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AN ENHANCER IN THE c-MOS LOCUS BINDS A NUCLEAR FACTOR 1-LIKE PROTEIN

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We recently identified a DNA sequence element located in the rat c-mos protooncogene which fulfills operational criteria for enhancers, increasing transcription from a heterologous promoter in fibroblasts over large distances and in an orientation-independent manner. Here we report that three mouse nuclear proteins bind to the enhancer. Several lines of evidence indicate that one is a Nuclear Factor 1 like activity. Oligonucleotide-directed mutagenesis of the NF-1 binding site destroys binding of the protein, but leaves enhancer activity unaffected. Tumor growth factor-β, which was shown to exert a stimulatory effect on the α_2(I) collagen promoter via its NF-1 binding site, has no effect on the activity of the enhancer. Thus, the NF-1 binding site of the c-mos enhancer is not essential for its activity in fibroblasts.

Protooncogenes are intimately involved in cell growth and differentiation. Inhibition of c-mos expression by injection of antisense oligonucleotides prevents progesterone-induced maturation of *Xenopus laevis* oocytes (1). Germ cells are the predominant sites of c-mos expression (2, 3, 4, 5). The restricted tissue specificity and the low level of expression have made the study of c-mos expression and its control difficult. Because the c-mos gene has a potent transforming activity in fibroblasts, these cells have been employed to define functional sequences in the mos gene locus that modulate expression directed by viral promoters. Sequences mediating negative regulation of accumulation (6, 7) and translation of RNA (8) have been described. We have also previously demonstrated that a DNA element located 2 kb upstream of the c-mos (rat) coding region exhibits enhancer activity in fibroblasts (9). While a possible role of this sequence in testis-specific expression is as yet unknown, we have data which suggests this 250 bp domain is located within the primary c-mos transcript of rat testis (van der Hoorn et al., manuscript submitted). We therefore refer to this sequence as the c-mos enhancer.

Recent characterizations of nuclear factors which bind to enhancers and mediate their activity demonstrate that enhancers may be composites of

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binding sites for multiple factors, some ubiquitous and some tissue specific (10, 11, 12). To begin elucidating the role of nuclear factors in the activity of the c-mos enhancer (at least in fibroblasts) we have investigated the binding of nuclear proteins to the c-mos enhancer in more detail.

MATERIALS AND METHODS

Cells and transfections: Ltk$^-$ cells were grown in α-MEM supplemented with 10% fetal calf serum (FCS) and antibiotics. The cells were transfected with constructs containing the reporter gene, chloramphenicol acetyl transferase (CAT), and CAT-activity was measured in cell lysates by a quantitative enzymatic/organic extraction assay as previously described (13). In some transfection experiments tumor growth factor-β_1 (TGF-β_1) was added to the cells 4 hour after transfection to a final concentration of 5 ng/ml. TGF-β_1 was added in α-MEM supplemented with 0.5% FCS and antibiotics instead of the normal 10% FCS (14).

DNA constructs and mutagenesis: Oligonucleotide-directed mutagenesis: The c-mos enhancer was cloned as an 0.31 kb PstI-XhoI fragment in the PstI-SalI sites of bacteriophage M13 mp11 DNA. The + strand was isolated then hybridized to an M13 sequencing primer and to the oligonucleotide 5′ GACTCCGTCGACAGTG 3′, which has 3 mismatches (underlined) with the wild-type enhancer and creates a SalI site in the enhancer. The annealed DNA was incubated with Klenow polymerase and T_4 DNA ligase for 1 hour at 16°C (15), digested with PstI and BamHI and separated on low-gelling-temperature agarose gels. The 0.31 kb band was isolated and ligated to PstI-BamHI digested pSP64 DNA. Colonies resulting from transformation of competent *E. coli* HB101 with the ligation products were transferred to nitrocellulose filters, denatured and screened with the mutagenic oligonucleotide labeled at the 5′ end using polynucleotide kinase. After hybridization the filters were washed at sequentially higher temperatures in order to distinguish mutant and wild type enhancer containing plasmids (15). The presence of all three base changes in the mutant enhancer mB1 was confirmed by detection of the introduced SalI site. The PstI site in both the mutant, pSP64-mB1, and the wild type enhancer plasmid, pSP64-WT, was converted to a BamHI site using BamHI linkers. The products are called pSP64-mB1-Bam and pSP64-WT-Bam, respectively.

CAT expression vectors: The wild-type and mutant enhancer DNAs were isolated as 0.31 kb BamHI fragments from pSP64-WT-Bam and pSP64-mB1-Bam respectively. The fragments were inserted into the unique BglII site of $pA_{10}CAT_2$ (16), resulting in pW-CAT-1 and -2 and in pM-CAT-1 and -2, respectively. -1 designates that the orientation of the enhancer with respect to the CAT gene is identical to the orientation of the enhancer with respect to the c-mos coding region in the natural situation (9). -2 designates an inverse orientation.

Nuclear protein extraction and DNAse I footprinting: Nuclear extracts were prepared from murine L929 cells and fractionated on Heparin-agarose columns as described previously (9). All nuclear protein preparations were stored at -70°C. Probes used in DNAse I footprinting experiments included an 0.31 kb XbaI-PstI fragment from pSP64-WT labeled with Klenow polymerase at the XbaI site on the non-coding strand and an 0.21 kb XbaI-HpaI fragment from pSP64-WT or pSP64-mB1 labeled with polynucleotide kinase at the XbaI site on the coding strand. DNAse I footprint assays were performed as described previously (9). Unlabeled DNAs containing either the mutant mB1 enhancer as a 0.21 kb XbaI-HpaI fragment or the 67 bp of the left end of adenovirus 5 DNA (origin of replication) were used in DNAse I footprint experiments as specific competitor DNAs where indicated. The source of the fragment of adenovirus origin of replication containing a Nuclear Factor I site was the plasmid pKB67-88 (17) a gift from Drs. E. O'Neill and T. Kelly.

RESULTS AND DISCUSSION

The c-mos enhancer has three binding sites for murine nuclear proteins: The c-mos enhancer, which is active in mouse fibroblasts although to a lower level than the SV40 enhancer, is located approximately 2 kb upstream of the c-mos coding region (9). To investigate the binding of nuclear proteins to the c-mos enhancer, we performed DNAse I footprinting experiments using high concentrations of crude nuclear proteins and enhancer DNA labeled at the coding or at the non-coding strand. These experiments revealed 3 protected regions on each strand, Figure 1A, which have been termed binding sites I, II and III. DNAse I hypersensitive sites are observed bordering site II and within site III (Figure 1A, arrows). The location of the binding sites and their nucleotide sequence are indicated in Figure 1B with respect to the boundaries of the c-mos enhancer (open arrows).

Binding sites I and II exhibit homology to the binding sites for two characterized, ubiquitous nuclear factors: site I contains the sequence ATGGAAAC which is homologous to the SV40 enhancer core element GTGG$(A/T)_3$G (18). This element has been identified in many viral and cellular enhancers. The sequence constitutes the binding site of activator protein 3 (AP-3) (19, 20) which is a 50 kD human nuclear protein. Another, small (20 kD) protein, EBP-20, which also binds to the SV40 enhancer core element has been identified in rat liver nuclei (21) and it has been speculated that AP-3 and EBP-20 are homologs (10). It is probable, though not experimentally tested, that AP-3 or a related factor binds to the c-mos enhancer binding site I.

Binding site II contains the sequence TTGGN_7CCAA, which conforms to a consensus binding site for Nuclear Factor I (NF-1). NF-1 was originally identified as a ubiquitous nuclear factor that binds to the adenovirus origin of replication and is required for maximal replication efficiency of adenovirus DNA *in vitro* (reviewed in 22). NF-1 appears to be a complex family of nuclear proteins (NF-1/CTF) which recognizes the sequences TTGGN_7CCAA or, in some cases, CCAAT in a large number of viral and cellular promoters (23). Further experiments described in this paper show that the nuclear protein which binds to site II indeed belongs to the NF-1/CTF family.

Binding site III is not homologous to any of the known consensus binding sites of nuclear proteins. Its identity remains to be determined.

An NF-1 like protein binds to binding site II: Binding site II contains the NF-1 consensus binding sequence TTGGN_7CCAA. Figure 2A shows a sequence comparison between binding site II ("c-mos"), the adenovirus NF-1 site in the origin of replication ("Ad5") and the NF-1 site present in the promoter of mouse mammary tumor virus ("MMTV") (24). The c-mos binding site shares significant homology with both the Ad5 and MMTV NF-1 binding sites. Fractionation of crude L929 nuclear extracts demonstrated that the protein which

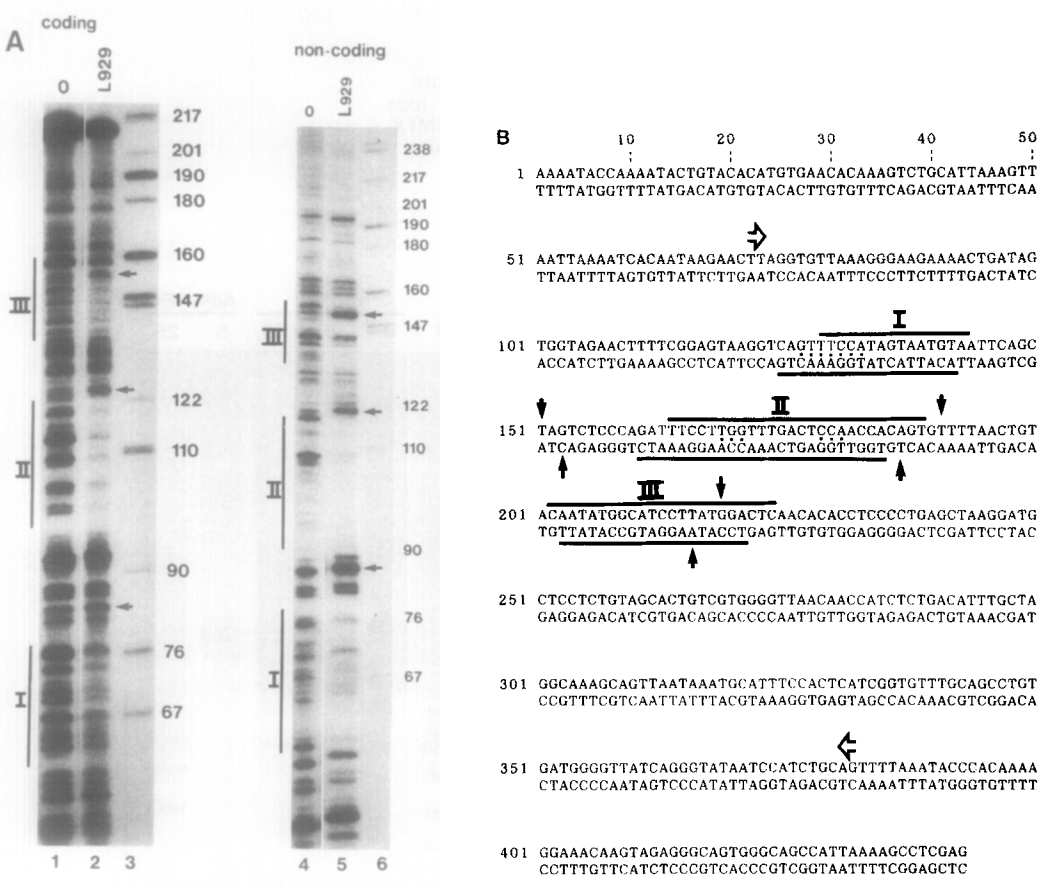

Figure 1. Binding of murine nuclear proteins to c-mos enhancer.

A) Binding sites of nuclear proteins on c-mos enhancer DNA were analyzed by DNAse I footprint experiments. Crude nuclear L929 extracts were incubated with labeled enhancer DNA treated with DNAse I, separated on 8% urea-polyacrylamide gels and exposed to Kodak XAR film. Lanes 1 and 4: no proteins added, lanes 2 and 5: 60 μg L929 crude nuclear proteins added, lanes 3 and 6: end-labeled HpaII digests of pBR322 serving as markers. Indicated are the protected regions (bars) and DNAse I hypersensitive sites (arrows).

B) The nucleotide sequence of the rat c-mos enhancer region is given. The boundaries of the enhancer fragment are indicated by the open arrows. Binding sites I, II and III are indicated by bars and DNAse I hypersensitive sites are indicated by vertical arrows. Dots mark sequences homologous to binding site consensus sequences of AP-3 (in binding site I) and NF-I (in binding site II).

binds to site II elutes at 0.5 M KCl from heparin-agarose columns which is consistent with the reported elution profile of NF-1 (24). To confirm the involvement of an NF-1 like activity, the NF-1 consensus sequence TTGGN$_7$CCAA was changed to TTGGN$_7$CCGT (Figure 2A). DNAse I footprints generated using fractionated nuclear proteins (0.5 M KCl heparin-agarose eluate) demonstrate that the mutation completely abolishes protein binding to binding site II (Figure 2B). This indicates that the protein probably belongs to the NF-1/CTF family of transcription factors. To further demonstrate the in-

A

TTATTTTGGATTGAAGCCAATA Ad5
TTtccTTGGtTTGActCCAAcc c-mos
TTcTTTTGGAaTctAtCCAAgt MMTV
gt g mB1

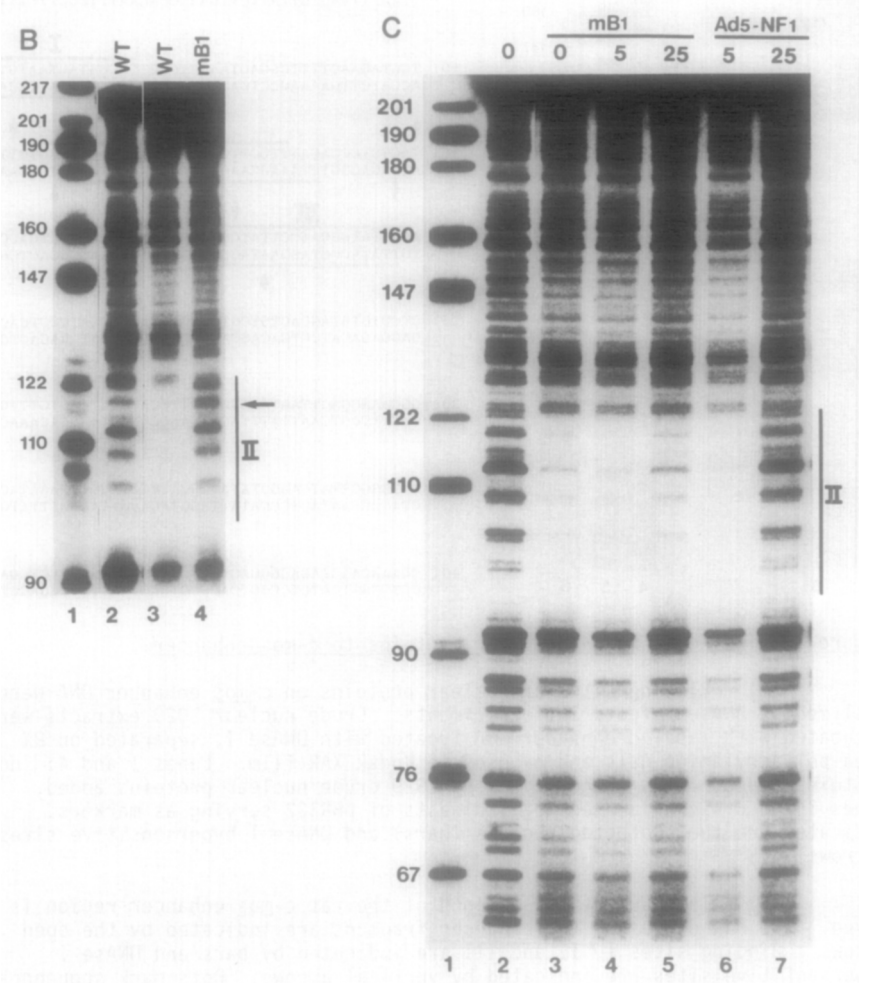

Figure 2. An NF-1 like protein interacts with binding site II.

A) Sequence comparison between the c-mos enhancer binding site II ("c-mos") and the NF-1 binding sites in the adenovirus 5 DNA origin of replication ("Ad5") and the mouse mammary tumor virus promoter ("MMTV"). Identical nucleotides are indicated in capitals and the NF-1 consensus binding site is underlined. Also indicated are the basepair changes introduced to generate mutant enhancer mB1.

B) DNAse I footprint analysis of wild-type (WT) (in the absence or presence of proteins, lanes 2 and 3) and mutant (mB1) (lane 4) c-mos enhancer DNAs using the 0.5 M KCl heparin-agarose fraction of L929 murine nuclear proteins. Binding site II is indicated by the vertical bar. The difference in digestion patterns between WT and mB1 enhancer DNAs, due to the introduced base changes in mB1, is indicated by the arrow.

volvement of an NF-1 like activity we performed DNAse I footprint experiments with labeled wild type c-mos enhancer in which increasing amounts of two specific competitor DNAs were included. One competitor DNA (mB1-competitor) is completely identical to wild-type enhancer except for three mutated base-pairs, the other (Ad5-NF-1 competitor DNA) is entirely different from wild-type enhancer except for the NF-1 site homology $TTGGN_7CCAA$. The results in Figure 2C, lanes 2-5, show that mB1-DNA, does not compete for binding of the nuclear protein present in the 0.5 M KCl heparin-agarose fraction. However, a 67 bp adenovirus 5 DNA fragment containing a strong NF-1 site, efficiently competes for the protein which generates footprint II (Figure 2C, lanes 6 and 7). These results demonstrate that NF-1 or a closely related nuclear protein belonging to the NF-1/CTF family binds to the c-mos enhancer at site II.

Mutation of the NF-1 binding site does not abolish c-mos enhancer activity: Detection of the interaction of an NF-1 like protein with the c-mos enhancer raises two questions which we wished to address: a) does NF-1 binding play a role in c-mos enhancer activity even though transcriptionally functional NF-1 sites have so far been reported only as basal promoter elements and b) does tumor growth factor-β (TGF-β)induce c-mos enhancer activity: TGF-β exerts a positive effect on α_2(I) collagen promoter activity mediated through an NF-1 binding site (14). Also, TGF-β is a member of a family (reviewed in 25) that includes Müllerian inhibiting substance (MIS) which like c-mos is specifically synthesized in the testis (26).

To investigate whether the NF-1 binding site plays a role in the activity of the c-mos enhancer, we compared the ability of the wild type enhancer and the mB1 mutant bearing a defective NF-1 binding site, to activate the truncated, enhancerless, SV40 early promoter linked to a chloramphenicol acetyl transferase (CAT) reporter gene. As shown in Table 1 the CAT-activity directed by transiently transfected expression plasmids bearing the mutant enhancer in either the forward or reverse orientation (pM-CAT-1 and pM-CAT-2, respectively) is identical to that of the wild type enhancer in either orientation (pW-CAT-1, pW-CAT-2). This indicates that a mutation which abolishes NF-1 binding has no effect on the c-mos enhancer activity present in Ltk^- mouse fibroblasts. Also, in agreement with our previous result using the wild type enhancer and a globin gene as reporter (9), enhancer activity

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C) The labeled, wild type 0.21 kb enhancer fragment was incubated with 0.5 M KCl heparin-agarose fraction of L929 nuclear proteins in the absence of specific competitor DNA (lane 3), in the presence of a 5 or 25 fold molar excess of 0.21 kb mutant enhancer (lanes 4 and 5) or with a 5 or 25 fold molar excess of the 67 bp origin of replication of adenovirus 5 DNA containing the NF-1 site (lanes 6 and 7). The DNAse I digestion pattern of the labeled enhancer in the absence of nuclear proteins is shown in lane 2. Binding site II is indicated by the bar. Lane 1 contains an end-labeled HpaII digest of pBR322 serving as markers.

Table 1
Enhancer Activities of Wild-Type and Mutant c-mos Enhancers

	-TGF-β_1	+TGF-β_1
$pA_{10}CAT_2$	1600[a]	3440
pW-CAT-1	6070	17400
pW-CAT-2	6100	N.T.[b]
pM-CAT-1	6400	N.T.
pM-CAT-2	6355	N.T.
pSV_2CAT	74190	155200

[a]Net CAT-activity is expressed as cpm. Figures have been corrected for 860 cpm background CAT-activity in mock-transfected cells. Each figure represents the mean of duplicate transfections.

[b]N.T., not tested.

is independent of orientation with respect to the promoter and reporter gene used.

To investigate whether TGF-β may activate c-mos enhancer activity through NF-1 as has been reported for the α_2(I) collagen promoter, Ltk- cells were transfected with $pA_{10}CAT_2$, pSV_2CAT and pW-CAT-1 in the absence or presence of 5 ng/ml TGF-β_1 (see Materials and Methods for details). Treatment with TGF-β, increased the activity from the enhancerless promoter as well as the two enhancer containing constructs (Table 1). However, this increase was similar (2-3 fold) in all cases suggesting that no specific activation through the c-mos enhancer occurs. Although we therefore conclude that TGF-β does not play a role in the regulation of c-mos enhancer activity as assessed in fibroblasts, it remains possible that a related molecule such as MIS may play such a role in the testis.

In conclusion, we demonstrate specific binding of nuclear proteins to the c-mos enhancer, one of which is identical or related to NF-1. Furthermore, mutation of the binding site II abolishes NF-1 binding, but has no effect on c-mos enhancer activity.

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