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5′ flanking and first intron sequences of the human β-actin gene required for efficient promoter activity

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ABSTRACT

We have identified a CCAAT box element that is required for the efficient transcription of the human β-actin gene. Both in vivo transient transfection assays in cultured HeLa cells and in vitro run-off transcription assays in HeLa whole cell extracts demonstrated the requirement of this element for efficient promoter activity. A gel mobility shift assay revealed a Hela nuclear factor that specifically interacted with the β-actin CCAAT element in vitro; mutation of the first three base pairs of the CCAAT pentanucleotide abolished binding of this factor. Competition gel shift experiments revealed that three sequence elements located within the β-actin promoter, each containing a $CC(A/T)_6GG$ motif similar to that contained within the c-fos serum response element, were able to bind a different nuclear factor, serum response factor (SRF). One of these $CC(A/T)_6GG$ motifs is contained within a first intron fragment that enhanced transcription from a heterologous promoter in vivo.

INTRODUCTION

Upstream and enhancer elements have been described for a large number of cellular and viral gene promoters, and have been shown to play a major role in governing the rate of mRNA initiation from RNA pol II genes (see refs. 1 and 2 for reviews). These cis-acting sequence elements exert their effects on transcription through interaction with specific trans-acting regulatory factors. The overall strength of the promoter, as well as its particular developmental and tissue-specific regulatory programme, is determined by the combination and spatial orientation of these elements, and by the presence and regulatory state of the nuclear factors that interact with them.

The human β-actin promoter provides a good example of the regulatory intricacy that may be achieved. β-actin is one of the most highly expressed proteins in all mammalian non-muscle cells and, together with γ-actin, is the principal component of microfilaments (3). The expression of β-actin is developmentally regulated; the level of β-actin mRNA decreases upon myogenic differentiation to barely detectable levels (4-7). This decrease has been

attributed to transcriptional (6) and possibly post-transcriptional (7) regulation by sequences within the 3' untranslated region (3' UTR). In contrast, the β-actin 5' flanking region and first intron demonstrate a high level of constitutive promoter activity (8-10). Indeed, it has been shown in a variety of human and rodent cell lines, that this activity is comparable to, or greater than, that of the SV40 early promoter (10). It would be of interest to understand the basis for this ubiquitous and constitutive high level of activity of the β-actin promoter. A recent report (11), has provided evidence for the existence of an enhancer element, located within an evolutionarily conserved region of the first intron (12), and described a factor present in HeLa whole cell extracts that interacts with it *in vitro*.

The expression of the β-actin gene is also regulated by serum growth factors (13-15). The kinetics of induction of β-actin transcription are similar to that observed for the cellular oncogene c-*fos* in response to serum in various cell types (14-15). The serum-responsiveness of the human c-*fos* promoter requires a dyad symmetry element (DSE) found within an enhancer element (16-17) located 300 base pairs (bp) 5' to the mRNA start site (18-19). The *Xenopus laevis* cytoskeletal actin gene contains an element highly homologous to the human c-*fos* DSE (20); these DSE sequences also confer serum-inducibility *in vivo* (21). The c-*fos* and *Xenopus* actin DSEs are binding sites for serum response factor (SRF), present in both human HeLa and *Xenopus* nuclear extracts (21). Competition gel shift experiments have also demonstrated that an SRF binding site exists within the rat β-actin gene (19). Taken together, these results suggest that the HeLa SRF may play a role in the serum response of the human β-actin gene.

In this report, we demonstrate the requirement of the β-actin CCAAT element for efficient transcription *in vitro* and *in vivo*. Moreover, we detect the specific interaction of a HeLa nuclear factor to this element *in vitro*. In addition, we identify three *in vitro* SRF binding sites, two of which are located in regions of the human β-actin promoter that we show are important for efficient transcription, suggesting that one or more of these sites may play a role in the constitutive and/or serum-regulated transcription from the β-actin promoter *in vivo*.

MATERIALS AND METHODS

Recombinants

The β-actin *in vivo* expression vector was constructed by insertion of the full-length murine T-cell receptor α chain cDNA from the helper T-cell line

FGB1 (Maurice Zauderer, University of Rochester Cancer Center, unpublished), via flanking XhoI and BamHI sites, into the SalI-BamHI sites of pHβAPr-1-neo (10). The resultant vector, pHβAPr-1-neo-*a* (herein referred to as pβEI*a*), contains the human β-actin promoter from approximately -3300 to +909 (12, 22-23; see Figs. 4A and 6A). Deletion mutants pβSSI*a*, pβSAI*a*, pβSMI*a* and pβXI*a* contain 5' deletions from the EcoRI site (~ -3300) to the SacI (-2012), SacI (-472), SmaI (-246) and XhoI (-52) sites, respectively. pβSCI*a* has the CfoI fragment from -102 to -82 deleted from pβSAI*a*. These vectors were designated the IVS1$^+$ series. Removal of the β-actin 5' UTR from +49 to +909, spanning the first intron, from the above resulted in the IVS1$^-$ series of vectors, pβSS*a*, pβSA*a*, pβSM*a*, pβX*a* and pβSC*a*, respectively.

pML15 (herein referred to as pMG_0) has been described in detail (24; see Fig. 5A). The EcoRI-HindIII polylinker fragment of pUC18 (25) was inserted, via a ClaI linker at the repaired EcoRI site, into the ClaI-HindIII sites of pMG_0, resulting in an enhancer-less construct, pMG_1. A NarI fragment of the human β-actin first intron (+690 to +822) was repaired and inserted into the SmaI site of pMG_1, in both orientations, resulting in vectors $p\beta NMG_1$ and $p\beta NMG_2$, respectively.

The β-actin *in vitro* expression vector was constructed by inserting human β-actin promoter restriction fragments into the pUC18 polylinker (25). pβSA, pβSM and pβX contain human β-actin sequences from -472 (SacI), -246 (SmaI) and -52 (XhoI), respectively, to the SphI site at +102 (Figs. 1A and 6A). pβSC was derived from pβSA by deletion of the CfoI fragment from -102 to -82.

Oligonucleotides

The synthetic oligonucleotides used in this study were produced using an Applied Biosystems Model 280A DNA synthesizer. Each oligonucleotide was purified by polyacrylamide gel electrophoresis as described (26). Two 39-mer oligonucleotides were synthesized complementary to the human β-actin promoter from -104 to -66 and containing either a wild-type or triple point mutant (underlined) CCAAT motif, 5'-CTTTCGGAACGGCGCACGCTGATTGGCCCCGCGCCGCTC-3' or 5'-CTTTCGGAACGGCGCACGCTGATATCCCCCGCGCCGCTC-3', respectively. A 12-mer primer was also synthesized, 5'-GAGCGGCGCGGG-3', complementary to the 3' ends of the two 39-mers. ^{32}p-labelled duplex probes were generated by primer extension (26). Wild-type and non-SRF binding mutant c-fos DSE fragments were prepared by isolating the SmaI-EcoRI fragment from either pAdSF or pAdSF23 (S. Leung and N.G.M., manuscript in preparation), containing the wild-type or mutant human c-fos DSE sequences, respectively, from -322 to -297 (27). ^{32}p-labelled DSE probes were prepared by 3' end repair at the EcoRI site (28).

Standard Gel Mobility Shift Assay

HeLa S-3 cells were cultured and harvested as described previously (29). Nuclear extracts were prepared according to a modified Dignam procedure (30) described in ref. 31, except that protease inhibitors (leupeptin, antipain, and pepstatin A (5 μg/ml each), and 0.5 mM PMSF (Sigma)) were added to all buffers and solutions. A ^{32}P-labelled DNA template (1-10 fmoles) was mixed with the indicated molar excess of unlabelled competitor DNA and 2 μg of a non-specific DNA mix of 1:1, poly(dI-dC)-poly(dI-dC):poly(dA-dT)-poly(dA-dT) or 1:1:0.5, poly(dI-dC)-poly(dI:dC):poly(dA:dT)-poly(dA:dT):MspI-digested pUC18. The latter DNA mix seemed to reduce non-specific interactions of nuclear protein with the probe much more efficiently than the former. The volume of the mix was brought to 10 μl, and contained 80 mM KCl and either 2 mM $MgCl_2$, 10 mM EDTA or 10 mM $MgCl_2$, for binding to SRF or the CCAAT-binding factor, respectively. An equal volume of HeLa nuclear extract (5-10 μg protein) diluted in nuclear extract buffer (20 mM Hepes (pH 7.9), 1 mM $MgCl_2$, 20 mM KCl, 2 mM DTT, 17% glycerol, and protease inhibitors) was added and the reaction mixture was incubated for 30 min at room temperature (RT). The reaction mixtures were loaded directly onto 4% polyacrylamide gels (30:1, acrylamide:bisacrylamide), pre-electrophoresed for > 1.5 h at 160 V at 4°C in 0.25 X TBE buffer (28). After electrophoresis for 2 to 4 h at 160 V at 4°C, the dried gel was autoradiographed with Kodak XAR film and a Dupont Cronex intensifying screen at -70°C.

In Vitro Transcription

Preparation of HeLa whole cell extracts (32) and in vitro run-off transcription assays (33) were as previously described.

Transfection analyses

1 X 10^6 HeLa cells were transfected with 20 μg plasmid plus 10 μg salmon sperm DNA as previously described (34); 10 μg total cellular RNA, isolated by the guanidium isothiocyanate-CsCl gradient method (35), was treated with 10 U DNase I (FPLC-purified, Pharmacia) in a 50 μl reaction containing 25 U RNAsin (Promega Biotec), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 50 mM Tris-HCl (pH 7.4) for 30 min at 37°C, and purified by phenol:$CHCl_3$ extraction followed by ethanol precipitation.

For northern blot analysis, the RNA was treated with 15% glyoxal for 60 min at 50°C, and electrophoresed through 1% agarose gels in 10 mM $NaPO_4$ (pH 7.0) at 70 V for 3 h with buffer circulation. RNA was transferred to Gene Screen Plus (NEN), and hybridization and washing were carried out according

to the supplier's specifications. Filters were autoradiographed as above. The probes, radio-labelled by nick translation (28), were neo, the HindIII-XhoI fragment from pC(P)Xneo, containing the BglII-XhoI fragment of the Tn5 neomycin gene (36), and α, the EcoRI fragment from pLCα, containing the XhoII-AvaII fragment of the murine T-cell receptor α-chain gene.

For primer extension analysis, the RNA was hybridized overnight at 42°C to a 5' ^{32}P-end labelled 34-mer oligonucleotide, complementary to the rabbit β-globin mRNA from +70 to +37 (37), as described (38). Samples were then diluted into a 90 μl volume containing 10 U AMV reverse transcriptase (Pharmacia), 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 10 mM DTT and 0.5 mM each dNTP, and incubated 60 min at 42°C. The ^{32}P-cDNA, purified by phenol:$CHCl_3$ extraction and precipitation, was analyzed on an 8% polyacrylamide-8.3M urea gel (40:2 acrylamide:bisacrylamide), and dried and autoradiographed as above.

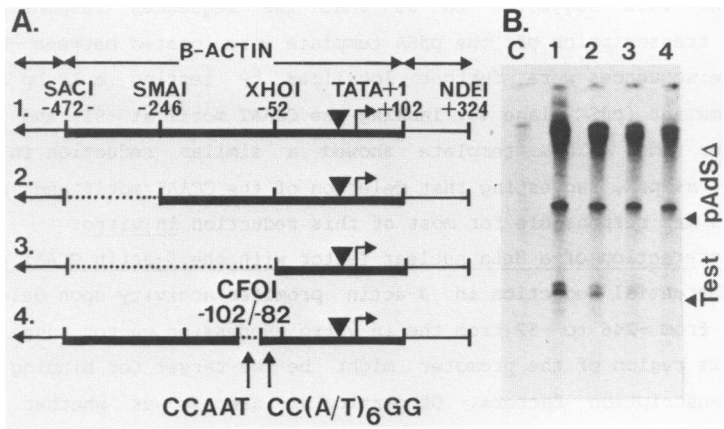


Fig. 1. In vitro run-off transcription analysis of the β-actin promoter. (A) Transcription templates. Heavy lines represent β-actin promoter fragments, cloned into pUC18 (thin lines); dotted lines represent deleted promoter sequences. Templates 1-4 correspond to pβSA, pβSM, pβX, and pβSC, respectively. (B) Representative run-off transcription assays. Lanes 1-4: 100 ng of the corresponding template 1-4 (shown in part A), linearized at the NdeI site (+324), were transcribed in vitro as previously described (33). 50 ng of the plasmid, pAdSΔ, linearized at the AatII site (+524), was pre-mixed with each template, and served as internal reference. pAdSΔ (29) contains the Ad2MLP (-52 to +33) cloned into the pUC12 polylinker. Lane C: transcription of 100 ng template 1, plus 50 ng pAdSΔ in the presence of α-amanitin (5 μg/ml). The expected β-actin and Ad2MLP run-off transcripts are indicated by test and pAdSΔ, respectively.

RESULTS

A CCAAT element is required for efficient β-actin promoter activity in vitro.

To determine sequences necessary for efficient β-actin promoter activity in vitro, run-off transcription assays were performed on a set of 5' flanking region deletion mutants in HeLa whole cell extracts. The DNA templates used in this study were comprised of the various β-actin promoter restriction fragments cloned into pUC18 (Fig. 1A). A typical result of this analysis is shown in Figure 1B. The minimal promoter tested (pβX, lane 3) contained sequences from -52 to +102, and included both the β-actin TATA box and mRNA start site. This template was poorly transcribed, suggesting that sequences necessary for efficient transcription in vitro were located upstream of -52. The largest promoter fragment tested contained sequences from -472 to +102 (pβSA, lane 1), and produced the greatest level of run-off transcript. Deletion to -246 (pβSM, lane 2) resulted in a 0-0.5-fold reduction, compared to pβSA, depending on template concentration and the particular extract preparation. This suggested to us that the sequences responsible for efficient transcription off the pβSA template were located between -246 and -52. These sequences were further localized by testing a 19 bp internal deletion mutant (pβSC, lane 4), lacking the CCAAT motif at -91, and several bp on either side. This template showed a similar reduction in run-off transcript as pβX, suggesting that deletion of the CCAAT motif and adjacent nucleotides was responsible for most of this reduction in vitro.

Specific interaction of a HeLa nuclear factor with the β-actin CCAAT element.

The substantial reduction in β-actin promoter activity upon deletion of sequences from -246 to -52 from the in vitro expression vector suggested to us that this region of the promoter might be the target for binding of cis-acting transcription factors. Of particular interest was whether nuclear factors could interact with the CCAAT motif, located at -91, and a $CC(A/T)_6GG$ motif located 30 bp downstream (see below). Therefore, a gel mobility shift assay (39-40) was used to determine whether the SmaI-XhoI fragment from -246 to -52 (denoted the SX fragment) could bind a specific nuclear factor(s) in vitro. The SX fragment was ^{32}P-labelled at the XhoI site (Fig. 2B, probe a), and incubated with a HeLa nuclear extract (Fig. 2A). Titration of the extract with increasing amounts of poly(dI-dC)-poly(dI-dC):poly(dA-dT)-poly (dA-dT) to minimize non-specific interactions of protein with the promoter fragment, revealed a specific nuclear factor-DNA complex (C_S). The formation of this complex was efficiently competed out by the inclusion of a 200-fold molar excess of unlabelled SX fragment in the reaction mixes (Fig. 2A, com-

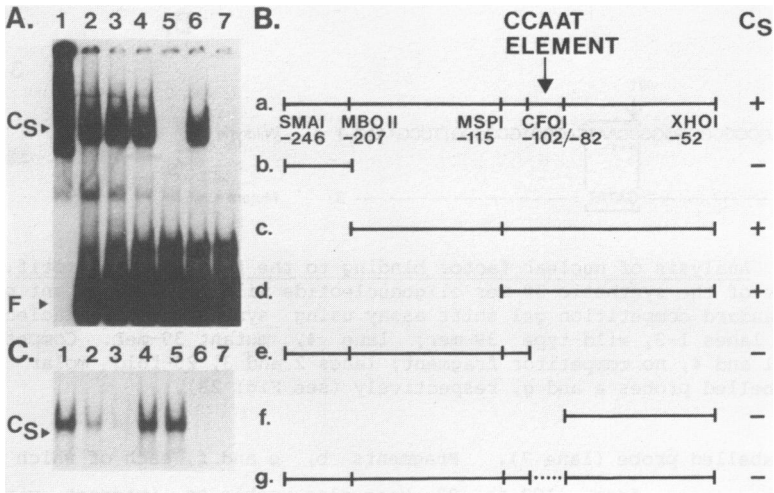


Fig. 2. Detection of specific nuclear factor binding to the SX fragment. (A) Titration of a nuclear extract with competitor DNA. ^{32}P-labelled SX fragment (probe a in B) was incubated with fixed amount of HeLa nuclear extract, and increasing amounts of a non-specific DNA mix (see Materials and Methods): 0, 0.1, 0.3, 1, 1, 2 and 2 μg in lanes 1-7, respectively. Lanes 5 and 7 also contained a 200-fold molar excess (25 ng) of the unlabelled SX fragment. F and C_S refer to the free probe and specific nuclear factor-DNA complex, respectively. (B) Deletion analysis of the nuclear factor binding site. Truncated probes, a-g, were ^{32}P-labelled and tested in a standard (2 μg non-specific DNA mix) gel shift assay for C_S formation, to localize binding on the SX fragment. (C) Standard competition gel shift assay using the SX fragment. Probes: lanes 1-6, SX fragment (probe a in B), and lane 7, 19 bp CCAAT deletion mutant (probe g in B). Competitors: lanes 1 and 7, no competitor fragment; lanes 2-3, 5- and 25-fold molar excess of unlabelled probe a; lanes 4-5, 5- and 25-fold molar excess of unlabelled probe g; lane 6, 25-fold molar excess of plasmid pβSA. Free probe is not shown.

pare lanes 4 and 6 with lanes 5 and 7, respectively), demonstrating that the interaction between the nuclear factor and the SX fragment was specific.

To further delineate the sequences necessary for factor binding, a set of 3' and 5' truncations of the SX fragment were produced for use both as radio-labelled probes and unlabelled competitor fragments in GMS competition assays (Fig. 2B, fragments a-g). An example of this analysis is shown in Fig. 2C for fragment g, which contained the 19 bp internal deletion from -102 to -82 spanning the CCAAT motif; this fragment failed to compete with radio-labelled wild-type SX fragment for binding to the factor in the gel shift assay when used as unlabelled competitor (compare lanes 4 and 5 with lanes 2 and 3), nor did the mutant SX fragment bind the factor when used as

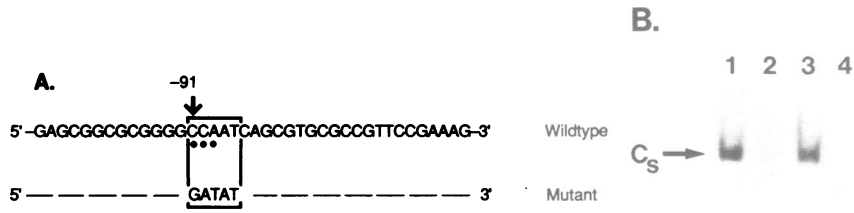


Fig. 3. Analysis of nuclear factor binding to the β-actin CCAAT motif. (A) Sequence of the synthetic 39-mer oligonucleotide wild-type and mutant probes. (B) Standard competition gel shift assay using synthetic oligonucleotides. Probes: lanes 1-3, wild-type 39-mer; lane 4, mutant 39-mer. Competitors: lanes 1 and 4, no competitor fragment; lanes 2 and 3, 25-fold molar excess of unlabelled probes a and g, respectively (see Fig. 2B).

radio-labelled probe (lane 7). Fragments b, e and f, each of which lacked these sequences from -102 to -82, were also unable to interact with the specific nuclear factor as judged by their failure to form the specific retarded band (C_S) in similar GMS competiton assays. These results are summarized in the right-hand side of Fig. 2B, and suggested to us that the specific complex seen in the gel shift assay was due to a factor binding at or near to the CCAAT element.

To determine whether the factor was indeed binding to the β-actin CCAAT element, a gel shift assay was performed on wild-type and triple point mutant (CCAAT → GATAT) synthetic oligonucleotide binding sites (Fig. 3A). As shown in Figure 3B, a wild-type 39-mer bound to the HeLa nuclear factor and was competed out by the wild-type, but not the 19 bp internal deletion mutant, SX fragment (lanes 1-3). In contrast, the mutant 39-mer was unable to form the specific complex (lane 4), demonstrating the importance of the CCAAT pentanucleotide for factor binding. Taken together with the previous *in vitro* transcription studies, these data strongly suggest that the HeLa nuclear factor is a *cis*-acting transcriptional activator whose effect is mediated through interaction with the CCAAT motif within the β-actin promoter.

The β-actin CCAAT element and first intron sequences are necessary for efficient promoter activity in cultured HeLa cells.

In order to demonstrate the requirement of the β-actin CCAAT element for efficient transcription *in vivo*, a β-actin *in vivo* expression vector (Fig. 4A) containing sequences of the human β-actin promoter from -3300 to +909, as well as various 5' flanking region deletion mutants, were transfected into HeLa cells. β-actin promoter activity was measured in transient expression assays by the level of the reporter T-cell receptor α chain mRNA, as detected

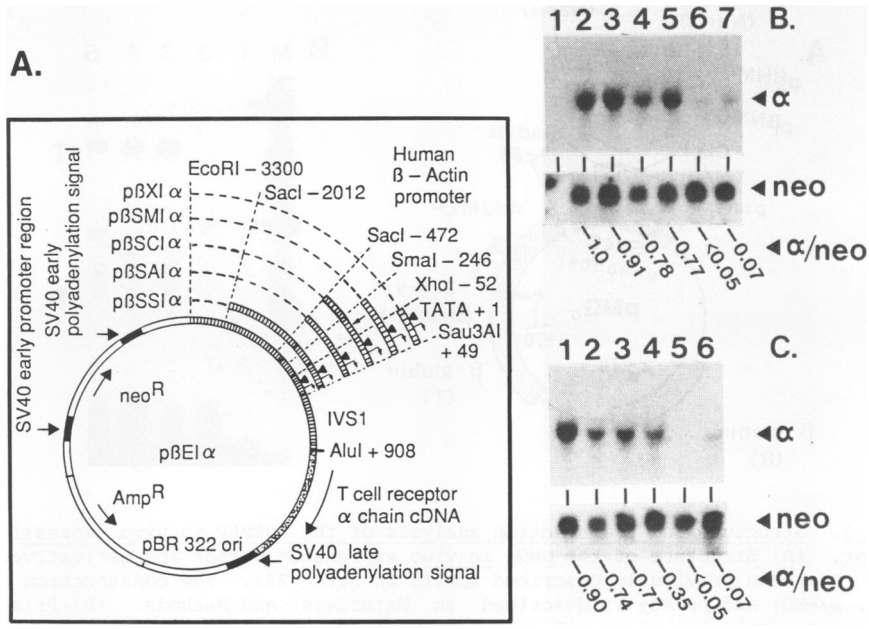


Fig. 4. Structure and transfection analysis of the human β-actin promoter in vivo expression vector. (A) Structure of the pβEIα in vivo expression vector and derivatives. Sequences of the β-actin promoter and murine T-cell receptor α-chain are indicated by hatched and stippled boxes, respectively. Other features of this vector have been described (10). 5' deletions are represented by the dashed lines. The arrow points to the location of the 19 bp internal deletion of the CCAAT motif within pβSCIα. (B and C) Northern blot analysis of transfected HeLa cell RNA probed with neo and α (see Materials and Methods). (B) IVS1$^+$ series. RNA from cells transfected with 30 μg salmon sperm DNA (lane 1) or with 10 μg salmon sperm DNA and 20 μg each of pβEIα, pβSSIα, pβSAIα, pβSMIα, pβXIα and pβSCIα (lanes 2-7). (C) IVS1$^-$ series. Lanes 1-6: RNA from cells transfected with 10 μg salmon sperm DNA and 20 μg each of pβSSIα, pβSSα, pβSAα, pβSMα, pβXα and pβSCα, respectively. α/neo: ratio of α chain and neo transcripts as determined by densitometric scanning of the autoradiograms shown in the Figure (normalized to 1.0 for pβEIα). Similar results were obtained in other experiments.

by northern blot analysis of total transfected HeLa RNA. Co-expression of neo mRNA, driven by the SV40 early promoter, was used as an internal reference to normalize for transfection efficiency. Two series of 5' deletion mutants were tested, one with the first intron retained (IVS1$^+$) and the other with these sequences deleted (IVS1$^-$). β-actin 5' flanking sequences upstream of -246 could be deleted with minor effects on promoter activity (Fig. 4B, lanes 2-5). Further deletion to -52 (pβXIα, lane 6), however, resulted in a

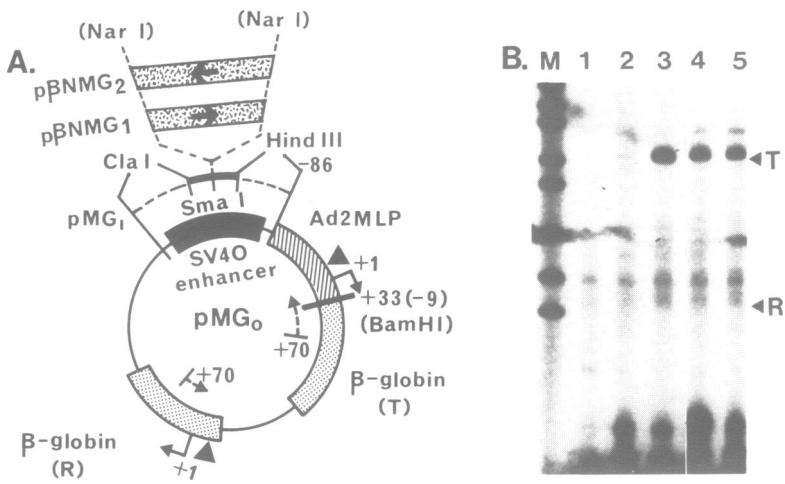
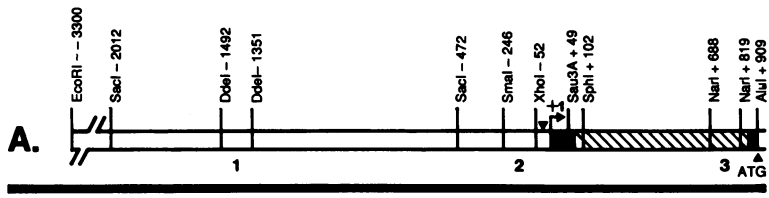


Fig. 5. Structure and transfection analysis of the Ad2MLP in vivo expression vector. (A) Structure of the pMG_0 in vivo expression vector and derivatives. pMG_0 has been previously described (pML15 in ref. 24). The construction of pMG_1, $p\beta NMG_1$ and $p\beta NMG_2$ is described in Materials and Methods. (B) Primer extension analysis of transfected HeLa cell RNA using as primer a rabbit β-globin 34-mer oligonucleotide complementary to the RNA from +70 to +37. RNA from cells transfected with 30 μg salmon sperm DNA (lane 1) or with 10 μg salmon sperm DNA and 20 μg each of pMG_1, pMG_0, $p\beta NMG_1$ and $p\beta NMG_2$ (lanes 2-5). M: MspI-digested pBR322 as size markers. T and R represent the expected primer extension products from the test and reference β-globin transcription units, respectively. The reference primer extension product was poorly represented in these constructs.

substantial reduction in levels of α chain mRNA, suggesting that sequence elements crucial for efficient β-actin promoter activity had been lost, as was also suggested by the run-off transcription analysis. The 19 bp internal deletion mutant of the CCAAT motif ($p\beta SCI\alpha$, lane 7) was also expressed at a much reduced level compared to its parent, $p\beta SAI\alpha$ (lane 4), yet at a somewhat greater level than $p\beta XI\alpha$. The relative levels of expression of the various deletion mutants of the $IVS1^-$ series (Fig. 4C), were similar to those seen with the $IVS1^+$ series (Fig. 4B), although the overall level of expression of this first intron-deleted series was generally reduced compared to the IVS1+ series, up to 2-fold for $p\beta SMI\alpha$ (Fig. 4B, lane 5) and $p\beta SM\alpha$ (Fig. 4C, lane 4). It is possible that the SV40 enhancer, used to drive the reference neo transcript, might have masked the effects of the β-actin promoter deletions; however, Wasylyk et al. (41) previously demonstrated that the SV40 enhancer preferentially activates the more proximal promoter, suggesting to us that



B.

	ELEMENT	FIRST NUCLEOTIDE	SEQUENCE			LAST NUCLEOTIDE
	C – FOS DSE	– 325	CACAGGATGT	CCATATTAGG	ACATCTGCGT	–296
	β– ACTIN:					
1	DISTAL 5'	–1440	GGTTCCATGT	CCTTATATGG	ACTCATCTTT	–1411
2	PROXIMAL 5'	–72	CCGAAAGTTG	CCTTTTATGG	CTCGAGCGGC	–43
3	INTRON 1	+752	CCAGTGTTTG	CCTTTTATGG	TAATAACGCG	+781

Fig. 6. Location and sequence of the human β-actin promoter $CC(A/T)_6GG$ motifs. (A) Restriction map of the human β-actin promoter. Black, hatched and open boxes depict 5' UTR, first intron and 5' flanking sequences, respectively. 1, 2 and 3 refer to restriction fragments containing the $CC(A/T)_6GG$ motif shown in B. (B) Sequences encompassing the c-fos DSE and three $CC(A/T)_6GG$ motifs within the human β-actin promoter.

the effects of the SV40 enhancer on the β-actin promoter in our constructs would be only minor. Nevertheless, these results clearly demonstrated that the CCAAT element and surrounding nucleotides were necessary for efficient transcription, in vivo as well as in vitro.

An enhancer has recently been identified within the first intron of the human β-actin gene, between +759 and +783, which interacts specifically with a HeLa factor (11). We also detected an enhancer activity within the β-actin first intron (Fig. 5). A NarI fragment (+690 to +822) was inserted, in either orientation, upstream of the adenovirus-2 major late promoter (MLP) in pMG_1, replacing the SV40 enhancer of pMG_0, resulting in $p\beta NMG_1$ and $p\beta NMG_2$ (Fig. 5A). Primer extension analysis of RNA from HeLa cells transfected with each of the above constructs showed that transcription from the MLP is barely detectable in the enhancerless construct (pMG_1, lane 2 in Fig. 5B). The NarI fragment (lanes 4 and 5) enhanced transcription from the MLP to levels similar to that observed for the SV40 enhancer (lane 3), in an orientation-independent manner. This data confirms the existence of the first intron β-actin enhancer; however, we detect a different nuclear factor interacting with this region than that described in ref. 11 (see below).

Specific interaction of SRF with three β-actin promoter binding sites.

Previous in vitro binding studies (19) demonstrated that a sequence(s) in the rat β-actin gene interacts with SRF, a sequence-specific DNA-binding protein thought to mediate serum induction of the c-fos and Xenopus actin transcription (18-21). Inspection of the human β-actin promoter sequences (12, 22-23) revealed three regions containing sequences similar to the c-fos and Xenopus actin SRF binding sites (DSEs). Binding studies were performed on three β-actin promoter fragments (Fig. 6A, probes 1-3), each of which contain a sequence motif similar to the DSE (Fig. 6B). By altering the binding conditions to that required for optimum SRF binding (see below), we

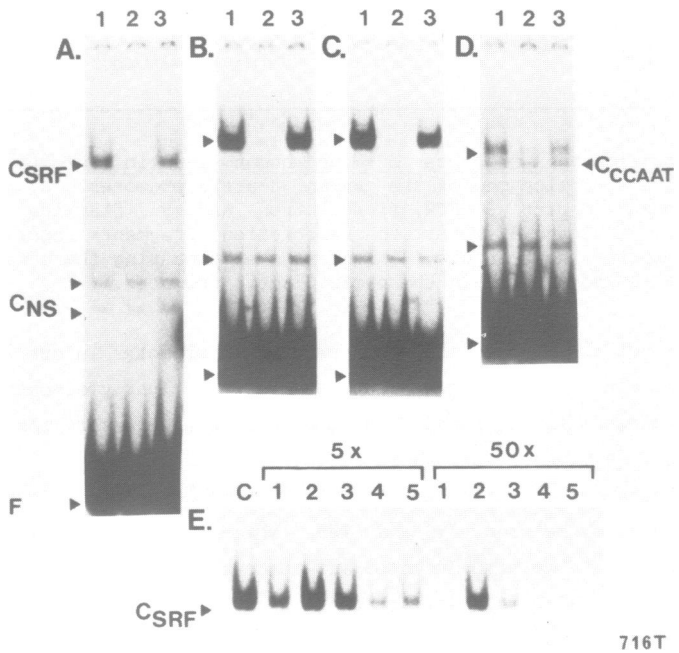


Fig. 7. Detection of three SRF binding sites within the β-actin gene. Standard gel shift assay using (A) the human c-fos DSE, and (B-D) the distal DdeI, intron 1 NarI, and SX promoter fragments, respectively, of the β-actin gene as radio-labelled probes. Lanes 1: no competitor, lanes 2-3: 50-fold molar excess of the wild-type and non-SRF binding mutated c-fos DSE, respectively (S. Leung and N.G.M., manuscript in preparation). C_{SRF}, C_{CCAAT}, C_{NS} and F indicate the SRF-DNA complex, β-actin CCAAT-binding factor-DNA complex, non-specific protein-DNA complex and free probe, respectively. (E) Competition gel shift assay using the c-fos DSE as radio-labelled probe, and 5- and 50-fold molar excesses of the following unlabelled competitors: Lanes 1-2, wild-type and mutant c-fos DSE; lanes 3-5, SX, DdeI, and NarI promoter fragments, respectively; lane C, no competitor.

observed that each of these fragments interact with SRF *in vitro* (C_{SRF} in Figs. 7A-D). These competition gel shift assays showed that the wild-type, but not a non-SRF binding mutant, c-*fos* DSE competed for factor binding (compare lanes 2 and 3 in these Figures). A competition gel shift assay, using a 38-mer wild-type c-*fos* DSE probe, demonstrated that the β-actin promoter fragments each bind to SRF with different affinity (Fig. 7E). The *Dde*I and *Nar*I promoter fragments (probes 1 and 3, lanes 4 and 5) competed most efficiently for SRF binding, moreso even than the c-*fos* DSE itself (lane 1), while the SX promoter fragment bound most poorly (probe 2, lane 3).

The higher mobility complex seen when using the proximal SX fragment as radio-labelled probe represents nuclear factor binding to the CCAAT element (C_{CCAAT} in Fig. 7D, and see Fig. 2), which is greatly inhibited under the reaction conditions used to optimize the interaction of SRF with this fragment. SRF binding is most efficient in the absence of $MgCl_2$ (42). Accordingly, the gel shift assays for SRF were performed in the presence of 5 mM EDTA; if an excess of Mg^{2+}, but neither Ca^{2+} nor Zn^{2+}, is added back to the reaction mix, efficient factor binding to the CCAAT element is restored and SRF binding to the DSE is dramatically reduced (data not shown). Thus, although both the CCAAT-binding factor and SRF are able to interact with the SX fragment of the β-actin promoter *in vitro*, the factors display different requirements for Mg^{2+} for optimal binding in our assay. Moreover, we have been unable to detect the simultaneous interaction of SRF and the CCAAT factor with this region of the β-actin promoter, based on our inability to detect a band of lower mobility in the gel shift assay which could represent a DNA-SRF-CCAAT factor complex; this suggests that, at least *in vitro*, the factors bind in a mutually exclusive manner.

DISCUSSION

In this report, we have described sequences within the 5' flanking region and first intron of the human β-actin gene that are necessary to achieve high levels of transcriptional activity in both cultured HeLa cells and whole cell extracts. Deletion of sequences encompassing a CCAAT motif at -91 greatly reduced promoter activity suggesting that these sequences are centrally important in directing efficient transcription. *In vitro* gel shift binding studies revealed a binding site for a HeLa nuclear factor, termed β-actin CCAAT-binding factor, centered on the CCAAT motif. *In vitro* binding of the nuclear factor to the promoter correlated with efficient transcription in Hela whole cell extracts.

Several eukaryotic RNA pol II genes contain a promoter element which is characterized by a pentanucleotide CCAAT motif, and various nuclear factors have been described that can bind to these elements in vitro (43-47 and refs. therein). The most extensively studied of these is NF-1, first identified as a site-specific DNA binding protein required for Adenovirus DNA replication in vitro (48). Recent studies have also implicated NF-1 and its cellular binding sites in the control of mRNA synthesis (29, 44). Based on extensive in vitro binding studies, as well as chromatographic data, it is clear that the β-actin CCAAT-binding factor is not NF-1 (R.M.F. and R.M. Gronostajski, unpublished data). Several other nuclear factors capable of binding to CCAAT motifs have been identified, the most notable including CBP/EBP20 (45), NF-Y (CP1) (46), and a factor binding to the mouse α-globin CCAAT-box (47). Preliminary studies using synthetic CCAAT-box binding sites from the HSV thymidine kinase, the murine MHC class II Eα, and the human α-globin genes (46), respectively, as competitor fragments in gel shift assays, suggest that either the β-actin CCAAT binding factor is distinct from the above proteins, or that the β-actin CCAAT element constitutes a much stronger binding site for one of the above factors (our unpublished data). A more extensive biochemical analysis of the factor is being conducted to distinguish between these two possibilities, and a study of the tissue distribution of this factor will allow us to determine the ubiquitousness of its expression.

A β-actin enhancer activity located in the first intron has recently been described (11), associated with a 25 bp sequence (+759 to +783), which binds a factor in HeLa whole cell extracts. This sequence contains a $CC(A/T)_6GG$ motif, yet the experiments presented in this report (11) suggest that the factor binding to the enhancer element in vitro is distinct from SRF, based on the inability of an unlabelled synthetic 27-mer c-fos DSE to compete for binding to the factor. However, this data is difficult to interpret in the absence of positive controls to demonstrate the existence of intact SRF binding activity in the whole cell extract, and that the synthetic DSE could interact with SRF under conditions of the binding assay. We clearly see binding of SRF to the NarI fragment of the first intron in our binding assay, as well as to two other fragments of the β-actin promoter, a distal DdeI fragment and the SX fragment, each of which contain $CC(A/T)_6GG$ sequence motifs (see Figs. 6 and 7). In Figure 5 we show that we can detect the first intron DSE-associated enhancer activity in the pMG_1 vector system; we have also used this system to detect enhancer-like activity associated with the SX and DdeI promoter fragments (Fig. 6A), both of which contain a DSE-like

element (N.G.M. and R.M.F., unpublished data). We are currently analyzing deletion mutants of these promoter fragments in the pMG_1 system to determine whether this activity is indeed associated with the $CC(A/T)_6GG$ motif. Taken together with the observation that the human β-actin gene is transcriptionally activated in tissue culture in response to serum with the same kinetics of induction as c-fos (14-15), it is suggested that one or more of these elements may serve as a functional in vivo target of SRF.

Although the β-actin intron and proximal DSEs share a high degree of sequence similarity, their affinities for SRF in vitro differ by ~ 10:1, respectively, presumably due to sequence differences within regions flanking these two motifs. The proximity of the CCAAT element to the SRF binding site on the SX fragment, coupled with our inability to detect the simultaneous binding of the two factors in the gel shift assay, suggests that there may be interference between their recognition factors. We are currently analyzing promoter mutants containing point substitutions in either the DSE or CCAAT element to determine whether loss of either of the nuclear factor binding sites can stimulate binding to the adjacent site on the SX fragment. These mutants will also allow us to address the function of these elements, specifically the DSE, as it relates to constitutive and/or serum responsive regulation of β-actin expression.

The human cytoskeletal γ-actin gene has recently been sequenced, and it has been suggested, based on conservation of intron/exon boundaries, as well as sequence similarities in the 5' UTR, 5' flanking and coding regions, between the β- and γ-actin genes, that gene duplication gave rise to the two cytoskeletal actin isoforms (49). Of particular relevance to this work, the authors also note the sequence conservation within the proximal 5' flanking region. Indeed, the sequences of the CCAAT and $CC(A/T)_6GG$ motifs, and the distance and sequences separating these two motifs, have been conserved between human γ- and β-actin, as well as between β-actin genes from human, rat and chicken, and the Xenopus borealis type I actin gene (12, 21, 49-51). Such strong conservation, together with our demonstration of two nuclear factor binding sites in vitro, and the requirement of at least the CCAAT element for efficient transcription in cultured HeLa cells and whole cell extracts, suggests that this region is centrally important in controlling coordinate cytoskeletal actin expression in vivo.

The γ- and β-actin proteins are expressed in all mammalian non-muscle cells, yet the relative expression of the two cytoskeletal actins varies considerably. Measurements of steady state protein in different rat tissue

types reveals a β/γ ratio of 0.5 to 3.4 (52-53), and the relative expression at the steady state mRNA level has recently been shown to vary up to 60-fold in a variety of mouse tissues (49). Interestingly, the distance between the $CC(A/T)_6GG$ motif and the TATA box differs by 30 bp between the γ- and β-actin promoters (49). Furthermore, inspection of sequences within the first intron of the human γ-actin gene reveals the absence of a sequence similar to the $CC(A/T)_6GG$ motif, present in the first intron of β-actin gene, and associated with an enhancer activity. It would be of interest to determine if the differential placement of the CCAAT element/proximal DSE relative to the TATA box, of the γ- and β-actin genes, or the apparent absence of an intron DSE and the associated enhancer activity in the γ-actin gene, may be contributing to the differential expression of these two cytoskeletal actin isoforms.

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