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Identification of a Candidate c-*mos* Repressor That Restricts Transcription of Germ Cell-Specific Genes

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The c-*mos* proto-oncogene is specifically expressed in female and male germ cells. Previous studies identified a negative regulatory element (NRE) upstream of the c-*mos* promoter that suppresses c-*mos* transcription in transfected NIH 3T3 cells. In this study, we used gel shift assays to detect proteins in nuclear extracts of NIH 3T3 cells that bind to the c-*mos* NRE in a sequence-specific manner. One protein was found to bind to a region of the NRE which was shown by site-directed mutagenesis to be required for suppression of c-*mos* transcription. This factor was present in nuclear extracts of several somatic cell lines and tissues but not in male germ cells in which c-*mos* is transcribed, suggesting that it is a somatic cell repressor of c-*mos* transcription. The binding site of the candidate repressor within the c-*mos* NRE consists of sequences related to putative NREs identified in two other male germ cell-specific genes (encoding protamine 2 and phosphoglycerate kinase 2). The c-*mos* repressor bound and could be UV cross-linked to these protamine 2 and phosphoglycerate kinase 2 gene sequences as a protein with an apparent molecular mass of ~30 kDa. The repressor binding site is also conserved in two other germ cell-specific genes (encoding testis-specific cytochrome *c* and heat shock-like protein 70), suggesting that the c-*mos* repressor may be generally involved in suppressing transcription of germ cell-specific genes in somatic cells.

The c-*mos* proto-oncogene encodes a cytoplasmic protein-serine/threonine kinase which is specifically expressed in germ cells and has been found to play a key role in meiotic maturation of both *Xenopus* and mouse oocytes. In *Xenopus* oocytes, microinjection of antisense oligonucleotides has demonstrated that Mos is required for the initiation of meiosis (28) as well as for progression from meiosis I to meiosis II (14). In addition, Mos has been identified as a component of cytostatic factor in *Xenopus* eggs and thus appears to be involved in the maintenance of metaphase II arrest (29). In contrast to *Xenopus* oocytes, mouse oocytes microinjected with antisense c-*mos* oligonucleotides developed normally through meiosis I (19, 20). These oocytes, however, failed to progress through meiosis II, instead re-forming nuclei and subsequently cleaving to two cells (19, 20). Recent experiments in which c-*mos* was disrupted by homologous recombination in mice further confirm that c-*mos*-deficient oocytes complete meiosis I normally but fail to arrest at metaphase II and instead undergo parthenogenetic activation (6, 13). Thus, c-*mos* is required for progression through meiosis II and maintenance of metaphase II arrest in both *Xenopus* and mouse oocytes.

Consistent with its role as a regulator of the meiotic cell cycle, c-*mos* is almost exclusively expressed in male and female germ cells (11, 18, 24, 25). In the mouse ovary, c-*mos* is transcribed in growing oocytes, and c-*mos* mRNA accumulates to approximately 100,000 copies per cell in fully grown oocytes arrested at the diplotene stage of meiotic prophase (11, 18). The oocyte c-*mos* mRNA lacks a detectable poly(A) tail and begins to be polyadenylated and translated following the resumption of meiosis (9, 10, 17). Like other maternal mRNAs in mice, c-*mos* mRNA is then degraded following fertilization (10, 15, 17). In mouse testes, c-*mos* transcripts are detectable in pachytene spermatocytes and reach the highest level in post-meiotic round spermatids (11, 18, 24). In contrast, the c-*mos* gene is generally not transcribed in somatic cells (25). Indeed, since abnormal expression of c-*mos* results either in cell death (22) or in neoplastic transformation (2), suppressing its transcription in somatic cells appears to be a critical aspect of c-*mos* regulation.

The c-*mos* gene is transcribed from two different promoters in spermatocytes and oocytes, located approximately 280 and 53 bp upstream from the c-*mos* translational initiation codon (21, 24). In somatic cells, transient-transfection assays have shown that c-*mos* transcription is suppressed by a negative regulatory element (NRE) extending from 392 to 502 bp upstream of the c-*mos* ATG (31). Further analysis of the NRE by site-directed mutagenesis revealed three regions (called boxes 1, 2, and 3) that contributed to suppression of c-*mos* transcription (31). Boxes 1 and 2 were first noted on the basis of sequence similarity to a region upstream of the mouse protamine 2 gene, which was reported to inhibit in vitro transcription in HeLa cell, but not male germ cell, extracts (4). Box 3 is a pyrimidine-rich sequence downstream of box 2. Mutations or deletions of any of these sequences within the NRE significantly reduced its suppressive activity, allowing transcription from the c-*mos* promoter in transfected NIH 3T3 cells (31). All three of these NREs are conserved in the mouse, rat, and human c-*mos* genes, and the mouse c-*mos* NRE is also active in transfected rat and human cells (31). The NRE region containing boxes 2 and 3 was also found to suppress transcription from a heterologous promoter, consistent with the NRE serving as a repressor binding site (31).

To analyze proteins involved in transcriptional regulation of c-*mos*, we have used gel shift assays to identify nuclear proteins that bind to the c-*mos* NRE. We report here the finding of a protein that specifically binds to the NRE box 2 sequence and is expressed in a variety of somatic cells and tissues but not in male germ cells. The binding site of this protein represents a consensus sequence of five germ cell-specific genes: the c-*mos*,

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protamine 2, phosphoglycerate kinase 2 (PGK-2), testis-specific cytochrome *c*, and heat shock-like protein 70 genes. Site-specific mutagenesis indicates that this consensus sequence is required for activity of the c-*mos* NRE in transfection assays. In addition, the candidate repressor binds and is UV cross-linkable to the conserved sequences from c-*mos*, protamine 2, and PGK-2 genes, implicating the protein that binds to these sequences as a somatic cell repressor of not only c-*mos* but also other germ cell-specific genes.

MATERIALS AND METHODS

Plasmids. Plasmids containing wild-type or mutated mouse c-*mos* upstream sequences linked to the chloramphenicol acetyltransferase (CAT) gene were previously described (31). Additional mutants of the c-*mos* NRE were generated by PCR using pmos731 as the template (31).

Preparation of nuclear extracts. Nuclear extracts were prepared from cell lines and tissues according to minor modifications of published procedures (1, 27). NIH 3T3, BALB 3T3, or HeLa cells maintained in Dulbecco's modification of Eagle's medium with 10% calf serum were harvested from monolayer cultures and collected by centrifugation. Livers, brains, and testes were obtained from BDF1 mice (Charles River Breeding Laboratories, Wilmington, Mass.). Liver and brain cells were prepared by dispersing the tissues in a Dounce homogenizer. Spermatogenic cells were prepared from adult mouse testis by disassociating the seminiferous tubules with collagenase and trypsin (26). The final preparation contained >90% pachytene spermatocytes and postmeiotic spermatids.

All cell pellets were washed once in cold phosphate-buffered saline, and all subsequent steps were performed at 4°C. The cell pellet was suspended in 10 volume of buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 2.5 μM leupeptin, 1 μM pepstatin, 1.5 μM aprotinin) and placed on ice for 10 min. After vigorous vortexing, nuclei were collected by centrifugation in a microcentrifuge for 10 s, resuspended in 2 volumes of buffer B (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 2.5 μM leupeptin, 1 μM pepstatin, 1.5 μM aprotinin), and placed on ice for 20 min. After centrifugation, the supernatant was saved, and small aliquots were frozen immediately in liquid nitrogen and stored at −70°C. Protein concentrations were determined by the method of Bradford (3), with bovine serum albumin as the standard.

Preparation of radiolabeled probes and nonradiolabeled competitor DNAs. DNA probes used for the gel shift assay were obtained by PCR amplification of the NRE region, using wild-type or mutant plasmids as templates. PCRs were performed by using a GeneAmp PCR reagent kit and AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). One micromolar each of two primers and 1 ng of template DNA were mixed in 100 μl of reaction mixture containing 200 μM each of four deoxyribonucleoside triphosphates and 2.5 U of polymerase. The amplification was carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 35 cycles of 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C. The probes were labeled either by direct incorporation of [α-^{33}P]CTP (NEN, Boston, Mass.) during PCR or by end labeling with [γ-^{33}P]ATP (NEN), using T4 polynucleotide kinase (Collaborative Research Inc., Waltham, Mass.). In addition, all other short probes (<20 bp) were made by annealing two synthesized complementary-strand oligonucleotides and labeled by T4 kinase. The DNA fragments used in the competition experiments were prepared as described above except that they were not radioactively labeled. All probes or competitor DNA fragments were then purified by polyacrylamide gel electrophoresis and subsequent elution from gel slices in 0.5 M ammonium acetate–1 mM EDTA.

Gel electrophoretic mobility shift assay. Nuclear extracts were mixed with 1 μg of poly(dI-dC) in buffer containing 10 mM HEPES (pH 7.5), 10% glycerol, 1 mM EDTA, and 100 mM NaCl and incubated for 15 min at room temperature. Approximately 20 fmol of radiolabeled DNA was added, and incubation was continued at room temperature for another 30 min. Samples were then layered onto 5% polyacrylamide gels and electrophoresed in 45 mM Tris–45 mM borate–1 mM EDTA buffer (pH 8.0) for 2.5 h at 200 V. They were then transferred to Whatman 3MM paper, dried, and exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, Calif.). Signals were analyzed in a PhosphorImager (Molecular Dynamics).

Transient-expression assay. Expression of Mos-CAT plasmids in transfected NIH 3T3 cells was assayed essentially as described previously (31). NIH 3T3 cells (5×10^5/60-mm-diameter dish) were transfected with 5 μg of plasmids carrying CAT reporter genes linked to c-*mos* regulatory sequences plus 15 μg of calf thymus DNA as a carrier. After 48 h, the transfected cells were lysed by repeated cycles of freezing and thawing. Cell extracts were incubated at 37°C overnight in 0.25 M Tris buffer (pH 7.8) with acetyl coenzyme A and [^{14}C]chloramphenicol as substrates. The CAT activity was assayed by thin-layer chromatography. The percent conversion of chloramphenicol to acetylated forms was quantified in a PhosphorImager, using the ImageQuant program (Molecular Dynamics).

UV cross-linking. The protein-DNA complexes were resolved in a gel shift assay as described above. The complexes on the gel were then irradiated in situ with a UV lamp emitting at 254 nm (relative intensity = 720 μW/cm^2 at 3 inches [ca. 7.6 cm]; UVP, Upland, Calif.) for 5 min from a distance of 1 cm. The gel strips containing the complexes were excised and soaked and boiled in sodium dodecyl sulfate (SDS) running buffer before being loaded onto an SDS–15% polyacrylamide gel (7).

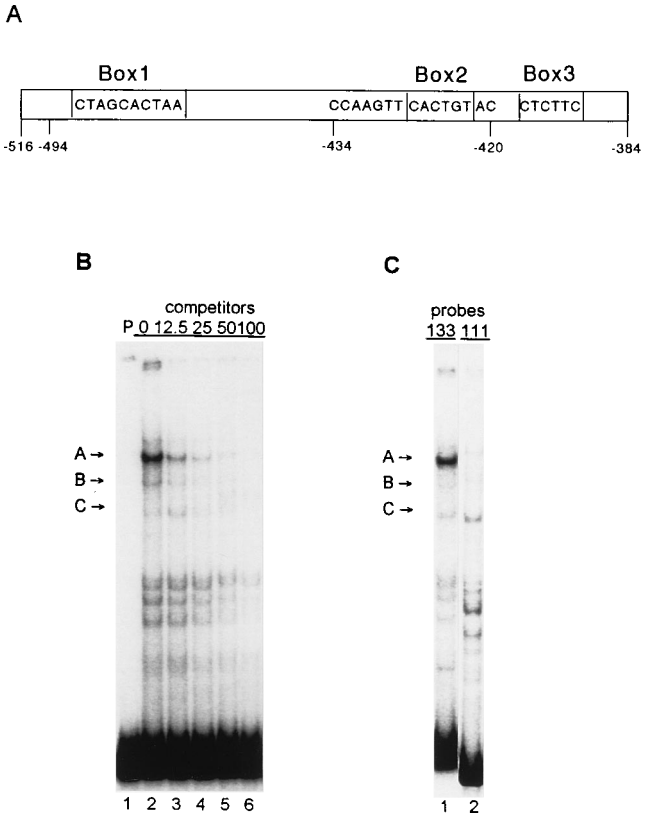


FIG. 1. Detection of protein binding to the c-*mos* NRE in nuclear extracts of NIH 3T3 cells. (A) Diagram illustrating (not to scale) the identified functional elements (boxes 1, 2, and 3 as well as the sequence surrounding box 2) within the c-*mos* NRE. The nucleotide positions shown correspond to the endpoints of various probes used in gel shift assays. (B) ^{33}P-labeled 133-bp DNA probe (extending from −384 to −516) was incubated with 2.5 μg of protein of NIH 3T3 nuclear extracts and 1 μg of poly(dI-dC) in the absence (lane 2) or presence (lanes 3 to 6) of a 12.5- to 100-fold molar excess of the same unlabeled 133-bp DNA fragment as a competitor. Lane 1 represents the probe alone. After electrophoresis of the mixture in a 5% nondenaturing polyacrylamide gel, the gel was dried and exposed to a phosphor screen. (C) Gel shift assays were performed as described for panel B, using either the 133-bp probe extending from −384 to −516 (lane 1) or a 111-bp probe extending from −384 to −494 (lane 2).

RESULTS

Detection of NIH 3T3 nuclear proteins that bind to the c-*mos* NRE. The suppression of c-*mos* transcription in somatic cells is due to an NRE extending from 392 to 502 bp upstream of the c-*mos* ATG (31). To identify nuclear proteins that bind specifically to the NRE, we performed gel shift assays with nuclear extracts of NIH 3T3 cells and a 133-bp probe covering the entire NRE region (extending from −384 to −516) (Fig. 1A). In the absence of nuclear extract (Fig. 1B, lane 1), the DNA probe migrated as a single band; in the presence of NIH 3T3 nuclear extracts (lane 2), several distinct bands with retarded mobility were observed. To determine whether those bands represented the formation of specific protein-DNA complexes, competition experiments with increasing amounts of unlabeled DNA were performed (lanes 3 to 6). The pres-

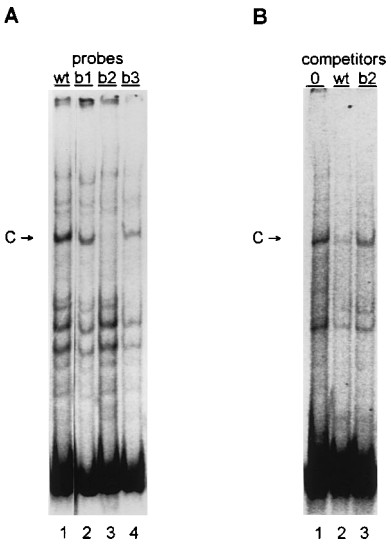


FIG. 2. Detection of specific protein binding to the NRE box 2 sequence. (A) Gel shift assays with nuclear extracts of NIH 3T3 cells were performed with labeled 111-bp NRE probes either corresponding to the wild-type (wt) NRE sequence (lane 1) or containing mutations in box 1 (b1; lane 2), box 2 (b2; lane 3), or box 3 (b3; lane 4). The mutations of these elements were previously described (31). (B) A labeled 111-bp wild-type NRE probe was incubated with NIH 3T3 nuclear extracts in the absence of competitor DNA (lane 1) or in the presence of a 100-fold molar excess of the unlabeled 111-bp fragment either corresponding to the wild-type NRE sequence (lane 2) or containing mutations in box 2 (lane 3).

ence of unlabeled DNA in the incubation clearly disrupted the formation of three complexes (A, B, and C), indicating that specific NRE-binding proteins were present in NIH 3T3 cells.

Further experiments were performed with a 111-bp probe which extended from −384 to −494 and therefore lacked the 5′ 22 bp of the 133-bp probe used in the previous experiment (Fig. 1A). Although this probe still contained all of the previously identified functional elements of the NRE (including boxes 1, 2, and 3), complex A was no longer formed with nuclear extracts of NIH 3T3 cells (Fig. 1C). Therefore, formation of complex A appeared to represent protein binding to sequences upstream of box 1. Further experiments demonstrated that complex A also failed to form with a probe extending from −384 to −502 (data not shown), indicating that complex A formation involved protein binding to sequences upstream of −502.

Previous deletion analysis indicated that sequences upstream of −502 were not required for NRE function in transient-transfection assays (31). Therefore, the protein-DNA interaction involved in complex A formation does not appear to be required for negative regulation of c-*mos* transcription. Instead, the major protein-DNA interaction involving potential functional sequence elements within the NRE appears to correspond to formation of complex C, which is the principal protein-DNA complex formed with the 111-bp probe extending from −384 to −494 (Fig. 1C).

Identification of protein binding to the box 2 NRE sequence. To test whether complex formation with the 111-bp NRE probe involved protein binding to the previously characterized functional elements of the NRE, probes containing mutations in either box 1, 2, or 3 were prepared from mutant plasmids (31) and used in gel shift assays with NIH 3T3 nuclear extracts (Fig. 2A). While probes with mutations in box 1 or box 3 formed protein-DNA complexes similar to those obtained with the wild-type probe, formation of complex C was completely disrupted when the box 2 mutant probe was used. Therefore, it appeared that formation of complex C required protein binding to the box 2 sequence, which had been previously shown in transient-transfection assays to be a functional element within the c-*mos* NRE.

To further confirm the specificity of this binding, competition experiments were performed with either an unlabeled wild-type or box 2 mutant DNA probe as a specific competitor in gel shift assays with a radiolabeled wild-type probe (Fig. 2B). The presence of unlabeled wild-type DNA clearly disrupted the formation of complex C (lane 2). In contrast, formation of complex C was unaffected by the presence of unlabeled box 2 mutant DNA (lane 3), further demonstrating that this protein-DNA complex represented specific protein binding to the box 2 sequence. Because mutagenesis and transfection studies have established box 2 as a functional component of the c-*mos* NRE, the box 2-binding protein is a candidate repressor of c-*mos* transcription.

The candidate repressor is present in several somatic cell types but not in male germ cells. The c-*mos* gene is not transcribed in somatic tissues and cell lines such as NIH 3T3 and BALB 3T3 (23, 25). Suppression of c-*mos* transcription by the NRE was detected in several different somatic cell lines in transient-expression assays (31). To determine whether the candidate repressor identified as a box 2-binding protein was present in somatic cells other than NIH 3T3 cells, we performed gel shift assays using an oligonucleotide probe covering the box 2 region (from −420 to −434) and nuclear extracts prepared from several somatic cell lines and tissues (Fig. 3). Protein binding was detected in nuclear extracts of BALB 3T3 (lane 2) and HeLa (lane 5) cells as well as mouse liver (lane 8) and brain (lane 11). In each case, the electrophoretic mobility of the protein-DNA complex was the same as that of complex C obtained with NIH 3T3 nuclear extracts (lane 1). Competition experiments further established the specificity of this protein-DNA interaction: an unlabeled oligonucleotide probe prevented complex C formation (lanes 3, 6, 9, and 12), whereas a nonspecific oligonucleotide did not (lanes 4, 7, 10, and 13). It

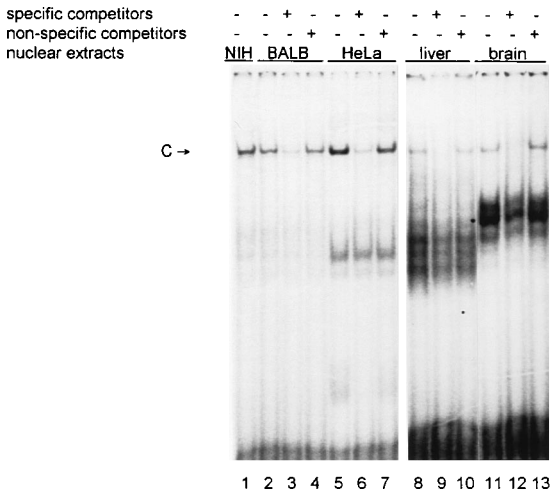


FIG. 3. Detection of the candidate repressor in somatic cells and tissues. A labeled 15-bp probe (extending from −420 to −434) was incubated with nuclear extracts of NIH 3T3 cells (lane 1), BALB 3T3 cells (lanes 2 to 4), HeLa cells (lanes 5 to 7), mouse liver (lanes 8 to 10), or mouse brain (lanes 11 to 13) in the absence of competitor DNA (lanes 1, 2, 5, 8, and 11) or in the presence of a 100-fold molar excess of either the same unlabeled 15-bp DNA fragment used as a probe (specific competitor; lanes 3, 6, 9, and 12) or an unrelated 36-bp DNA fragment (nonspecific competitor; lanes 4, 7, 10, and 13).

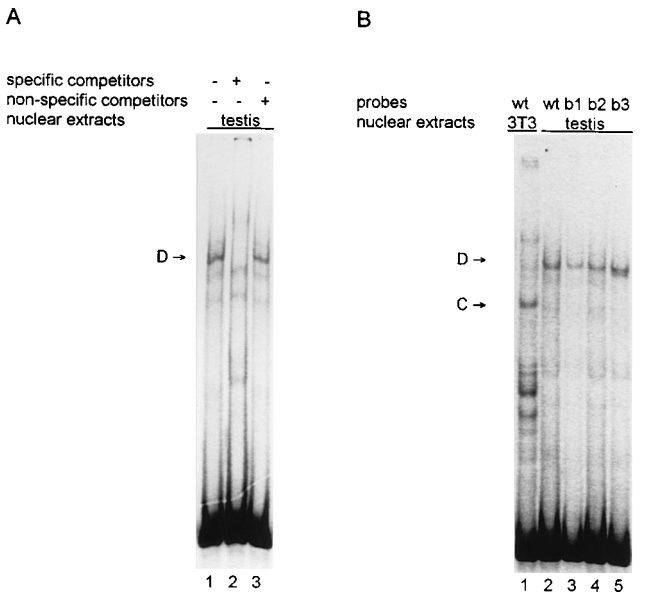


FIG. 4. The candidate repressor is not present in male germ cells. (A) Nuclear extracts of male germ cells prepared from testes of adult mice were incubated with a labeled 111-bp NRE probe either in the absence of competitor (lane 1) or in the presence of a 100-fold molar excess of either the same unlabeled 111-bp DNA fragment used as a probe (specific competitor; lane 2) or an unrelated 36-bp DNA fragment (nonspecific competitor; lane 3). (B) Gel shift assays were performed with nuclear extracts of either NIH 3T3 cells (lane 1) or male germ cells (lanes 2 to 5), using a labeled 111-bp NRE probe either corresponding to the wild-type (wt) NRE sequence (lanes 1 and 2) or containing mutations in box 1 (b1; lane 3), box 2 (b2; lane 4), or box 3 (b3; lane 5).

thus appeared that the putative repressor was detectable in several mouse cell lines and tissues as well as in HeLa cells (a human carcinoma cell line), consistent with the previously reported transcriptional inactivity of the c-*mos* gene in somatic tissues and cells (23, 25), as well as the activity of the mouse c-*mos* NRE in both rodent and human cell lines (31).

We next sought to determine whether the putative repressor was present in male germ cells, in which c-*mos* is actively transcribed. Nuclear extracts were prepared from male germ cells of adult mice, consisting of pachytene spermatocytes and spermatids, since c-*mos* has been shown to be expressed at these stages of spermatogenesis (11, 18, 24). Binding of male germ cell nuclear extracts to the NRE region was then assessed by gel shift assays using a labeled NRE probe extending from −384 to −494 (Fig. 4). The male germ cell extracts formed one protein-DNA complex with the NRE probe (labeled complex D), and complex formation was competed for by addition of an unlabeled NRE probe but not nonspecific DNA (Fig. 4A). However, complex D formed with male germ cell extracts clearly differed in mobility from complex C formed with nuclear extracts of NIH 3T3 cells (Fig. 4B; compare lanes 1 and 2). In addition, male germ cell extracts formed complex D not only with the wild-type NRE probe but also with probes containing mutations of either box 1, box 2, or box 3 sequence (Fig. 4B, lanes 3 to 5).

These results indicate that formation of complex D represents the binding of a male germ cell-specific protein to distinct sequences within the NRE region. This male germ cell factor may play a role in activating c-*mos* transcription in spermatocytes. On the other hand, male germ cells clearly lack the box 2-binding protein responsible for complex C formation in extracts of somatic cells, consistent with the box 2-binding protein being a somatic cell repressor of c-*mos* transcription.

The candidate repressor binds and can be UV cross-linked to the putative NREs of two other germ cell-specific genes. The box 2 sequence of the c-*mos* NRE was initially identified on the basis of similarity with the putative negative regulatory sequence of the germ cell-specific protamine 2 gene (31). More recent studies of another male germ cell-specific gene, encoding PGK-2, identified a putative repressor binding site (8), which is also similar to the box 2 sequence (Fig. 5). To directly test whether the c-*mos* repressor can bind to the NREs of these two genes, we performed gel shift assays comparing the levels of binding of the repressor to the related c-*mos*, protamine 2, and PGK-2 sequences. The same protein-DNA complexes were formed with nuclear extracts of NIH 3T3 cells and oligonucleotide probes corresponding to either the c-*mos*, protamine 2, or PGK-2 sequence (Fig. 5, lanes 1 to 3). To determine whether this resulted from specific binding of the same protein to each probe, competition experiments were performed with excess amounts of either unlabeled c-*mos* (lanes 4, 8, and 12), protamine 2 (lanes 5, 9, and 13), PGK-2 (lanes 6, 10, and 14), or nonspecific DNA (lanes 7, 11, and 15) sequence. In each case, the c-*mos*, protamine 2, and PGK-2 gene sequences specifically cross-competed, indicating that the same protein bound to the related sequences from all three of these germ cell-specific genes.

To further confirm these results, we tried to cross-link the repressor to its binding sites by in situ UV irradiation of the complexes formed in a gel shift assay (Fig. 6). The repressor could be cross-linked upon UV irradiation to the binding sites from either the c-*mos*, protamine 2, or PGK 2 gene. In all

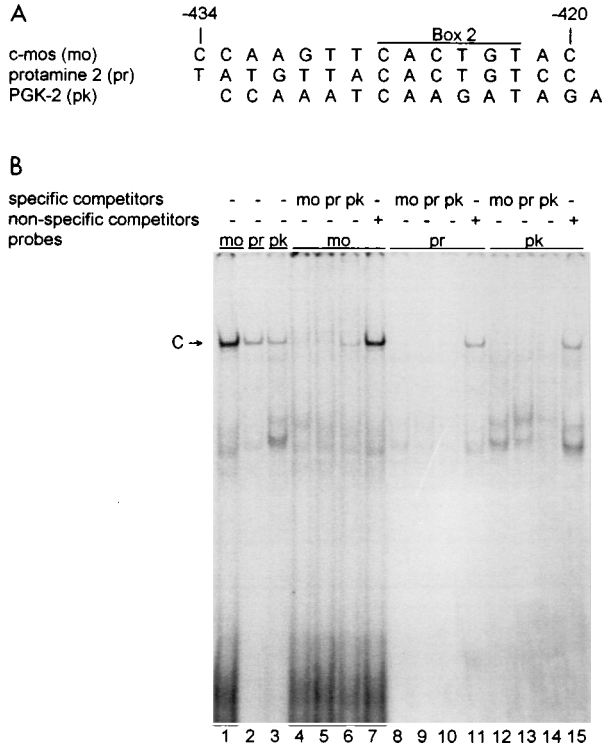


FIG. 5. The c-*mos* repressor binds to the related NREs of protamine 2 and PGK-2 genes. (A) The c-*mos* sequence from −420 to −434 with box 2 sequence overlined is aligned with the related NRE sequences of protamine 2 and PGK-2 genes. (B) Labeled 15-bp probes from either c-*mos* (mo), protamine 2 (pr), or PGK-2 (pk) genes as shown at the top were incubated with NIH 3T3 cell nuclear extracts in the absence of competitor (lanes 1 to 3) or in the presence of the indicated specific or nonspecific competitor oligonucleotides (lanes 4 to 15).

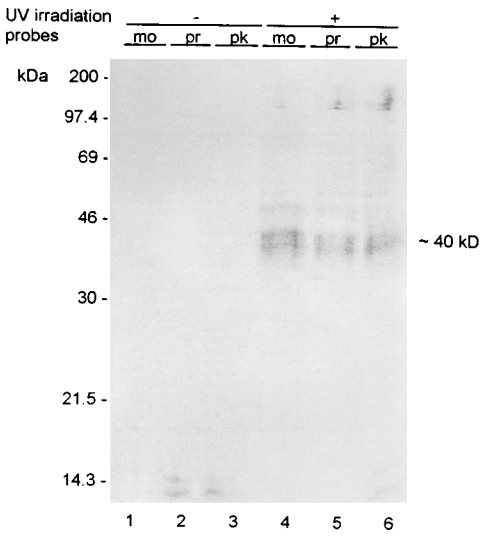


FIG. 6. UV irradiation cross-links the same repressor to the related NRE sequences of c-*mos*, protamine 2, and PGK 2 genes. UV cross-linking was performed by in situ irradiation of the protein-DNA complexes formed in a gel shift assay. The protein-DNA complexes were then cut out and denatured in SDS buffer before being run on a 15% denaturing gel. Lanes 4 to 6 contain proteins resolved from UV-irradiated complexes formed with labeled 15-bp probes from either the c-*mos* (mo; lane 4), protamine 2 (pr; lane 5), or PGK-2 (pr; lane 6) gene as shown in Fig. 5A. Lanes 1 to 3 represent controls in which the respective protein-DNA complexes were not irradiated.

cases, the major protein-DNA complex was indistinguishable in electrophoretic mobility and had an apparent molecular mass of around 40 kDa, corresponding to a protein molecular mass of approximately 30 kDa.

Mapping the candidate repressor binding site. To further define the repressor binding site, we generated mutations in the 5′ (−434 to −428) and 3′ (−427 to −420) halves of the 15-bp oligonucleotide used as a probe in gel shift assays. The effects of these mutations on c-*mos* NRE activity were then determined in transient-expression assays in which a CAT reporter gene was linked to c-*mos* upstream sequences. As previously reported, a plasmid containing c-*mos* upstream sequences extending to −746 (including the NRE) was not expressed in transfected NIH 3T3 cells (Fig. 7). In contrast, a truncated plasmid containing c-*mos* upstream sequences extending only to −392 (and therefore lacking the NRE) yielded significant CAT expression. Plasmids including c-*mos* upstream sequences to −746 but containing point mutations of either the box 2 sequence (pmos7180) or the upstream sequence (pmos7777) yielded comparable levels of CAT expression, which were about 50% of that obtained following deletion of the entire NRE. Therefore, the upstream sequence, as well as box 2, is a functional element of the c-*mos* NRE.

In addition, we tested the effects of mutations in the upstream sequence on repressor binding. In these experiments, the wild-type labeled NRE was used as probe and unlabeled mutant fragments were used as competitors (Fig. 8). The unlabeled wild-type NRE probe effectively competed for formation of complex C (lane 2). In contrast, unlabeled NRE probes containing mutations in either box 2 (pmos7180) or the upstream sequence (pmos7777) failed to compete for complex C formation (lanes 3 and 4). These results indicate that the sequence immediately upstream of box 2 is included in the binding site of the repressor. However, most of this upstream sequence was not conserved among the c-*mos*, protamine 2, and PGK-2 genes (Fig. 8). We therefore tested repressor binding to the 15-bp fragment in which only the two conserved T's immediately upstream of box 2 were mutated (pmos7777a). This unlabeled fragment also failed to compete for complex C formation (lanes 5 to 7), indicating that these two conserved nucleotides immediately upstream of box 2 were required for repressor binding. The repressor binding site therefore contains a 10-bp element, including box 2 and two upstream nucleotides, which is conserved in the c-*mos*, protamine 2, and PGK-2 genes.

DISCUSSION

The specific expression of the c-*mos* proto-oncogene in male and female germ cells provides a novel example of tissue-specific transcriptional regulation. Such regulation is important not only in achieving appropriate c-*mos* expression in germ cells but also in preventing c-*mos* expression in somatic cells, which can either be cytocidal or induce neoplastic transformation (2, 22). Previous studies identified an NRE upstream of the c-*mos* spermatocyte promoter that suppressed c-*mos* transcription in somatic cells (31). Analysis of the NRE by site-directed mutagenesis indicated that it consisted of at least three functional elements (boxes 1, 2, and 3), which were highly conserved in rodent and human c-*mos* genes. We report here the identification of a candidate somatic cell repressor that binds to the c-*mos* NRE sequences.

The candidate repressor was identified as a protein in nuclear extracts of NIH 3T3 cells that specifically bound to box 2 sequences within the c-*mos* NRE. Mutations of box 2 both abolished protein binding and abrogated activity of the NRE, allowing transcription from the c-*mos* promoter in transfected somatic cells. The box 2-binding protein was also present in several additional somatic cell lines and tissues, including HeLa cells, consistent both with the general lack of transcription of c-*mos* in somatic cells and with the activity of the NRE in suppressing transcription from the mouse c-*mos* promoter in

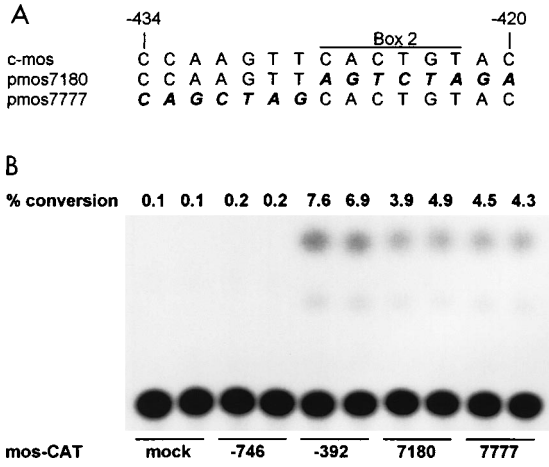
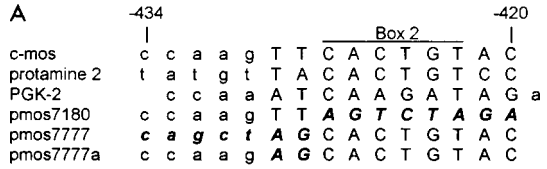


FIG. 7. Effects of mutations in the upstream sequence of box 2 on activity of the c-*mos* NRE. (A) The wild-type sequence of the c-*mos* NRE extending from −420 to −434 is shown on the top line, above the sequences of plasmids pmos7180 and pmos7777 containing the indicated mutations (in boldface italics). (B) NIH 3T3 cells were transfected with 5 μg of Mos-CAT plasmids and collected after 40 h of incubation at 37°C. CAT activity was assayed by thin-layer chromatography, and the percent conversion of chloramphenicol to acetylated forms was quantified by the ImageQuant program. Duplicate transfections were performed with each plasmid. Mock indicates transfection with a control plasmid lacking a CAT gene. The Mos-CAT plasmids designated −746 and −392 contain c-*mos* sequences extending 746 and 392 bp, respectively, upstream of the c-*mos* ATG. The Mos-CAT plasmids designated 7180 and 7777 contain the indicated mutations introduced into c-*mos* upstream sequences extending to −746.



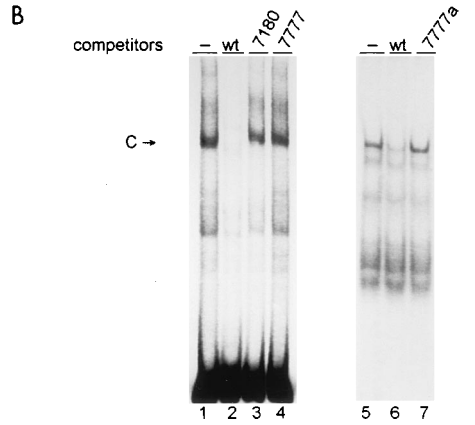


FIG. 8. The dinucleotide upstream of box 2 is included in the binding site of the candidate repressor. (A) The sequence of the c-*mos* NRE extending from −420 to −434 is shown above the related sequences of the protamine 2 and PGK-2 genes, along with mutated sequences of pmos7180, pmos7777, and pmos7777a indicated in boldface italics. The sequences conserved among the c-*mos*, protamine 2, and PGK-2 genes are shown in uppercase. (B) A labeled 111-bp wild-type c-*mos* NRE probe was incubated with nuclear extracts of NIH 3T3 cells either in the absence of competitor DNA (lane 1) or in the presence of a 100-fold molar excess of the same 111-bp fragments either corresponding to the wild-type (wt) sequence (lane 2) or containing mutations in box 2 (7180, lane 3) or the upstream sequence (7777; lane 4). A labeled 15-bp c-*mos* oligonucleotide probe was incubated with nuclear extracts of NIH 3T3 cells either in the absence of competitor DNA (lane 5) or in the presence of a 100-fold molar excess of the same 15-bp fragments either corresponding to the wild-type sequence (lane 6) or containing substitution of AG for TT (7777a; lane 7).

human as well as rodent cell lines (31). In contrast, the candidate repressor was not detected in nuclear extracts of male germ cells in which c-*mos* is normally transcribed. Although it is likely that c-*mos* regulation is complex, involving interactions between multiple proteins, the fact that mutations of the box 2 sequences suffice to abrogate activity of the NRE and allow transcription from the c-*mos* promoter in somatic cells attests to the functional importance of the box 2 element as a repressor binding site. The box 2-binding protein detected specifically in somatic cells, but not in male germ cells, is thus a strong candidate for a repressor of c-*mos* transcription.

Although nuclear extracts of male germ cells lacked the box 2-binding protein implicated as a c-*mos* repressor, they instead formed a distinct protein-DNA complex with the 111-bp NRE probe. This complex did not involve protein binding to the NRE sequences (boxes 1, 2, and 3) previously implicated in repression of c-*mos* in somatic cells but was formed by specific protein binding to another sequence element within the NRE. The binding of a male germ cell-specific protein to this regulatory region upstream of the c-*mos* promoter suggests the possibility that it is an activator of c-*mos* transcription in spermatocytes. Interestingly, the formation of two distinct tissue-specific protein-DNA complexes within the c-*mos* NRE is similar to the protein-DNA interactions which have been reported to occur within a 40-bp region upstream of the germ cell-specific PGK-2 promoter (8). Proteins in extracts of nonexpressing somatic cells bind to PGK-2 gene sequences which are similar to those identified within the candidate repressor binding site of the c-*mos* NRE (discussed below). In addition, extracts of male germ cells bind to a distinct upstream site in the PGK-2 regulatory region, which has been suggested to activate transcription in spermatocytes (8). It is thus possible that the PGK-2 and c-*mos* genes are subject to similar schemes of transcriptional regulation, involving tissue-specific repression in somatic cells and activation in spermatocytes.

c-mos	T	T	C	A	C	T	G	T	A	C
protamine 2	T	A	C	A	C	T	G	T	C	C
PGK-2	A	T	C	A	A	G	A	T	A	G
Cyt Ct	T	T	C	C	C	T	G	T	A	C
Hst 70	T	T	C	A	C	A	G	T	C	C
Consensus	T_4	T_4	C_5	A_4	C_4	T_3	G_4	T_5	A_3	C_4

FIG. 9. The c-*mos* repressor binding site is conserved among the regulatory sequences of five germ cell-specific genes. The conserved sequences of the indicated germ cell-specific genes are aligned, and the consensus is shown below. Cyt Ct, testis-specific cytochrome *c*; Hst 70, heat shock-like protein 70.

The box 2 sequence within the c-*mos* NRE was initially identified by similarity to sequences within a region upstream of the protamine 2 gene which suppressed in vitro transcription in HeLa cell, but not male germ cell, extracts (4). In the present study, we have found that the binding site of the candidate repressor within the c-*mos* NRE houses a 10-bp consensus sequence containing box 2 and flanking nucleotides, which is conserved in both protamine 2 and PGK-2 genes. The c-*mos* repressor also bound and could be UV cross-linked to the protamine 2 and PGK-2 gene sequences in gel shift assays, demonstrating that the same repressor (apparent molecular mass of around 30 kDa) bound to conserved NRE sequences from these three germ cell-specific genes.

Sequences related to the consensus repressor binding site are also found in the 5′ regulatory regions of another germ cell-specific gene, the heat shock-like protein 70 gene (30), and in a testis-specific promoter of the cytochrome c gene (12). The binding site of the candidate c-*mos* repressor is thus related to sequences present in five genes which are specifically transcribed during spermatogenesis (Fig. 9), suggesting that the c-*mos* repressor may play a general role in suppressing the expression of germ cell-specific genes in somatic cells. A similar case in which a repressor in nonneuronal cells binds to the common silencer elements of four neuron-specific genes has recently been reported (5, 16). It thus appear that tissue-specific repression is an important aspect of transcriptional regulation in both the nervous and reproductive systems.

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