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Xenopus cytoskeletal actin and human c-*fos* gene promoters share a conserved protein-binding site

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***Xenopus laevis* cytoskeletal actin gene promoters contain a 20-bp sequence homologous to the serum response element (SRE) required for transient human c-*fos* gene transcription in response to serum factors. Both sequences bind the same factor in HeLa cell extracts, as shown by binding competition, DNase I and dimethylsulphate (DMS) protection and DMS interference assays. A similar protein is present in *Xenopus laevis* oocytes. Sequences containing the SRE homology are essential for constitutive activity of the actin promoter in both *Xenopus* and mouse cells, and a synthetic SRE functions as a promoter element in these cells. In mouse cells, transcription of both transfected *Xenopus* actin and actin/c-*fos* fusion genes is activated following serum stimulation. These data suggest that the SRE and its cognate protein form part of a regulatory pathway that has been highly conserved during evolution.**

Key words: actin/c-*fos*/transcription/DNA-binding proteins/growth factors

Introduction

Actins form a major component of the cytoskeleton of eukaryotic cells. In vertebrates the two major cytoskeletal actins, β and γ, are encoded by a pair of evolutionarily related genes which are expressed in all cell types. Recently, it was demonstrated that the mammalian cytoskeletal actin genes are members of a set of genes whose transcription is rapidly and transiently activated following stimulation of susceptible cells with growth factors and mitogens (Elder *et al.*, 1984; Greenberg and Ziff, 1984; Lau and Nathans, 1985). Transcription of these 'rapid response' genes, which include the proto-oncogene c-*fos* (Curran *et al.*, 1982, 1983; Verma, 1986), can be induced by diverse agents depending on the cell type (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984; Greenberg *et al.*, 1985; Curran and Morgan, 1985; Mitchell *et al.*, 1985; Zullo *et al.*, 1985). In the case of the actin genes, the molecular mechanisms underlying increased transcription following exposure to growth factors is unknown. However, in the case of the human c-*fos* gene (c-*fos*^{H}), transient transcriptional activation by serum is mediated by a short conserved sequence element, the serum response element (SRE) that forms the binding site for a protein, presumably a transcription factor, termed serum response factor (SRF; Treisman, 1985, 1986; Gilman *et al.*, 1986).

In the amphibian *Xenopus laevis*, a family of cytoskeletal actin proteins has been identified by peptide analysis. These proteins are synthesized in varying proportions depending upon the tissue type (Vanderkerckhove *et al.*, 1982; Mohun *et al.*, 1984). Two *X. laevis* cytoskeletal actin genes, encoding the type 5 and type 8 actins, have been isolated and sequenced (T.J.Mohun, unpublished results). The promoters of these genes, but not those encoding muscle-specific actin isoforms, contain a region of striking homology to the c-*fos*^{H} SRE. The *X. laevis* SRE homology is located 75 bp 5′ to the mRNA cap site, whereas in the c-*fos*^{H} gene it is located at position −300. In this paper we have investigated the role of the SRE-like sequence in the transcriptional regulation of the *X. laevis* type 5 cytoskeletal actin gene.

Results

The X. laevis actin gene promoter binds HeLa cell SRF

The structure of the *X. laevis* type 5 cytoskeletal actin gene promoter is shown in Figure 1. The promoter contains homologies to several higher eukaryote promoter elements: the CCAAT box (Efstradiatis *et al.*, 1980; nucleotides −121 to −125), the TATA box (Goldberg, 1979; nucleotides −33 to −36), and the consensus binding site for Sp1 protein (Dynan and Tjian, 1983, nucleotides −57 to −48). In addition, nucleotides −94 to −75 form an imperfect match to the SRE required for serum-induced transient transcription of the human cellular proto-oncogene, c-*fos*^{H}.

The DNA binding–gel electrophoresis assay (Fried and Crothers, 1981; Garner and Revzin, 1981; Strauss and Varshavsky, 1984) was used to monitor the formation of DNA–protein complexes between the actin gene promoter and HeLa cell nuclear extract proteins. A short radiolabelled DNA fragment containing the *Xenopus* SRE homology (see Figure 1) was incubated with HeLa cell nuclear extract under conditions known to favour

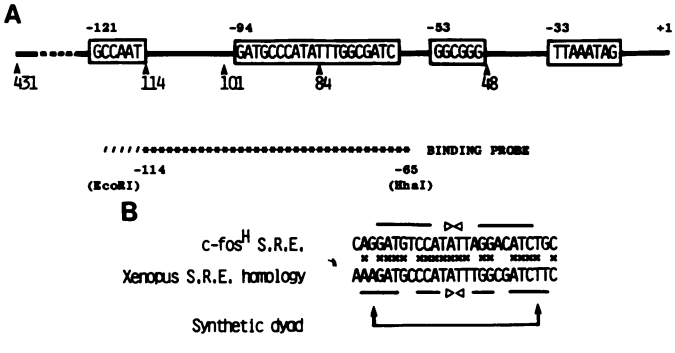

Fig. 1. A. Structure of the *X. laevis* (type 5) cytoskeletal actin gene promoter. The CCAAT, TATA and SRE-like sequences are shown boxed, as is the putative Sp1 binding site. Positions relative to the start of transcription are shown above. Arrows below represent the 5′ ends of deletion mutants. The first nucleotide of each (relative to the cap site) is indicated. Also shown is the DNA fragment used in binding studies (see Figure 2). This is derived from pA114CAT, extending from a *Hha*I site within the actin gene promoter to an *Eco*RI site in the plasmid polylinker sequence adjacent to the truncated 5′ end of the promoter. **B.** Comparison of the *Xenopus* SRE-like sequence with the c-*fos*^{H} SRE. Identical nucleotides are indicated by asterisks. The imperfect dyad structure of each sequence is indicated by broken arrows. The extent of the synthetic *Xenopus* SRE used in subsequent experiments is also shown.

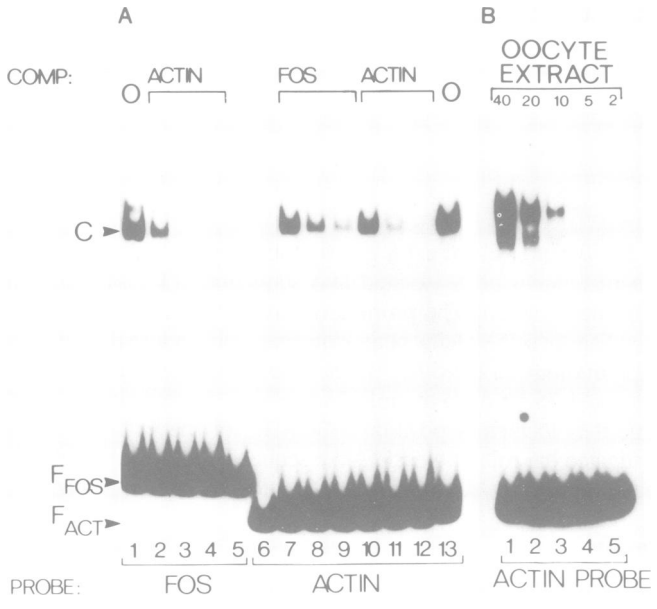

Fig. 2. A. The *Xenopus* actin promoter binds HeLa cell SRF. DNA binding assays were performed as described in Materials and methods. The actin probe is shown in Figure 1, and the c-*fos*^{H} probe was probe B (Treisman, 1986). The assay contained 0.25 ng probe as indicated and 10 μg HeLa cell nuclear extract. **Lanes 1, 13:** no competition. **Lanes 5,6:** probe alone. **Lanes 2, 3, 4,** and **10, 11, 12** contain a 10-, 30- and 100-fold molar excess of actin promoter plasmid, and **lanes 7, 8, 9** contain 10-, 30- and 100-fold molar excess of the c-*fos*^{H} SRE plasmid pDYAD, respectively. **B.** *Xenopus* oocytes contain an SRF-like factor. An S100 extract of *Xenopus* oocytes (see Materials and methods) was used in binding assays with the *Xenopus* actin probe shown in Figure 1A. Assays contained from 2 to 40 μg of protein extract as indicated. Other minor complex species with higher mobility than the principal complex result from partial proteolysis of the protein during extract preparation (unpublished observations). Arrows indicate the positions of unbound probe (F_{ACT} or F_{FOS}) and probe−DNA complex (C) resolved by electrophoresis.

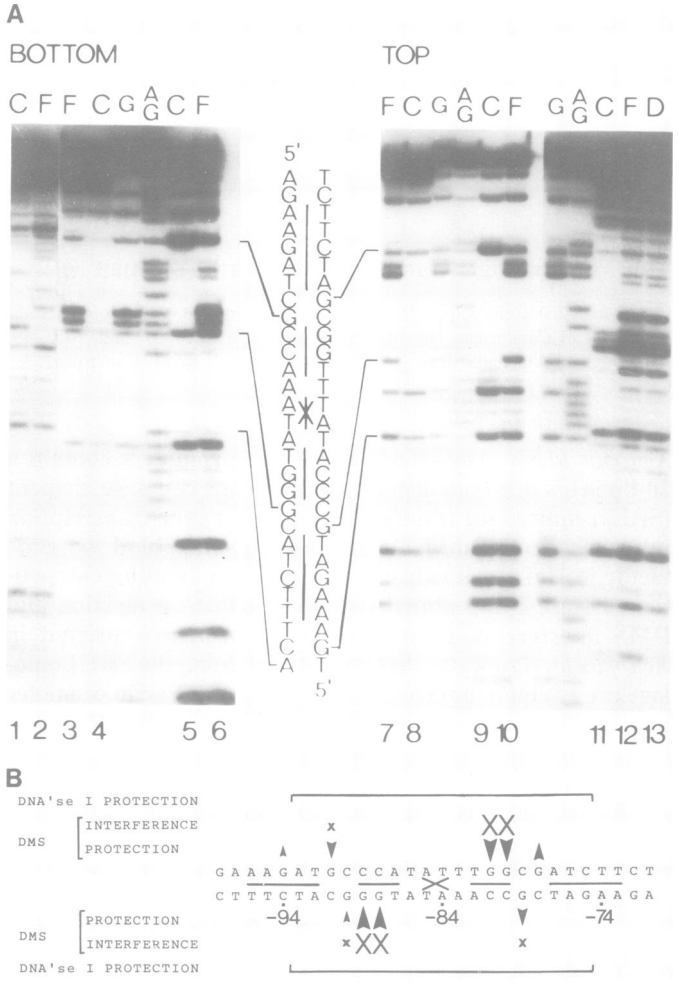

Fig. 3. A. Delineation of the factor binding site at nucleotide resolution on both 'bottom' **(lanes 1−6)** and 'top' **(lanes 7−13)** strands of the actin promoter. **Lanes F** and **C** represent DNA recovered from free and complexed DNA bands, and **lanes G** and **AG** are markers comprizing partial chemical degradation products cleaved at guanines and purines respectively. **Lane D** shows partial DNase I cleavage in the presence of bovine serum albumin only. **Lanes 1, 2** and **11, 12, 13:** DNase I protection; **lanes 3, 4** and **7, 8:** DMS interference; **lanes 5, 6** and **9, 10:** DMS protection. **B.** Summary of data from **A** with the limits of affected DNase I reactivity shown by square brackets. Methylation interference is shown by crosses. Protection or enhancement of DMS reactivity is shown by arrowheads pointing towards or away from the sequence, respectively. Symbol sizes are proportional to the magnitude of the effect; effects on reactivity of adenines are omitted.

complex formation with c-*fos*^{H} DNA (Treisman, 1986), and DNA−protein complexes separated from unbound probe on low ionic strength polyacrylamide gels (Figure 2A). A well-defined complex band is resolved under these conditions (lane 13, band C). This represents a specific complex, since the inclusion of increasing amounts of unlabelled actin promoter DNA in the binding reaction results in the progressive reduction of the amount of radioactivity in the complex (Figure 2A, lanes 10−12). To test whether this complex is related to that formed with the c-*fos*^{H} SRE, a binding competition experiment was performed. Increasing amounts of actin DNA were included in binding reactions containing a c-*fos*^{H} probe, and increasing amounts of c-*fos*^{H} SRE DNA were included in reactions containing the actin probe. In both cases, competition for binding was observed, with the actin promoter appearing to bind factor slightly more avidly than the c-*fos*^{H} SRE [compare Figure 2A, lanes 2−4 (actin competitor) and 7−9 (c-*fos*^{H} competitor)].

In order to identify the exact location of the SRF binding site within the actin promoter, DNase I and dimethylsulphate (DMS) protection and interference assays were used. In the DNase I experiments, binding reactions were treated with the enzyme, complexes resolved on gels, and the eluted free and complexed DNA displayed on sequencing gels. Both actin DNA strands showed differences in the pattern of DNase I cleavage within the region of the SRE homology [Figure 3A compare lanes 1 with 2 (bottom strand) and lanes 11 with 12 (top strand)]. The pattern of altered cleavage is similar to that observed with the c-*fos*^{H} SRE (Treisman, 1986). We used DMS interference (Siebenlist and Gilbert, 1977) and protection (Johnsrud, 1978) to examine interactions at specific nucleotides throughout the region. On the bottom strand, methylation of G residues at positions −79, −88, −89 and −90 interferes with complex formation (Figure 3A, compare lanes 3 and 4). When pre-formed complexes are methylated, residues G-88 and G-89 are essentially inert as expected from the interference data; however, residue G-79, whose prior methylation reduces complex formation, exhibits increased reactivity in pre-formed complexes [Figure 3A, compare lanes 5 and 6 (protection) and lane 3 with 4 (interference)]. On the top strand, methylation of G residues at positions −91, −81 and −80 interferes with factor binding; methylation at positions −81 and −80 appears to block binding quantitatively (Figure 3A, compare lanes 7 and 8). When complexed DNA is treated with DMS, reactivity of these residues is also affected, with residue

Table I. The CAT enzyme activity in *Xenopus* and mouse cells after transfection or microinjection of various actin−CAT chimeric genes

Experiment	*X. laevis* oocytes	*X. laevis* renal cells			Mouse NIH/3T3 cells		
	1	2	3	4	5	6	7
pA431 CAT	100.0	100.0	100.0	100.0	–	100.0	100.0
pA114 CAT	–	58.5	38.9	32.9	–	96.8	84.1
pA48 CAT	3.6	4.0	0.8	0.3	0.36	5.2	2.4
pDYAD 48 CAT	–	35.7	15.1	8.4	35.8	38.7	10.9
p3DYAD 48 CAT	196.7	46.6	92.7	35.6	100.0	62.7	100.1
pACT.D 48 CAT	–	–	–	4.9	8.8	7.7	16.4

Each experiment represents the results obtained for a single set of injections/transfections performed simultaneously using the same batch of cells. [Acetyl-^{14}C]chloramphenicol synthesized was quantitated by scintillation counting. The values were normalized to the activity of pA431 CAT observed in each experiment (except for experiment 5 in which the level of p3DYAD 48 CAT was used).

−91 slightly reactive and residues −81 and −80 essentially inert (Figure 3A, compare lanes 9 and 10). Reactivity of residues G-78 and G-94, whose prior methylation does not interfere with binding, is increased (Figure 3A, lanes 9 and 10). The data are summarized in Figure 3B. Overall, the patterns of protection and interference are similar to those observed with the c-*fos*^{H} SRE, with residues symmetrically disposed about the dyad centre exhibiting similar interactions with the protein (see Discussion).

Xenopus cells contain a factor that binds the SRE

To test whether *Xenopus* cells contain protein factors that bind to the SRE homology, a 100 000 *g* supernatant fraction was prepared from *X. laevis* oocytes (Dignam *et al.*, 1983) and used in binding reactions with the *Xenopus* actin promoter probe. The results are shown in Figure 2B (lanes 1−5). Increasing amounts of the extract generate increasing amounts of a specific complex when incubated with this probe. Again, inclusion of excess unlabelled actin or c-*fos*^{H} SRE DNA in the binding reaction reduces the yield of labelled complex; moreover, synthetic variant SREs with different affinities for HeLa cell SRF display the same range of affinities for the oocyte S100 binding activity (data not shown). These data suggest that the sequence specificity of the oocyte SRE-binding acivity and the HeLa cell SRF are identical, suggesting the existence of an amphibian counterpart to the mammalian SRF.

The actin SRE is a promoter element in X. laevis cells

To ascertain the role of the SRE homology in *Xenopus* actin gene expression, intact and mutant actin promoters were examined in *Xenopus* oocytes and cultured *Xenopus* renal cells. For these experiments a plasmid was constructed in which the *Xenopus* type 5 actin gene promoter is joined to the bacterial chloramphenicol acetyl transferase (CAT) gene (Gorman *et al.*, 1982). In this chimeric gene, which contains 452 nucleotides of actin 5′-flanking sequences, the fusion point with the CAT transcription unit is within the actin 5′-untranslated region, at position +21. When this plasmid is microinjected into oocytes or transfected into renal cells, CAT enzyme activity is readily detected after 24 h. Deletion of sequences 5′ to position −114 permits expression of the chimeric gene at levels within 2- to 3-fold of the full sized actin/CAT plasmid (Table I, experiments 2−4). Deletion to position −48, however, results in the loss of virtually all activity (Table I), indicating the presence of constitutive promoter elements between −114 and −48. This region includes both the SRE homology and the putative SP1 factor binding site (see Figure 1A). To test directly whether the actin SRE possesses transcription stimulatory activity in the absence of other promoter elements, a synthetic actin SRE oligonucleotide was inserted into the fusion gene containing 48 bp of 5′-flanking sequence. In this construction the actin SRE homology is brought exactly 10 bp closer to the TATA box, and the conserved GC-rich region is replaced by linker sequences. We also constructed derivatives in which a synthetic c-*fos*^{H} SRE (Treisman, 1986) is inserted at the same position. These were then tested for expression in oocytes and cultured renal cells. The results in Table I show that transcription of an actin−CAT fusion gene is substantially increased by a single copy of the c-*fos*^{H} SRE and that three copies restore transcription to within 2-fold of wild-type levels. The synthetic actin SRE homology also stimulates transcription (Table I, experiment 4) although its effect is less than that found with the c-*fos*^{H} sequence. These results indicate that in both *Xenopus* cell types, the SRE homology constitutes part of the actin gene

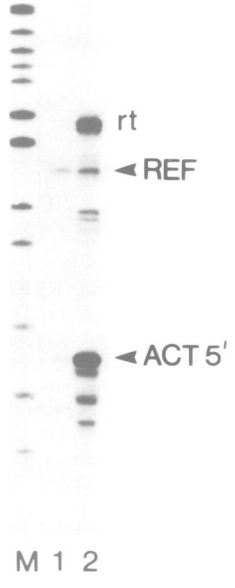

Fig. 4. The *Xenopus* cytoskeletal actin gene is serum-inducible in transfected mouse cells. A pBR322 derivative carrying the entire actin gene was transfected along with a reference α-globin gene plasmid into mouse NIH/3T3 fibroblasts which were subsequently deprived of serum for 24 h and then stimulated with serum-containing medium. Total cellular RNA was prepared before stimulation **(lane 1)** and 40 min following stimulation **(lane 2)**. 30 μg of each RNA was analysed by RNase mapping using probes that span the entire first exons of the actin and α-globin genes. Authentic actin transcripts give rise to the major protected band of 76 nucleotides arrowed (ACT 5′). Heterogeneity in the precise 5′ end of transcripts gives rise to several smaller bands. These are also detected in *Xenopus* RNA samples (T.J.Mohun, in preparation). A number of larger protected fragments (rt) presumably result from the presence of readthrough transcripts. Fragments representing human α-globin transcripts are indicated (REF). Markers (M) are 3′ end-labelled *Msp*I fragments of pBR322 DNA.

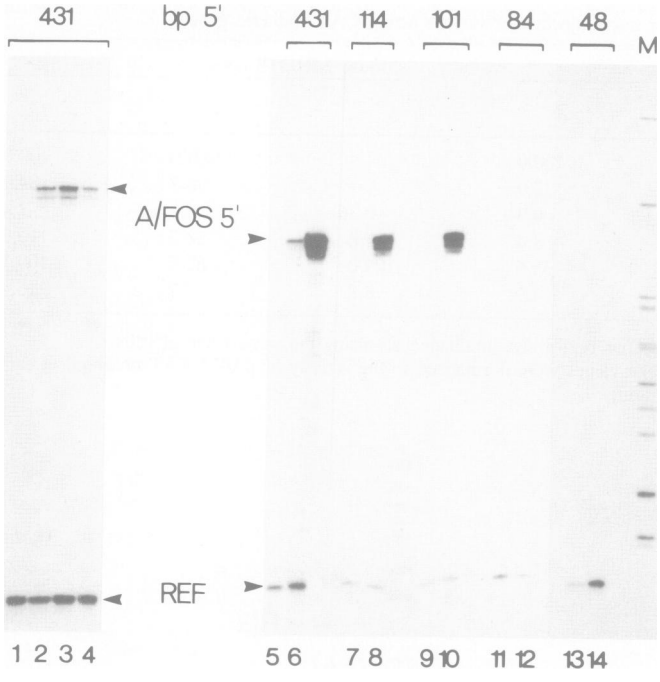

Fig. 5. Serum inducibility of actin/FOS fusion genes carrying varying amounts of 5′-flanking sequences. Mouse NIH/3T3 fibroblasts were transfected with the fusion genes and α-globin reference gene plasmids, deprived of serum and re-stimulated with medium containing serum 24 h later. Total cellular RNA was prepared before serum stimulation **(lanes 1, 5, 7, 9, 11, 13)** 40 min following stimulation **(lanes 2, 8, 10, 12, 14)** 90 min following stimulation **(lane 3)** or 240 min following stimulation **(lane 4)**. 30 μg of each RNA was analysed by RNase protection mapping. The extent of 5′-flanking sequence is indicated at the top of each set of lanes. Positions of fragments representing the actin/FOS 5′ exon and the reference α-globin 5′ exon are indicated (A/FOS 5′ and REF respectively). Markers (M) are 3′ end-labelled fragments of pBR322 DNA.

promoter, and that in these cells the SRE can function as a constitutive upstream promoter element.

The Xenopus actin promoter is serum inducible in mouse cells

In mammalian c-*fos* genes, the SRE is located several hundred nucleotides upstream of the gene promoter and mediates their transcriptional stimulation by serum factors. Since mammalian cytoskeletal actin genes are also responsive to serum stimulation (Elder *et al.*, 1984; Greenberg and Ziff, 1984) it seemed plausible that transcription of the *Xenopus* cytoskeletal actin genes might also be regulated by serum factors. Attempts to test this notion were unsuccessful owing to the unavailability of clearly responsive *Xenopus* cell lines. We therefore tested whether the *Xenopus* cytoskeletal actin genes were regulated by serum stimulation following their transfection into mouse 3T3 cells. The experiment shown in Figure 4 demonstrates that a substantial increase in RNA transcribed from the transfected gene can be detected after serum stimulation. The majority of transcripts are correctly initiated and give rise to a cluster of protected bands, as indicated. This microheterogeneity in the 5′ termini of transcripts is also observed in RNA from *Xenopus* tissues and is presumably inherent to the cytoskeletal actin gene promoter (T.J.Mohun, in preparation).

The sequences necessary for serum responsiveness of the actin gene lie between positions −431 and +21, since a fusion gene comprising this DNA fragment attached to the c-*fos*^{H} transcription unit is also serum responsive when tested in a transient serum stimulation assay (Figure 5A, lanes 1−4). The fusion gene

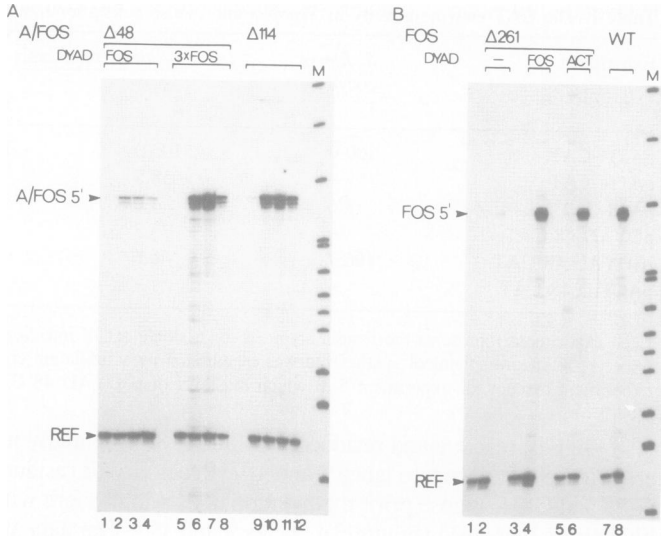

Fig. 6. A. The c-*fos*^{H} SRE alone will confer serum inducibility. Mouse NIH/3T3 cells were transfected with either the actin/FOS fusion gene pA114FOS **(lanes 9−12)** or gene pA48FOS carrying one **(lanes 1−4)** or three **(lanes 5−8)** copies of the c-*fos*^{H} SRE. After serum deprivation the cells were re-stimulated as described in Figure 5. Total cell RNA was prepared either before **(lanes 1, 5, 9)** 40 min after **(lanes 2, 6, 10)** 90 min after **(lanes 3, 7, 11)** and 240 min after **(lanes 4, 8, 12)** stimulation with 15% serum, and 30 μg of each RNA was analysed by RNase protection mapping. **B.** The actin SRE homology will restore serum inducibility to an inactive c-*fos*^{H} deletion mutant. Mouse NIH/3T3 cells were transfected with either the intact c-*fos*^{H} plasmid pF711 ('WT', **lanes 7, 8**) the uninducible 5′ deletion mutant pF261 **(lanes 1, 2)** or PF261 derivatives carrying a single copy of the c-*fos*^{H} SRE **(lanes 3, 4)** or the actin SRE homology **(lanes 5, 6)**. Total cellular RNA was prepared either before **(lanes 1, 3, 5, 7)** or 40 min after **(lanes 2, 4, 6, 8)** stimulation of starved cells with serum, and 30 μg was analysed by RNase protection mapping. Positions of protected fragments representing the actin/FOS 5′ exon and the reference α-globin first exon are indicated. Markers (M) are 3′ end-labelled *Msp*I fragments of pBR322 DNA.

RNA accumulates rapidly following serum stimulation reaching a peak level within 40 min. The transcript levels begin to decline by 4 h following serum stimulation, somewhat slower than the intact c-*fos*^{H} gene. To map sequences required for this induction a series of 5′ deletion derivatives of the actin/c-*fos*^{H} fusion gene was constructed and analysed. A gene containing only 101 bp of 5′-flanking sequence remains fully inducible (Figure 5B, lanes 9,10); truncation to position −84, which removes half of the SRE homology, abolishes inducibility (Figure 5B, lanes 11,12). Deletion to position −84 also significantly decreases preincubation transcript levels (Figure 5; data not shown). To confirm that the SRE alone can confer serum inducibility on a linked TATA box, synthetic copies of the c-*fos*^{H} SRE were joined to the inactive −48 actin/*fos*^{H} deletion mutant. Figure 6A shows the results of a transient serum stimulation assay using plasmids carrying either one or three copies of the synthetic c-*fos*^{H} SRE (lanes 1−4 and 5−8, respectively). Although a single copy of the SRE functions poorly, three copies of the SRE restore inducibility to wild-type levels (Figure 6A, lanes 9−12).

To assay constitutive SRE activity in mammalian cells, actin/CAT fusion genes were transfected into NIH/3T3 cells. The results (Table I, experiments 6 and 7) show that in these cells the SRE also functions as a constitutive promoter element in the absence of serum stimulation. Synthetic copies of either the *Xenopus* SRE homology or the c-*fos*^{H} SRE restore constitutive expression to an actin−CAT fusion gene containing only 48

nucleotides of actin promoter sequence (Table I experiments 5–7). We note that in two of the experiments the *Xenopus* SRE sequence functions poorly compared with its c-fos^H counterpart. The reason for this variation is unclear.

The Xenopus SRE homology will activate a c-fos^H gene in mouse cells

Finally, we tested whether a synthetic copy of the actin SRE could substitute for the authentic c-fos^H SRE. A single copy of the actin SRE was inserted into plasmid pF261, which carries a c-fos^H gene lacking sequences necessary for serum induction (Treisman, 1986). In this plasmid, the *Xenopus* SRE is located upstream from the c-fos^H promoter at position −261 in contrast to its natural location within the *Xenopus* actin promoter, close to the actin gene TATA box. At this distant location, which is similar to the location of the c-fos^H SRE in the normal human c-*fos* gene, the *Xenopus* SRE restores serum inducibility to the c-fos^H gene. Figure 6 compares the serum inducibility conferred on plasmid pF261 by the c-fos^H SRE (lanes 3 and 4) and the *Xenopus* SRE (lanes 5 and 6) with that shown by the wild-type c-fos^H gene (lanes 7 and 8). The *Xenopus* SRE entirely restores serum inducibility to the c-fos^H gene.

Discussion

The data presented here show that a *Xenopus* cytoskeletal actin gene promoter can bind a factor in HeLa cell nuclear extracts indistinguishable from the factor, termed SRF, that recognizes the prototype c-fos^H SRE (Treisman, 1986). A binding factor with the same specificity is present in *X. laevis* cells and a synthetic factor binding site functions as a promoter element in these cells. The presence of the SRE and its cognate protein in *X. laevis* therefore suggests that these proteins are highly conserved through evolution.

The presence of a sequence structurally and functionally homologous to the c-fos^H SRE in a cytoskeletal actin gene is of interest since in mammalian cells the transcription of cytoskeletal actin genes, like that of the c-fos^H gene, is inducible by serum factors (Elder *et al.*, 1984; Greenberg and Ziff, 1984). Indeed, when transfected into mouse cells, the *Xenopus* actin gene promoter can also be activated by serum stimulation; this activation is dependent on the SRE homology. This suggests that SRE homologies may regulate many serum responsive genes. A search of other published cytoskeletal actin gene sequences (Kost *et al.*, 1983; Nudel *et al.*, 1983; Bergsma *et al.*, 1985; Nakajima-Iijima *et al.*, 1985; Cross *et al.*, 1986) revealed that although the sequences are clearly related to the *X. laevis* genes in the vicinity of the TATA box, the SRE homology is not conserved at this position. However, the promoters of all the vertebrate cytoskeletal and muscle-specific actin genes sequences to date do include sequences related to the SRE, 5′ CC(A/T)$_6$GG 3′ (Minty and Kedes, 1986; Mohun *et al.*, 1986). No function has yet been assigned to this element. Binding studies with the *X. laevis* cardiac actin gene promoter indicate that this element does not compete efficiently for SRF binding *in vitro* (R.Treisman and T.J.Mohun, unpublished observations). Thus, although these promoters have not been studied *in vivo*, it seems likely that serum inducibility of other cytoskeletal actin genes may be mediated by SRE homologies located elsewhere within their 5′-flanking sequences.

DMS protection and interference experiments showed that the variation in sequence between the actin and c-fos^H SREs gives rise to subtle variations in the interaction of the protein with the DNA. For example, the 5′ half of the actin SRE dyad contains only one difference from the c-fos^H sequence, at position −90 (actin, C-G; c-fos^H, T-A). This is sufficient to cause residue G −91, which is conserved in both elements, to make much closer contact with the protein in the actin SRE. Clearly, the nucleotides within a binding site cannot always be varied independently of each other. The 3′ half of the actin SRE dyad is also different from its counterpart in the c-fos^H SRE, but no changes in protein–DNA interaction were detectable with DMS protection and interference techniques. Together, the changes cause the actin SRE to bind HeLa cell SRF more tightly *in vitro* than the c-fos^H SRE, while both sequences function with equal efficiency *in vivo*.

In both *Xenopus* and mouse cells, 5′ deletions of the actin promoter that remove the SRE homology significantly lower than the level of constitutive transcription from these promoters. This contrasts with the c-fos^H gene where removal of the SRE has only a slight effect on constitutive promoter activity (Treisman, 1985; Deschamps *et al.*, 1985; Gilman *et al.*, 1986). This most likely reflects the different location of the SRE in the two genes: in the c-fos^H gene other promoter elements are located between the SRE and the TATA box (Deschamps *et al.*, 1985; Gilman *et al.*, 1986). The pre-stimulation activity of the SRE and constitutive promoter elements, and the stability of the RNA transcript, will therefore all contribute to the degree of induction observed at the RNA level in a given cell type. This may explain the apparent difference in behaviour of cytoskeletal actin genes in mouse AKR2B (Elder *et al.*, 1984) and rat PC12 cells (Greenberg *et al.*, 1985) following stimulation with EGF and NGF, respectively.

We confirmed that the SRE can act as a constitutive promoter element in *Xenopus* and mammalian cells by using synthetic SRE oligonucleotides to restore constitutive activity to an inactive truncated actin promoter lacking sequences 5′ to position −48. The resulting promoters are also serum responsive when transfected into mouse cells. The SRE can therefore act in the absence of other promoter upstream elements. Although a single SRE functions poorly in these circumstances, three copies are significantly more effective. This perhaps reflects cooperative interactions in the establishment or activity of a protein complex on the promoter. Similar phenomena have been observed with the SV40 enhancer (Herr and Gluzman, 1985; Herr and Clarke, 1986), and the heat shock regulatory element (Dudler and Travers, 1984; Pelham, 1985; Bienz and Pelham, 1986). Alternatively, the low efficiency observed may be a result of the altered SRE–TATA box spacing in these promoters.

Taken together, our experiments show that the SRE is associated with both constitutive and serum-inducible transcription. A simple interpretation of the data is that the same transcription factor is responsible for both modes of transcription, and that this corresponds to the SRF detected in the binding assays. Transcriptional activation following serum stimulation would occur via modification of the factor or proteins that interact with it. The behaviour of SRE sequences carrying G–C transversions at protein contact points (position −81 and −88) is consistent with this interpretation. These mutations prevent SRF binding *in vitro*, and abolish constitutive and inducible transcription (unpublished observations). It remains possible, however, that there are two factors which recognize the SRE and which are separately responsible for constitutive and inducible transcription. The resolution of this question and the understanding of the properties of the SRE will require the purification of SRF and the isolation of both the genes encoding it and related proteins.

Materials and methods

Extracts

HeLa cell nuclear extracts were prepared from HeLa S3 cells as previously described (Dignam and Roeder, 1983). A S100 fraction was prepared from *X. laevis* ovaries as described by Dignam *et al.* (1983). To reduce proteolytic damage, 0.5 mM phosphomethylsulphonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin were included in buffer C.

Oligonucleotides

The oligonucleotides 5′ AATTAGATGCCCATATTTGGCGATCT 3′ and 5′ AATTAGATCGCCAAATATGGGCATCT 3′ were synthesized by Terry Smith with an Applied Biosystems model 380B DNA synthesizer. Phosphorylation and cloning of annealled oligonucleotides were as described (Treisman, 1986).

Plasmids

The actin/CAT chimeric gene constructs contain the bacterial CAT transcription unit from pSVoCAT (Gorman *et al.*, 1982) fused to the *Xenopus* type 5 cytoskeletal actin gene promoter at position +21 within the first exon. Transcription of the fusion gene proceeds in the same direction as the ampicillin resistance gene of the pUC12 vector. The constructs possess actin promoter fragments progressively truncated from positions −431 to −48, (denoted pAnnnCAT) and were obtained as follows.

The bacterial CAT gene was subcloned as a *Bam*HI−*Hin*dIII fragment into pBR322 (pBRCAT). The *Xenopus* type 5 cytoskeletal actin gene fragment used in the fusions comprized a 523-bp *Eco*RI−*Sal*I fragment extending from −491 to +21 relative to the cap site and including 12 bp of polylinker sequence remaining from an insertion into the first exon used to obtain the *Sal*I site. The actin gene fragment was blunt-end ligated into the *Hin*dIII site of pBRCAT after both were filled in with the Klenow fragment of DNA polymerase. The resulting chimeric gene was used to obtain a 5′ deletion series using *Bal*31 nuclease digestion from the unique *Cla*I site. These were subcloned into pUC12 between the *Sal*I and *Sma*I sites. Deletion endpoints were determined by direct sequencing with collapsed supercoil DNA templates (Sanger *et al.*, 1977; Chen and Seeburg, 1985).

The basic actin/c-*fos*^{H} fusion gene was constructed from pA431CAT by excision of the CAT sequences and their replacement with c-*fos*^{H} sequences from +45 (octamer *Bgl*II linker) to the 3′-flanking *Bam*HI site. This fragment derives from pNOTBgl (Treisman, 1985). Actin 5′ deletion derivatives were constructed in the same way from appropriate derivatives of pA431CAT, and are denoted pAnnnFOS.

To construct synthetic oligonucleotide promoters, oliginucleotides comprizing the c-*fos*^{H} or actin SREs were inserted into the pUC12 *Eco*RI site, creating pDYAD (Treisman, 1986), p3DYAD (three copies of c-*fos*^{H} SRE), and pACT.D (one copy of actin SRE). CAT and c-*fos*^{H} fusion gene derivatives of these plasmids were constructed by insertion of *Sac*I−*Sal*I fragments from plasmids pA48CAT and pA48FOS. In these plasmids, the SRE is located 10 bp closer to the actin TATA box than in the wild-type actin gene, and nucleotides −63 to −49 are replaced by heterologous linker DNA.

An oligonucleotide derivative of plasmid pF261, which contains a truncated c-*fos*^{H} gene that lacks an SRE (Treisman, 1985, 1986), was constructed by insertion of the actin SRE oligonucleotide at this position to generate plasmid pF261AD+.

DNA binding−gel electrophoresis assay

All DNA binding experiments were performed exactly as previously described (Treisman, 1986). The c-*fos*^{H} binding probe was probe B (Treisman, 1986). The actin DNA binding probes were derived from pA114CAT, and comprised nucleotide −114 (joined to the pUC12 *Sma*I site, extending through the *Sac*I site to the labelled *Eco*RI site) to −63 (natural *Bst*NI). For top strand analysis the *Eco*RI site was 5′ end-labelled with [γ-^{32}P]ATP (3000 Ci/mmol); for bottom strand analysis the site was labelled with [α-^{32}P]dATP using reverse transcriptase. The presence of N^6-methyladenine at positions −76 and −77 owing to growth of the plasmid in *dam*$^+$ bacterial strains has no effect on complex formation (data not shown).

Expression assays

Microinjection of uncentrifuged *X. laevis* oocytes was performed as described by Gurdon and Wakefield (1986). Each oocyte germinal vesicle was injected with 20 nl of solution containing 10 ng of circular plasmid DNA. Oocytes were then cultured for 16−20 h at 19°C before freezing. Frozen oocytes were homogenized directly in ice-cold 0.25 M Tris-HCl (pH 7.8) using 30−50 μl of buffer per oocyte or embryo. Homogenates were spun at 12 000 r.p.m. for 10 min in the cold and the yolk-free supernatant stored at −70°C.

X. laevis renal cells (XLKE cells: line A6, American Type Culture Collection, Rockeville, MD) were cultured in 75% Dulbecco's modified Eagles medium/10% fetal calf serum at 23°C. Cell cultures were re-fed 12 h prior to transfection. Calcium phosphate precipitates containing 5 μg of DNA per 60 mm dish were left on the cells for 8−12 h and then removed and replaced with medium containing 20% glycerol. After 2 min, cultures were washed extensively in fresh medium and maintained for a further 48 h. Cells were then harvested, washed in phosphate-buffered saline, and spun down. The cell pellets were frozen and thawed in 0.25 M Tris-HCl, pH 7.8 (100 μl per dish) three times and centrifuged briefly at 12 000 r.p.m. in the col. The aqueous supernatant was stored at −70°C and used for CAT enzyme assays. Extracts prepared from each of two plates transfected with the same precipitate gave identical CAT activities.

Enzyme assays were performed as described by Gorman *et al.* (1982) and analysed by t.l.c. Levels of [acetyl-^{14}C]chloramphenicol synthesized were measured by scintillation counting and values corrected for variations in the protein concentration of the extracts.

Culture, transfection and serum stimulation of mouse NIH/3T3 cells were performed as previously described (Treisman, 1985). Preparation and analysis of RNA with anti-sense RNA probes were as described (Treisman, 1985). Cultures used for CAT assays were harvested by scraping in phosphate-buffered saline and extracts prepared as for *Xenopus* renal cells.

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Note added in proof

The human γ actin gene contains the SRE-related sequence 5′ AGATCGCCATATATGGACATGT 3′ at an analogous position to the *Xenopus laevis* SRE described here [H.Erba and L.Kedes, personal communication; Erba,H. (1986) *Ph.D. Thesis*, Stanford University].