Identification of COUP-TF as a Transcriptional Repressor of the c-mos Proto-oncogene*

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The c-mos proto-oncogene is specifically expressed in the male and female germ cells of the mouse and other vertebrates. We previously identified a 15-base pair sequence element (B2) as the binding site of a candidate repressor of c-mos transcription in somatic cells. In the present study, we used the yeast one-hybrid system to isolate HeLa cell cDNAs encoding proteins that specifically bound to the c-mos B2 element. Nucleotide sequencing identified several of the clones isolated in this screen as the orphan nuclear receptors COUP-TFI and COUP-TFII. A COUP-TF-binding site was then identified within the B2 sequence. Complexes formed between purified COUP-TFs and the c-mos B2 probe comigrated in electrophoretic mobility shift assays with those formed using whole nuclear extracts of NIH 3T3 or HeLa cells. Moreover, the complexes formed with NIH 3T3 nuclear extracts and B2 probe were supershifted with antibody against COUP-TF, identifying COUP-TF as the candidate repressor previously detected in these somatic cell extracts. Substitution of a consensus COUP-TF-binding site for the c-mos negative regulatory element suppressed expression from the c-mos promoter in transfected somatic cells, demonstrating the functional activity of COUP-TF as a repressor of c-mos transcription.

The c-mos proto-oncogene, which encodes a protein-serine/threonine kinase, is unusual in its highly restricted pattern of tissue-specific expression. In contrast to other proto-oncogenes, which are generally expressed in a wide range of cell types, c-mos is specifically expressed in the male and female germ cells of the mouse and several other species (1–8). Studies utilizing both microinjection of antisense oligonucleotides and inactivation of c-mos by homologous recombination have demonstrated that Mos is required for normal oocyte meiosis, including progression from meiosis I to meiosis II and maintenance of metaphase II arrest (9–15). Although a role for Mos in male germ cells remains to be demonstrated, its specific expression in spermatocytes suggests that Mos may also function in spermatogenesis.

Transcription of c-mos is initiated from different promoters in murine spermatocytes and oocytes, located approximately 280 and 53 base pairs upstream of the c-mos translation initiation codon, respectively (5, 16). Additional sequences involved in tissue-specific regulation of c-mos have been identified within a negative regulatory region, located approximately 100 to 200 nucleotides upstream of the mouse c-mos spermatocyte promoter, that suppresses c-mos transcription in somatic cells (17). Deletion of these sequences allows expression of reporter constructs driven by the c-mos promoter in transfected NIH 3T3 cells and other somatic cell types (17). Further analysis by site-directed mutagenesis identified three sequence elements (designated B1, B2, and B3) within this region that functioned to repress c-mos transcription and that were conserved in the mouse, rat, and human c-mos genes (17). The c-mos negative regulatory region was further found to suppress transcription from a heterologous promoter and to function in cells of human and rat, as well as mouse, origin (17).

These studies suggested that c-mos transcription in somatic cells was suppressed by repressors that recognized conserved sequences within the c-mos negative regulatory region. Using electrophoretic mobility shift assays and UV cross-linking, we identified a candidate repressor protein that bound to one of the c-mos negative regulatory elements (B2) (18). Mutations of the B2 element both abolished protein binding and allowed transcription from the c-mos promoter in transfected somatic cells. The candidate repressor was present in nuclear extracts of several mouse cell lines and somatic tissues, as well as in HeLa cells, consistent with the general repression of c-mos in somatic cells and with the activity of the mouse c-mos negative regulatory sequence in cells of human as well as mouse origin.

In contrast, nuclear extracts of testicular germ cells, in which c-mos is transcribed, formed a distinct protein complex with the c-mos negative regulatory sequence. The protein identified by binding to the c-mos B2 element thus appeared to be a strong candidate for a somatic cell repressor of c-mos transcription.

In the present study, we have used the yeast one-hybrid system (19, 20) to isolate molecular clones encoding this candidate repressor of c-mos transcription. Our results identify this c-mos regulatory protein as COUP-TF, an orphan member of the nuclear receptor family that has previously been shown to function as a transcriptional repressor of a variety of target genes (21, 22).

Experimental Procedures

Yeast One-Hybrid System—A yeast one-hybrid system (CLONTECH) was used to screen a HeLa cell cDNA library to identify proteins that bound to the c-mos negative regulatory sequence. A c-mos/HIS3 reporter construct was generated by inserting three tandem repeats of the c-mos 15-base pair box 2 (B2) negative regulatory element (CCAGTTTACGTGACGTC) (18) in the SacI and SacII sites of the pHIS integration vector. In this vector, the HIS3 gene is expressed from a minimal promoter that allows a low level of HIS3 expression. The yeast strain YM4271 (his3Δ leu2Δ) was transformed with the c-mos/HIS3 construct and transformants were selected for growth on medium lacking histidine (His− medium). A transformant that grew on His− medium but failed to grow on His+ medium supplemented with 30 mM 3-amo-
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1,2,4-triazole (3-AT), added to suppress growth resulting from low level HIS3 expression from the minimal promoter, was then isolated and used as the c-mos/HIS3 reporter strain for library screening.

A hybrid library in which HeLa cell cDNAs were fused to the GAL4 activation domain (CLONTECH) was then screened by transformation of the reporter strain carrying the c-mos/HIS3 construct. The library vector also contained a LEU2 selectable marker, allowing selection of all transformants on Leu− medium and of transformants expressing hybrid proteins that activated transcription of the c-mos/HIS3 reporter construct on Leu−/His−/3-AT medium.

Preparation of COUP-TFI and TFII—Native COUP-TFI and COUP-TFII were overexpressed using recombinant vaccinia viruses in HeLa cells (23). Each was also overexpressed with amino-terminal polyhistidine construct on LEU selectable marker, allowing selection of all transformants on Leu− medium and of transformants expressing hybrid proteins that activated transcription of the c-mos/HIS3 reporter construct on Leu−/His−/3-AT medium.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from NIH 3T3 and HeLa cells as described previously (18). Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed as described (18). Purified COUP-TFs or nuclear extracts were mixed with 1 μg of poly(dl-dC) in 10 mM HEPES (pH 7.5), 10% glycerol, 1 mM EDTA, 100 mM NaCl and incubated for 15 min at room temperature. Radiolabeled DNA was then added and incubation was continued at room temperature for another 30 min. Samples were then electrophoresed in 5% polyacrylamide gels and analyzed using a PhosphorImager (Molecular Dynamics). In competition experiments, the indicated amount of unlabeled oligonucleotide was added at the same time as the radiolabeled DNA. For supershift experiments, polyclonal antibody against COUP-TF (24) (a generous gift of Ming-Jer Tsai, Baylor College of Medicine) was mixed with nuclear extracts in the presence of poly(dl-dC) and incubated for 30 min at room temperature prior to addition of radiolabeled DNA.

Plasmids—Plasmids containing mouse c-mos upstream sequences linked to the chloramphenicol acetyltransferase (CAT) gene were previously described (17). The c-mos−/746 plasmid contains a 731-base pair c-mos fragment (−16 to −746 with respect to the mouse c-mos ATG) that includes the negative regulatory sequence. The negative regulatory sequence has been deleted from the c-mos−/392 plasmid, which contains sequences from −16 to −392 with respect to the mouse c-mos ATG. The c-mos−/380/DR1 plasmid was constructed from c-mos−/746 by removing the sequences upstream of the BclI site at −380 (including the negative regulatory sequence) and adding a synthetic oligonucleotide containing a single copy of the consensus COUP-TF binding sequence DRI (CAGTTCACAGGTCAGA).

Transient Expression Assays—NIH 3T3 cells (5 × 10⁵ per 60-mm plate) were transfected with 5 μg of CAT plasmid DNA (CaCl₂ gradient purified) and 15 μg of calf thymus DNA as carrier using calcium phosphate (17). Cells were harvested 48 h after transfection and lysed in 100 μl of 0.25 M Tris/HCl (pH 7.8) by 3 cycles of freezing and thawing. Cell lysates were clarified by centrifugation and protein concentration was determined using the Bio-Rad protein assay. Extracts (50 or 100 μg of protein) were incubated overnight at 37 °C in 0.25 μl Tris/HCl (pH 7.8) with acetyl-coenzyme A and [³⁵S]chloramphenicol as substrates. The conversion of chloramphenicol to acetylated forms was assayed by thin-layer chromatography and quantified using a PhosphorImager with ImageQuant software.

RESULTS

Molecular Cloning of Proteins That Bind to the c-mos Negative Regulatory Element—We previously identified a 15-base pair sequence (termed B2 or B2) as the binding site of a candidate repressor of c-mos transcription (18). To identify the candidate repressor, we used the yeast one-hybrid system (19, 20) to isolate cDNA clones encoding DNA-binding proteins that specifically recognized this sequence. The reporter construct used in this screen consisted of three concatenated copies of the 15-base pair c-mos B2 negative regulatory element inserted upstream of the HIS3 gene expressed from a low activity minimal promoter. The yeast strain YM4271 (his3/leu2) was transformed with the reporter plasmid and transformants that expressed low levels of HIS3 were selected for the ability to grow in the absence of histidine but not in the presence of 30 μM 3-AT, which suppresses growth resulting from transcription from the weak HIS3 promoter. A hybrid expression library consisting of HeLa cell cDNAs fused to the GAL4 activation domain was then screened to identify proteins that bound the c-mos B2 sequence and activated HIS3 transcription from the reporter construct. The CDNA library vector also carried a LEU2 selectable marker, allowing selection of all transformants on Leu− medium and double selection of transformants in which HIS3 expression was activated on Leu−/His−/3-AT medium.

From an initial screen of approximately 6 million LEU+ transformants, we isolated 200 colonies that survived the double Leu−/His−/3-AT selection. Plasmids recovered from these initial transformants were then tested by retransformation of the reporter strain, and seven were found to reproducibly induce HIS3 expression in this secondary screen. Nucleotide sequencing identified one of these 7 positive plasmids as COUP-TFI and three as COUP-TFII (22), indicating that the COUP-TFs can bind to the c-mos B2 negative regulatory element. The specificity of this interaction is illustrated in Fig. 1. Transformation with plasmids expressing either COUP-TFI or COUP-TFII fusion proteins allowed growth of yeast carrying the c-mos/HIS3 reporter construct on Leu−/His−/3-AT selective medium, but not of yeast carrying a similar p53/HIS3 reporter construct in which four copies of the binding site for p53 were inserted upstream of HIS3. Conversely, an expression plasmid for a p53/GAL4 fusion protein activated transcription from the p53/HIS3 reporter construct but not from the c-mos/HIS3 reporter. The transcription factors COUP-TFI and COUP-TFII thus appeared to specifically recognize the 15-base pair c-mos B2 negative regulatory element, activating HIS3 transcription when expressed as GAL4 fusions in the yeast one-hybrid system.

Binding of COUP-TF Transcription Factors to the c-mos Negative Regulatory Element in Electrophoretic Mobility Shift Assays—The COUP-TFs bind as homodimers to paired nuclear receptor hexamer-binding sites (A/G/G/T/TCA (22, 25, 26) (Fig. 2). The highest affinity and most common motif among identified COUP-TF regulatory site targets is a direct repeat of the hexamer AGGTCA with single base pair spacing (DR1).
However, the COUP-TFs exhibit relatively promiscuous binding and function on paired hexamers arranged as direct, inverted, or everted repeats with different spacing (25), in contrast to other members of the nuclear receptor superfamily whose functional specificity is largely determined by hexamer spacing and orientation (21). Within the c-mos B2 negative regulatory element, we identified a candidate nuclear receptor hexamer-binding site AGTTCA. This is separated by 1-base pair from a second inverted hexamer AGTACA, which deviates from consensus by a single residue at the permissive fourth position (Fig. 2). Based on the yeast one-hybrid results, this sequence appeared likely to represent a COUP-TF-binding site within the c-mos negative regulatory element. Consistent with this, mutations of the c-mos negative regulatory element that had previously been shown to abrogate binding of the candidate repressor and allow expression of c-mos promoter constructs in transfected NIH 3T3 cells (17, 18) destroyed one or both of these COUP-TF half-sites (Fig. 2).

To further characterize COUP-TF binding to the c-mos regulatory sequence, we compared purified COUP-TFs with proteins present in nuclear extracts of NIH 3T3 and HeLa cells in electrophoretic mobility shift assays using both the c-mos negative regulatory element DR1 and NRRE-1 (Fig. 2) as probes. The purified COUP-TFs formed complexes of similar electrophoretic mobility with both the DR1 and NRRE-1 COUP-TF-binding sites and with the c-mos negative regulatory element B2 probe (Fig. 3). In addition, nuclear extracts of both NIH 3T3 and HeLa cells formed complexes of similar electrophoretic mobility with the c-mos B2, DR1, and NRRE-1 probes. As expected, neither purified COUP-TFI nor NIH 3T3 nuclear extract exhibited significant binding to a probe consisting of only a single nuclear receptor half-site.

The specificity of COUP-TF binding to the c-mos negative regulatory element was further tested by competition experiments (Fig. 4). Both purified COUP-TFII and nuclear extract from NIH 3T3 cells formed similar complexes with B2 probe of the c-mos negative regulatory element. In both cases, binding was competed by an excess of unlabeled probe corresponding either to the c-mos B2 sequence or to the DR1 COUP-TF-binding site. In contrast, binding was not affected by an unrelated competitor corresponding to the binding site of a distinct transcription factor, LSF (27). It is noteworthy that DR1 was a more effective competitor than the c-mos B2 sequence for both purified COUP-TFII and NIH 3T3 nuclear extract. This is consistent with the higher affinities of COUP-TFs for the consensus DR1 elements as opposed to inverted sequences, such as that present in the c-mos B2 sequence (22, 25).
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The results above not only indicate that COUP-TFs bind to the c-mos B2 negative regulatory element, but also that COUP-TF complexes with the c-mos probe comigrate with complexes formed with proteins detected in nuclear extracts of NIH 3T3 and HeLa cells. This is consistent not only with COUP-TFs being able to bind to the c-mos negative regulatory element, but also with the hypothesis that COUP-TFs correspond to the candidate repressor previously identified in these somatic cell extracts. To further test this hypothesis, we used antibody against COUP-TFs in supershift experiments (Fig. 5). Addition of COUP-TF antibody (which recognizes both COUP-TFI and COUP-TFII) supershifted the complex formed between nuclear extract of NIH 3T3 cells and probes corresponding to either the 15-base pair B2 element of the c-mos negative regulatory sequence or to the entire 111-base pair c-mos negative regulatory sequence (411 probe) originally defined by deletion analysis (17). As expected, no protein binding was detected to a 111-base pair c-mos probe containing the 7180 mutation, which destroys the COUP-TF-binding site (see Fig. 2). Antibody against COUP-TF thus recognizes the c-mos negative regulatory element-binding protein detected in somatic cell nuclear extracts, identifying this candidate repressor of c-mos transcription as COUP-TF.

COUP-TF-binding Sites Function as Negative Regulatory Elements of the c-mos Promoter—Previous studies defined three functional elements (designated B1, B2, and B3) within the 111-base pair c-mos negative regulatory region (17). Mutations affecting the previously defined B2 element destroy one or both of the consensus nuclear receptor-binding sites (see Fig. 2) and allow expression of c-mos promoter/CAT constructs in transfection assays in NIH 3T3 cells (17, 18), indicating that the COUP-TF-binding site is one of the functional elements within the c-mos negative regulatory sequence.

To further determine whether a consensus COUP-TF-binding site can functionally replace the B2 negative regulatory element, we investigated the activity of c-mos promoter constructs in which a consensus DR1 COUP-TF-binding site was substituted for the normal c-mos negative regulatory sequences (Fig. 6). In these experiments, the entire c-mos negative regulatory region (including B1, B2, and B3 sequences) was deleted in the c-mos/−392 construct, and replaced with a consensus DR1 COUP-TF-binding site in the c-mos/−380/DR1 plasmid. Addition of a COUP-TF-binding site to this truncated promoter in the c-mos/−380/DR1 construct resulted in a 2–3-fold reduction in CAT expression, to a level about twice that obtained with the full-length c-mos/−746 promoter. The complete negative regulatory sequence contained within the c-mos/−746 construct includes the B1 and B3 elements in addition to B2, and the partial repression obtained with the c-mos/−380/DR1 construct is similar to that seen with other constructs containing only the B2 element (17). It thus appears that a consensus COUP-TF-binding site can functionally substitute for the c-mos B2 negative regulatory element, further indicating that COUP-TF can repress c-mos transcription in transfected somatic cells.

FIG. 5. Antibody to COUP-TF recognizes the c-mos B2 element-binding protein in NIH 3T3 nuclear extracts. Nuclear extracts of NIH 3T3 cells were incubated with c-mos B2 probe, with a probe containing 3 tandem repeats of the B2 sequence (3XB2), with c-mos 411 probe (consisting of the entire 111-base pair c-mos negative regulatory region), or with c-mos 411 probe containing the 7180 mutation (see Fig. 2). Nuclear extracts were preincubated for 30 min with antibody against COUP-TFs in the lanes designated +Ab. Lane 1 is a reaction in which B2 probe was incubated with antibody in the absence of nuclear extract.

FIG. 6. Effect of a consensus COUP-TF-binding site on transcriptional activity of the c-mos promoter. NIH 3T3 cells were transfected with the indicated c-mos/CAT constructs and harvested 48 h post-transfection for assays of CAT activity. The c-mos/CAT constructs contained upstream c-mos sequences extending to either −746 (including the entire negative regulatory region) or to −392 (from which the negative regulatory region has been deleted). A DR1 consensus COUP-TF-binding site has been added in place of the c-mos negative regulatory region in the plasmid designated −380/DR1. The results of five independent transfection assays are presented individually in panel A, with CAT activities expressed relative to the c-mos/−746 construct, and as mean ± S.D. in panel B. The difference in activities of the −392 and −380/DR1 constructs was statistically significant (p < 0.001).

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. Because aberrant expression of c-mos in somatic cells results in either oncogenic transformation or cell death (15, 28, 29), the normal transcriptional
silencing of c-mos is a critical aspect of its control. Previous studies delineated 3 negative regulatory elements (designated B1, B2, and B3) within a 111-base pair region upstream of the c-mos spermatocyte promoter (17) and identified a candidate repressor that bound to the B2 element, suppressing c-mos transcription in somatic cells (18). In the present study, we have identified this somatic cell repressor of c-mos transcription as the orphan nuclear receptor COUP-TF.

Mice and humans encode two closely related members of the COUP-TF family, designated COUP-TFI and COUP-TFII (22). We initially isolated clones of both COUP-TFI and COUP-TFII, which have indistinguishable DNA-binding specificities (22), in a yeast one-hybrid screen for proteins that bound to the c-mos B2 negative regulatory element. A COUP-TF-binding site was then identified within this element. Notably, the complexes formed between purified COUP-TFs and the c-mos B2 probe in electrophoretic mobility shift assays comigrated with those formed using whole nuclear extracts of NIH 3T3 or HeLa cells, suggesting that COUP-TF was the candidate repressor previously detected in extracts of these somatic cells (18). The identity of the candidate repressor previously detected in NIH 3T3 and HeLa cells as COUP-TF was then established by demonstrating that antibody against COUP-TFs supershifted the complex formed between the c-mos B2 probe and NIH 3T3 or HeLa cell nuclear extracts. Consistent with the role of COUP-TF as a transcriptional repressor, mutations of the B2 element that destroyed the COUP-TF-binding site allowed expression from the c-mos promoter in transfected somatic cells (17, 18). Conversely, substitution of a consensus COUP-TF-binding site in place of the c-mos promoter allowed repression of c-mos expression from the c-mos promoter in transfected somatic cells. Additional proteins that bind to the B1 and B3 elements of the c-mos negative regulatory region (17) as well as the Cux/CDP homoeodomain protein, which binds to an enhancer in the rat c-mos locus (30), may also contribute to repression of c-mos in somatic cells.

The identification of COUP-TFs as somatic cell repressors of c-mos is consistent with several known properties of these proteins. COUP-TFI and COUP-TFII are closely related proteins that bind DNA as homodimers and repress transcription in somatic cells. Additional proteins that bind DNA as homodimers and repress transcription in somatic cells. Additional proteins that bind DNA as homodimers and repress transcription in somatic cells. Additional proteins that bind DNA as homodimers and repress transcription in somatic cells.