4. Jones, D. A., Glad, J., Silson-Shaw, D., Hahn, W. E., and Sikela, J. M. (1991) FEBBS Lett. 289, 105–109
5. Ohnsiedel, C., Bland, M. M., Merrill, B. M., and Sahyoun, N. (1993) Proc. Natl. Acad. Sci. U. S. A. 88, 5784–5788
6. Sun, Z., Sassone-Corsi, P., and Means, A. R. (1995) Mol. Cell. Biol. 15, 561–571
7. Betz, A. G., Millstein, C., Gonzalez-Fernandez, A., Pannell, R., Larson, T., and Neuberger, M. S. (1994) Cell 77, 299–308
8. Brooks, A. R., Nabby, B. P., Taylor, S., Simonet, W. S., Taylor, J. M., and Levy-Wilson, B. (1994) Mol. Cell. Biol. 14, 2243–2256
9. Gomper, R. S., Holst, B. D., Wood, I. C., Jones, F. S., and Edelman, G. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7985–7989
10. Hambor, J. E., Menonne, J., Coon, M. E., Hanke, J. H., and Kavathas, P. (1993) Mol. Cell. Biol. 13, 7056–7070
11. Henke, G., and Brown, M. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7377–7414
12. Herv, W., and Clarke, J. (1986) Cell 46, 461–470
13. Schirm, S., Jirny, J., and Schaffner, W. (1987) Genes & Dev. 1, 65–74
14. Peterlin, B. M. (1991) Res. Immunol. 142, 193–199
15. Lefranc, G., and Lefranc, M. P. (1999) Biochimie (Paris) 72, 7–17
16. Morris, B. J. (1992) J. Hypertens. 10, 337–342
17. Birtstein, P., McKay, J., Liska, D. J., Apone, S., and Devaruyalu, S. (1988) Mol. Cell. Biol. 8, 4851–4857
18. Mitchell, P. J., and Tjian, R. (1989) Science 245, 371–378
19. Imai, E., Miner, J. N., Mitchell, J. A., Yamanoto, K. R., and Granner, D. K. (1993) J. Biol. Chem. 268, 5353–5356
20. Delegeane, A. M., Ferland, L. H., and Mellon, P. L. (1987) Mol. Cell. Biol. 7, 3994–4002
21. Fouligues, N. S., Melström, B., Benusiglio, E., and Sassone-Corsi, P. (1992) Nature 355, 80–84
22. Delmas, V., van der Hoorn, F., Melström, B., Jegou, B., and Sassone-Corsi, P. (1993) Mol. Endocrinol. 7, 1502–1514