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Nucleic Acids Research, Vol. 19, No. 24 6913–6922

Transcription factor binding and spacing constraints in the human β-actin proximal promoter

Sandra L.Danilition, Robert M.Frederickson+, Catherine Y.Taylor and Neil G.Miyamoto*
Division of Cellular and Molecular Biology, The Ontario Cancer Institute/Princess Margaret Hospital, and Department of Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, Ontario M4X 1K9, Canada

Received July 22, 1991; Revised and Accepted October 31, 1991

ABSTRACT

The human β-actin promoter, including its 5′ flanking region and 5′ untranslated region, is ubiquitously active in mammalian cells in culture. In this report we investigated the transcriptional activity of, and the protein-DNA interactions that occur within, the proximal region of the human β-actin promoter. Efficient β-actin promoter activity in transfected human HeLa cells requires only 114bp of 5′ flanking sequences. Two of the cis-acting regulatory elements within this region of the β-actin promoter, the CCAAT box and proximal CCArGG box, are specific *in vitro* binding sites for the transcription factors, nuclear factor Y (NF-Y) and serum response factor (p67^{SRF}), respectively. These two elements are required together to stimulate *in vivo* transcription from the homologous as well as a heterologous promoter. Finally, a particular spatial alignment between the CCAAT box and proximal CCArGG box is required for trans-activation *in vivo*. The above provides strong evidence for a functional interaction between NF-Y and p67^{SRF} when bound to their respective binding sites in the β-actin promoter.

INTRODUCTION

Changes in the level of gene expression often involve the activation or repression of the promoter. Cell cycle, developmental, hormonal, and tissue-specific regulation of a promoter are dictated by the combination and spatial arrangement of its cis-acting sequence elements, in addition to the presence and regulatory state of the trans-acting protein factors that interact with it (1,2,3). Trans-acting factors are believed to regulate RNA polymerase II-dependent mRNA synthesis by influencing the assembly of the pre-initiation or initiation complex at the TATA box and mRNA start site region of the promoter (4,5). Transcription is thus regulated by trans-acting factors through protein-protein interactions with other trans-acting factors, co-activators, and/or the general transcription factors and RNA polymerase II (6,7).

Not only is the presence of particular cis-acting elements in a promoter important, but also the specific arrangement or location of the elements relative to each other and to the mRNA start site is often vital for efficient initiation of transcription. Variation in distances between certain elements in the early promoter of SV40 showed a periodicity in transcriptional activity correlating with insertions of integral turns of the DNA helix (8,9,10), suggesting a requirement for specific alignment between these elements for optimal promoter function. In a number of promoters with variable distances between cis-acting elements and TATA boxes, however, periodicity in transcriptional activity has not been observed (11,12,13,14).

We have chosen the human β-actin gene promoter as a model to study the control of transcription initiation by RNA polymerase II. As one of the principal components of microfilaments, β-actin is ubiquitously and abundantly expressed in non-muscle cells (15). An expression vector containing the 5′ flanking and 5′ untranslated region sequences from ~ −3300 to +909 of the human β-actin gene demonstrates a high level of transcriptional activity when transfected into a variety of human and rodent cell lines and, in fact, this expression vector is active in myotubes, which do not express the endogenous β-actin gene (16). The β-actin gene is also a member of the 'immediate-early' genes transiently induced at the transcriptional level upon exposure of quiescent cells to serum (17,18,19). Thus, the β-actin promoter provides a unique opportunity to investigate both constitutive and inducible gene transcription.

The human β-actin promoter consists of a consensus TATA box and at least four cis-acting elements: three CCArGG boxes and one CCAAT box (20,21,22,23). Two of the CCArGG boxes are located within the 5′ flanking sequences, one distal and one proximal to the mRNA start site, while the third is located within the first intron. We demonstrated that restriction fragments spanning each of these CCArGG boxes interacted specifically with serum response factor (p67^{SRF}) (23). p67^{SRF} is a sequence-specific DNA binding protein first identified as the factor that binds to the serum response element (SRE) of the c-*fos* promoter (24) and is believed to mediate serum induction of that gene (25).

* To whom correspondence should be addressed

+ Present address: McGill Cancer Center, Department of Biochemistry, McIntyre Medical Sciences Building, McGill University, 3655 Drummond Avenue, Montreal, Quebec H3G 1Y6, Canada

More recent studies have shown that $p67^{SRF}$ can also negatively regulate transcription from the c-*fos* promoter (26,27,28). Another protein, $p62^{TCF}$, which binds the c-*fos* SRE through interaction with $p67^{SRF}$, is also required for serum inducibility of the c-*fos* promoter (29,30). Our previous study also showed that the CCAAT box is required for activity of the human β-actin promoter both *in vivo* and *in vitro* and binds a factor termed β-actin CCAAT-binding factor (23). Similar results were obtained with the chicken β-actin promoter (31).

In this report the dual requirement of the CCAAT box and proximal CCArGG box for optimal transcriptional activity from the human β-actin promoter *in vivo*, as described previously (23,32,33), is examined in detail. *In vitro* binding studies establish that the β-actin CCAAT and proximal CCArGG elements specifically interact with the transcription factors, nuclear factor Y (NF-Y) and $p67^{SRF}$, respectively. The requirement for a particular spatial alignment between the CCAAT box and proximal CCArGG box is demonstrated, strongly suggesting that NF-Y and $p67^{SRF}$ (and possibly $p62^{TCF}$) functionally interact when bound to their respective sequence elements.

MATERIALS AND METHODS

Plasmid Constructions

A β-actin promoter *in vivo* expression vector was constructed by insertion of the SacI-Sau3AI restriction fragment of the human β-actin gene (20,21,22) into the pUC18 polylinker region of pGβ (26). The resultant vector, pβAGβ.SA, contains β-actin promoter sequences from −472 to +49, relative to its mRNA start site, driving expression of a promoter-less rabbit β-globin reporter gene (23,26).

A 5′ deletion series was constructed in pβAGβ.SA using the available restriction sites from SacI (−472) to the StuI (−320), SmaI (−246), NaeI (−114), BamHI (−76), and XhoI (−52) restriction sites to generate constructs pβAGβ.ST, pβAGβ.SM, pβAGβ.N, pβAGβ.B, and pβAGβ.X, respectively (see Fig. 1A). pβAGβ.B and pβAGβ.N were derived from pβAGβ.B0 and pβAGβ.N0, respectively, using restriction sites created by oligonucleotide (oligo)-directed, site-specific mutagenesis (see below).

Point mutations were introduced into the β-actin promoter by oligo-directed, site-specific mutagenesis as previously described (26) except that the template used was mp19βSA, which contains the β-actin promoter SacI-SphI restriction fragment from −472 to +102. Each oligo used generates point mutations resulting in one of the unique, new restriction sites of BamHI (−76), BglII (−56), EcoRV (−91), and NaeI (−114). Appropriately mutated mp19βSA derivatives were subcloned back into the pβAGβ.SA vector generating constructs pβAGβ.B0, pβAGβ.BG, pβAGβ.EV, and pβAGβ.N0, respectively. The mp19βSA derivative with the BamHI restriction site was subsequently used as the template to create point mutants containing additional EcoRV (pβAGβ.EB), BglII (pβAGβ.BB) and NaeI (pβAGβ.NB) restriction sites.

A series of chimeric β-actin promoter/adenovirus-2 major late promoter (Ad2MLP) fusions were constructed. SmaI-XhoI restriction fragments, containing the β-actin promoter from −246 to −52 from pβAGβ.SA, pβAGβ.BG, and pβAGβ.EV, were cloned into the pUC18 polylinker region upstream of the Ad2MLP and rabbit β-globin reporter gene in $pMG_1\beta$, which was derived from pMG_1 (23) by removal of the internal control β-globin gene. These constructs are designated $pMG_1\beta$.SX, $pMG_1\beta$.BG, and $pMG_1\beta$.EV, respectively.

Mutants with insertion or deletion of nucleotides between the CCAAT box and proximal CCArGG box were constructed (see Fig. 6A). Ten base pair (bp) insertions at the BamHI restriction site of pβAGβ.B0 and pβAGβ.EB were created by introduction of a 10bp BamHI-XmaIII adapter of sequence 5′-GATCCGGCCG-3′, generating plasmids pβAGβ.B10 and pβAGβ.EB10, respectively. Two 4bp insertion mutants were created by linearization of pβAGβ.B0 with BamHI (pβAGβ.B4) or XhoI (pβAGβ.X4). For each, the 4bp 5′ overhangs were filled in with T4 DNA polymerase (Pharmacia) followed by blunt end ligation. A 2bp insertion mutant, pβAGβ.B2, resulted from incomplete repair of BamHI digested pβAGβ.B0. A 4bp deletion mutant, pβAGβ.BL4, was obtained by removing the 4bp 5′ overhangs of BamHI linearized pβAGβ.B0 with S1 nuclease (Boehringer Mannheim) followed by blunt end ligation. A 10bp deletion mutant was obtained by site-specific mutagenesis of mp19βSA with an oligo spanning both the CCAAT box and proximal CCArGG box but with 10bps deleted in between them. The resultant construct, pβAGβ.BL10, lacks β-actin promoter sequences from −81 to −72. The β-actin promoter 5′ flanking sequences to ~ −114 of all the above constructs were confirmed by dideoxy sequencing of supercoiled plasmids (34).

The synthetic oligos described in this study were generated with an Applied Biosystems Model 280A DNA synthesizer and purified by polyacrylamide gel electrophoresis as described previously (35). All DNA manipulations were performed using standard protocols (36).

In Vivo Transfection Analysis

1×10^6 HeLa cells cultured in 100mm dishes in α-medium plus 10% fetal bovine serum (HyClone) were transfected by the calcium phosphate-DNA co-precipitation method (37) with 15μg of test plasmid, 1 to 3μg of the internal control plasmid, pRGβ (26), plus salmon sperm DNA to a total of 30μg DNA. Total cellular RNA was isolated, and primer extension analysis of 10 or 20μg of RNA was performed, as described previously (23). Specific primer extension products were quantified using a Molecular Dynamics 300A computing densitometer on autoradiograms of appropriate exposure.

Radiolabelled Probes

The wild-type β-actin CCAAT 39-mer double-stranded (ds) oligo, ^{32}P-labelled and utilized as probe, was described previously (23). The ^{32}-P-labelled β-actin CCAAT box probe in Figure 3 was prepared by subcloning the NaeI-BamHI fragment (containing β-actin promoter sequences from −114 to −76) from pβAGβ.NB into the SmaI-BamHI restriction sites of pUC18, followed by its excision with EcoRI and HindIII and subsequent 3′ end repair with the large fragment of DNA polymerase I (BRL/Gibco) in the presence of [α-^{32}P]-dATP. The ^{32}P-labelled wild-type murine MHC class II Eα Y-box probe was prepared by first subcloning the Eα 22-mer ds oligo (38) into the SmaI restriction site of pTZ18R, followed by excision with EcoRI and HindIII and subsequent 3′ end repair as described above. The ^{32}P-labelled β-actin wild-type and BglII point mutant proximal CCArGG box probes were prepared by subcloning the BamHI-NarI fragments (containing β-actin promoter sequences from −76 to −36) from pβAGβ.B0 and pβAGβ.BB into the BamHI-AccI restriction sites of pUC18, followed by their excision with BamHI and HindIII and subsequent 3′ end repair as described above.

Gel Mobility Shift Assays

In gel mobility shift (GMS) assays ^{32}P-labelled template DNA was mixed with HeLa nuclear extract, 1μg each of poly(dI:dC)-poly(dI:dC) and poly(dA:dT)-poly(dA:dT) (Pharmacia) and the indicated fold molar excess of unlabelled competitor DNA. The competitor DNA fragments used in this study are listed in Table 1. The volume was brought to 20μl in a final reaction mix containing 12mM Hepes (pH 7.9); 70mM NaCl or KCl; 10% glycerol; and 1.2mM DTT. Assays for NF-Y or p67^{SRF} also contained 5mM $MgCl_2$ or 5mM EDTA, respectively. After incubation at room temperature (RT) for 30 min, reactions were loaded directly onto a 4% polyacrylamide gel (30:1, acrylamide:bisacrylamide) that had been pre-electrophoresed for ~ 2 h at 160V at RT in 0.25×TBE buffer. Following electrophoresis for 2 h at 160V, the gel was dried and autoradiographed with Kodak XAR film and a Dupont Cronex intensifying screen at −70°C.

Nuclear Extracts

HeLa S-3 cells were purchased from the Massachusetts Institute of Technology Cell Culture Facility. HeLa cell nuclei, salt extracts and chloroquine intercalation extracts were prepared as

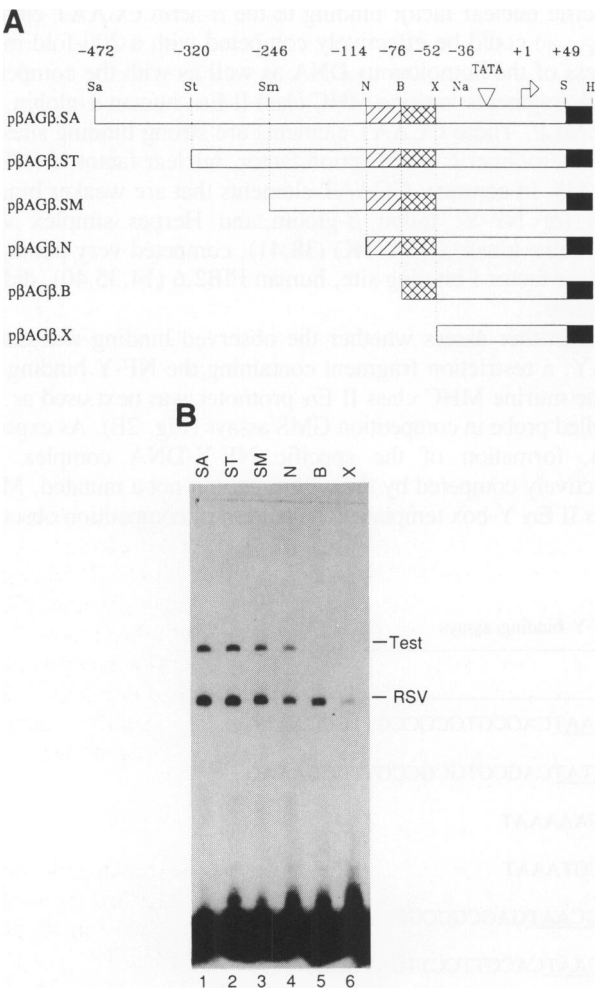

Figure 1. Transcriptional activity of human β-actin promoter 5′ deletion mutants in transfected HeLa cells. (A) Diagrammatic representation of β-actin promoter sequences inserted into the promoter-less expression vector pGβ, generating pβAGβ.SA and the 5′ deletion series. The diagonally lined and cross-hatched boxes represent regions of the promoter containing the CCAAT box and proximal CCArGG box, respectively. The filled box denotes pUC18 polylinker sequences. The positions of restriction sites in the β-actin promoter are indicated: Sa:SacI, St:StuI, Sm:SmaI, N:NaeI, B:BamHI, X:XhoI, Na:NarI, S:Sau3AI, H:HindIII. The inverted triangle and arrow indicate the positions of the TATA box (−29 to −23) and mRNA start site (+1), respectively. (B) Primer extension analysis of total cellular RNA isolated from HeLa cells transiently transfected with the constructs depicted in (A). Test and RSV indicate the positions of the 166 and 123 bases primer extension products, corresponding to the rabbit β-globin transcripts initiated from the β-actin promoter and RSV LTR (pRGβ), respectively. Lane 1:pβAGβ.SA, lane 2:pβAGβ.ST, lane 3:pβAGβ.SM, lane 4:pβAGβ.N, lane 5:pβAGβ.B, and lane 6:pβAGβ.X.

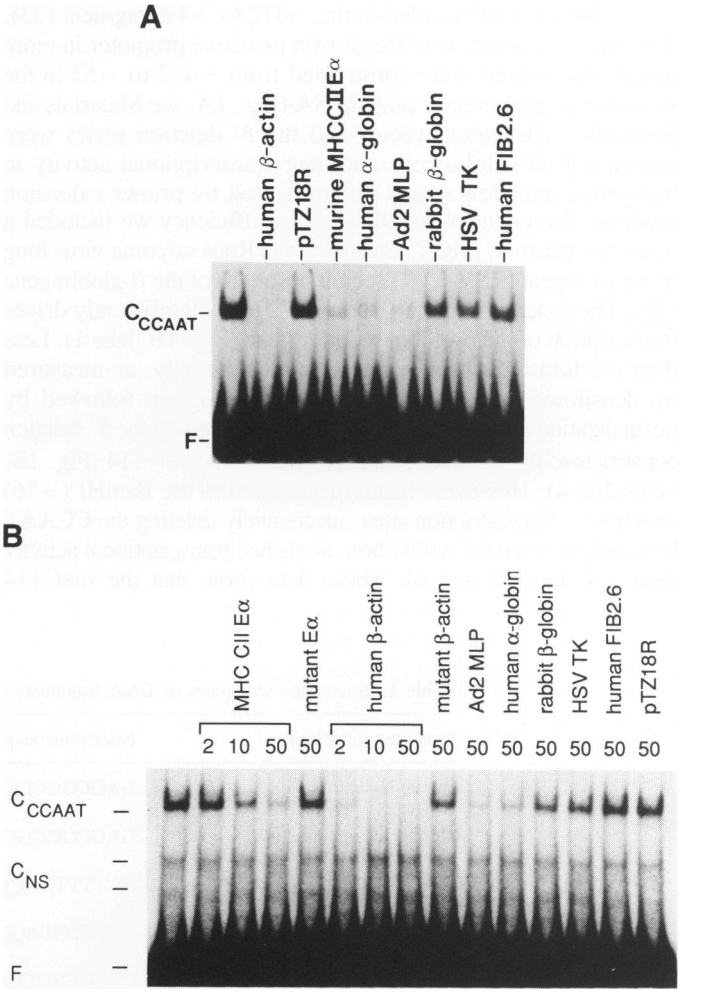

Figure 2. Specific interaction of a HeLa nuclear protein with CCAAT elements of the (A) human β-actin and (B) murine MHC class II Eα promoters. (A) Competition of specific nuclear factor binding to the human β-actin promoter CCAAT 39-mer template with CCAAT box containing DNA fragments. Radiolabelled β-actin CCAAT 39-mer probe was incubated with 2.5μg HeLa nuclear extract, non-specific DNA mix, and a 200-fold molar excess of unlabelled competitor DNA fragment, indicated at the top of each lane. No specific competitor DNA was included in the first lane. The nucleotide sequences of the competitor DNA fragments used are shown in Table 1. pTZ18R, the non-CCAAT box containing EcoRI-HindIII restriction fragment from pTZ18R, serves as a negative control. C_{CCAAT} and F indicate the positions of the specific NF-Y-DNA complex and unbound probe, respectively. (B) Competition of specific nuclear factor binding to the murine MHC class II Eα template with CCAAT box containing DNA fragments. Radiolabelled MHC class II Eα probe was incubated with 5μg HeLa nuclear extract, non-specific DNA mix, and a 2- to 50-fold molar excess of unlabelled competitor DNA fragment, indicated at the top of each lane. No specific competitor DNA was included in the first lane. Symbols are as described in (A). C_{NS} indicates the position of non-specific protein-DNA complexes.

previously described (39), except that the protease inhibitors, PMSF, leupeptin, antipain, and pepstatin A, were added to all buffers just prior to use (23). All other nuclear extracts described in this study were prepared by salt extraction of nuclei isolated from NP-40 lysed cells (40).

RESULTS

Efficient β-actin promoter activity requires the first 114 nucleotides upstream of the mRNA start site

Previously, we showed that in transfected HeLa cells greater than 70% of the activity of the human β-actin promoter from ~ −3300 to +909 resided in the −472 to +49 fragment (23). To examine this region of the β-actin proximal promoter in more detail, 5′ deletions were constructed from −472 to −52 in the *in vivo* expression vector pβAGβ.SA (Fig. 1A, see Materials and Methods). The parent vector and the 5′ deletion series were introduced into HeLa cells and their transcriptional activity in transient expression assays was measured by primer extension analysis. As a control for transfection efficiency we included a reference plasmid, pRGβ, containing the Rous sarcoma virus long terminal repeat (RSV LTR) region upstream of the β-globin gene (26). The β-actin promoter from −472 to +49 efficiently drives transcription of the β-globin reporter gene (Fig. 1B, lane 1). Less than a 2-fold reduction in transcriptional activity, as measured by densitometric scanning of the autoradiogram followed by normalization to the pRGβ control, was detected by the 5′ deletion constructs to the fabricated NaeI restriction site at −114 (Fig. 1B, lanes 2 to 4). However, further truncation to the BamHI (−76) or XhoI (−52) restriction sites, successively deleting the CCAAT box and proximal CCArGG box, abolished transcriptional activity (Fig. 1B, lanes 5 and 6). These data show that the first 114 nucleotides 5′ to the mRNA start site, spanning the CCAAT box and proximal CCArGG box, are sufficient for β-actin promoter activity *in vivo*.

The β-actin CCAAT binding factor is equivalent to NF-Y

Previously, we demonstrated that the CCAAT box was central to β-actin promoter activity both *in vivo* and *in vitro* and we identified a factor in HeLa nuclear extracts that can specifically interact with the β-actin CCAAT element (23). To determine if this β-actin CCAAT binding factor is related to any other previously described CCAAT binding proteins, we performed competition GMS assays using the wild-type β-actin CCAAT 39-mer ds oligo (23) as probe (Fig. 2A). The various CCAAT containing DNA fragments used here are shown in Table 1. Specific nuclear factor binding to the β-actin CCAAT element (C_{CCAAT}) could be effectively competed with a 200-fold molar excess of the homologous DNA as well as with the competitor DNA fragments: murine MHC class II Eα, human α-globin, and Ad2MLP. These CCAAT elements are strong binding sites for the heterodimeric transcription factor, nuclear factor Y (NF-Y) (38,41). In contrast, CCAAT elements that are weaker binding sites for NF-Y: rabbit β-globin and Herpes simplex virus thymidine kinase (HSV TK) (38,41), competed very poorly. A nuclear factor I binding site, human FIB2.6 (14,35,40), did not compete.

To further assess whether the observed binding was due to NF-Y, a restriction fragment containing the NF-Y binding site in the murine MHC class II Eα promoter was next used as ^{32}P-labelled probe in competition GMS assays (Fig. 2B). As expected (38), formation of the specific NF-Y-DNA complex was effectively competed by the wild-type, but not a mutated, MHC class II Eα Y-box template. The pattern of competition observed

Table 1. Nucleotide sequences of DNA fragments used in NF-Y binding assays

Promoter (position)	Nucleotide sequence
Human β-actin[1] (−104 to −66)	GAGCGGCGCGGGGCCAATCAGCGTGCGCCGTTCCGAAAG
Mutant human β-actin[1] (−104 to −66)	GAGCGGCGCGGGGGATATCAGCGTGCGCCGTTCGCAAAG
Murine MHC class II (CII) Eα[2] (−44 to −65)	ACTTTTAACCAATCAGAAAAAT
Mutant murine MHC CII Eα[2] (−44 to −65)	TCTTTGTAGCCAGAACGTAAAT
Human α-globin[2] (−85 to −56)	CGGGCTCCGCGCCAGCCAATGAGCGCCGCC
Adenovirus-2 major late[3] (−62 to −96)	CCTACACCTATAAACCAATCACCTTCCTTGATGCC
HSV thymidine kinase (TK)[2] (−76 to −98)	CGAATTCGCCAATGACAAGACGC
Rabbit β-globin[4] (−100 to −10)	CTGCAG...GGTGTTGGCCAATCTACACACGG...GGGCAG
Human FIB2.6[5] (unknown)	AGGTCTGGCTTTGGGCCAAGAGCCGC

Double-stranded oligos or restriction fragments were inserted into the SmaI restriction site in the polylinker region of pTZ18R, and isolated by acrylamide gel purification of the EcoRI-HindIII restriction fragment (except for HSV TK, which was isolated as a SacI-HindIII restriction fragment). The CCAAT box is underlined.

[1]Synthetic single-stranded oligos were made double-stranded as described (23) and were purified by polyacrylamide gel electrophoresis.

[2]Double-stranded oligos kindly provided by D. Mathis and C. Benoist (see ref. 38).

[3]The HaeIII restriction fragment of the Ad2MLP from pM97 (50).

[4]The PstI-PvuII restriction fragment of the rabbit β-globin gene from pMG_0 (23).

[5]Double-stranded nuclear factor I binding site oligo provided by J. Knox (35).

for the other DNA fragments was: Ad2MLP, human α-globin > rabbit β-globin, HSV TK > human FIB2.6. Interestingly, the wild-type human β-actin CCAAT 39-mer ds oligo competed significantly better on a molar basis than the homologous MHC class II Eα fragment; the mutant β-actin CCAAT ds oligo (EcoRV mutation, see Fig. 4A and ref. 23) did not compete. Therefore, the β-actin CCAAT element likely represents a very strong binding site for NF-Y.

A variety of biochemical analyses were performed on the β-actin CCAAT binding factor, based on previous characterization of NF-Y (42). Following treatment of HeLa cell nuclear salt or chloroquine extracts by a) heat, b) cation chelation, c) NaCl addition, and d) ortho-phenanthroline, standard GMS assays were performed using the β-actin CCAAT box containing fragment as radiolabelled probe. Specific complex formation is a) heat labile (5 min at 56°C), b) insensitive to EDTA (10mM), c) sensitive to NaCl (binding optimum at 50 to 100mM; and strongly reduced at 170mM) and d) sensitive to ortho-phenanthroline (5mM) (43). In addition, proteinase K treatment (20ng/μl) following DNA binding yields a protease resistant protein-DNA complex (43). The β-actin CCAAT binding factor appears to be ubiquitously expressed, being present in nuclei of a wide variety of human and rodent cell types (44). These properties of the β-actin CCAAT binding factor are identical to those of NF-Y (42).

Finally, to confirm the identity of the β-actin CCAAT binding factor as NF-Y, we performed GMS assays in the presence of anti-NF-Y antibodies (45). As shown in Fig. 3, when antibodies directed against either subunit of NF-Y, NF-YA (lane 1), or NF-YB (lane 2) were included in standard GMS assays with HeLa nuclear extract and radiolabelled β_{CCAAT} fragment, super-shifted complexes ($C_{CCAAT}SS$) were observed. No super-shift was seen upon the addition of anti-$p67^{SRF}$ antibody (46) (lane 3).

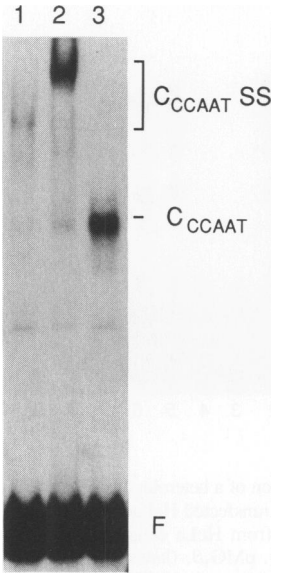

Figure 3. Specific interaction of anti-NF-Y antibodies with the HeLa nuclear protein-C_{CCAAT} DNA complex. Radiolabelled β-actin CCAAT box containing fragment was incubated with HeLa nuclear extract and 3μl of rabbit polyclonal antisera raised against peptides present in NF-YA (lane 1), NF-YB (lane 2), or $p67^{SRF}$ (lane 3). Symbols are as described in Figure 2. $C_{CCAAT}SS$ refers to the super-shifted complexes of the β-actin CCAAT box and NF-Y by antibody.

$p67^{SRF}$ interacts directly with the β-actin proximal CCArGG box

Our previous *in vitro* binding studies (23) indicated that three CCArGG box containing restriction fragments of the human β-actin promoter interacted with the transcription factor, serum response factor ($p67^{SRF}$). We noted that the proximal CCArGG box containing fragment, which also encompasses the NF-Y binding site, appeared to be a poor binding site for $p67^{SRF}$. Conversely, Kawamoto *et al.* (32,47) reported that a factor(s) binding to radiolabelled probes spanning the proximal or intron CCArGG boxes was competed poorly or not at all by the c-*fos* $p67^{SRF}$ binding site. To confirm that $p67^{SRF}$ specifically binds to the β-actin proximal CCArGG box, we performed GMS assays with an anti-$p67^{SRF}$ antibody and ^{32}P-labelled templates containing the wild-type proximal CCArGG box, but not the CCAAT box sequence (Fig. 4). As a control, we prepared a point mutated CCArGG box probe (BglII mutation, see Fig. 5A) with nucleotide substitutions in positions known to be important for $p67^{SRF}$ binding *in vitro* (26). Two specific complexes (CI and CII) were observed upon the addition of HeLa nuclear extract to the β-actin CCArGG box wild-type probe (Fig. 4A, lane 1) but not with the BglII mutant probe (Fig. 4A, lane 2). Both complexes are competed by a 50-fold molar excess of the homologous, but not the mutant, competitor (Fig. 4B, lanes 2 and 1, respectively). Each of the complexes, CI and CII, are

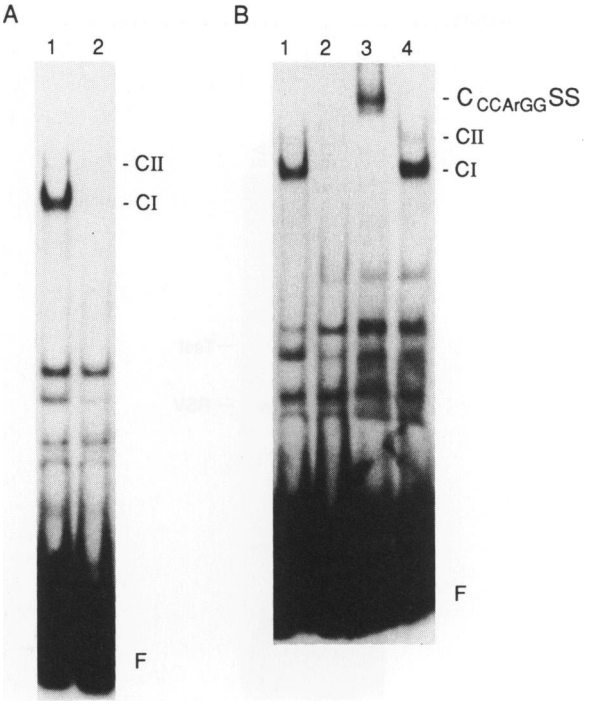

Figure 4. Specific interaction of serum response factor ($p67^{SRF}$) with the proximal CCArGG element of the human β-actin promoter. (A) Radiolabelled β-actin CCArGG box probes, wild-type (lane 1) and point mutated (lane 2) were incubated with HeLa nuclear extract. CI and CII are specific protein-DNA complexes observed. F refers to the free probe. (B) Radiolabelled wild-type β-actin CCArGG box probe was incubated with HeLa nuclear extract and either a 50-fold molar excess of unlabelled homologous (lane 2) or point mutated (lane 1) CCArGG box fragment or antibodies directed against $p67^{SRF}$ (lane 3) or NF-YB (lane 4). $C_{CCArGG}SS$ refers to the super-shifted complexes of the β-actin CCArGG box and $p67^{SRF}$ by antibody.

super-shifted by the addition of antibodies directed against p67^{SRF} but not against NF-YB (Fig. 4B, lanes 3 and 4, respectively). The above clearly demonstrates that p67^{SRF} interacts directly with the β-actin CCArGG box. Moreover, in GMS assays using reticulocyte lysate produced recombinant p67^{SRF} (48) a specific p67^{SRF}-DNA complex corresponding to CI (see Fig. 4) was observed on the wild type but not point mutated β-actin proximal CCArGG box template (49). The two complexes observed here are reminiscent of the p67^{SRF} containing complexes identified with the c-*fos* SRE (29,30).

The CCAAT box and proximal CCArGG box are required together for trans-activation

To investigate the role of NF-Y and p67^{SRF} in regulating human β-actin promoter activity a series of point mutations were created in pβAGβ.SA (Fig. 5A, and see Materials and Methods). pβAGβ.B0, containing point mutations between the CCAAT box and proximal CCArGG box, expressed similar transcriptional activity to pβAGβ.SA in HeLa cell transient expression assays (Fig. 5B, compare lanes 1 and 2). However, point mutations in either the CCArGG box (pβAGβ.BG) or the CCAAT box (pβAGβ.EV), resulting in loss of specific p67^{SRF} (Fig. 4) and NF-Y (Fig. 2) binding *in vitro*, respectively, severely reduced transcriptional activity (Fig. 5B, lanes 3 and 4). The above results confirm previous work (23,32) showing that the CCAAT and proximal CCArGG boxes are essential components of the β-actin proximal promoter and strongly suggest that NF-Y and p67^{SRF} regulate β-actin transcription *in vivo*.

To determine if NF-Y and p67^{SRF} can trans-activate independently of each other, but in cooperation with a heterologous promoter, the β-actin promoter from −246 to −52, wild-type and point mutated, was inserted into the chimeric promoter *in vivo* expression vector, pMG$_1$β (23). This vector contains Ad2MLP sequences from −86 to +33, driving expression of the β-globin reporter gene. It should be noted that the Ad2MLP in this vector contains the TATA element and binding sites for the transcription factors USF/MLTF (50) and NF-Y/CP1 (38,41), but lacks an enhancer. In transient expression assays in HeLa cells the Ad2MLP activity (Fig. 6, lanes 3 and 4) is stimulated by the SV40 enhancer (lanes 1 and 2) and by the β-actin promoter fragment (lanes 5 and 6). This enhancement of transcription by the β-actin promoter fragment was abolished by mutation of either the CCAAT box (lanes 7 and 8) or CCArGG box (lanes 9 and 10). Thus, the same pattern of transcriptional activity is observed for the series of chimeric β-actin promoter/Ad2MLP constructs (Fig. 6) as for the native β-actin promoter constructs (Fig. 5). This result implies that the CCAAT and proximal CCArGG boxes are unable to trans-activate individually and suggests that the two cis-acting elements function together as a unit to stimulate β-actin promoter activity *in vivo*.

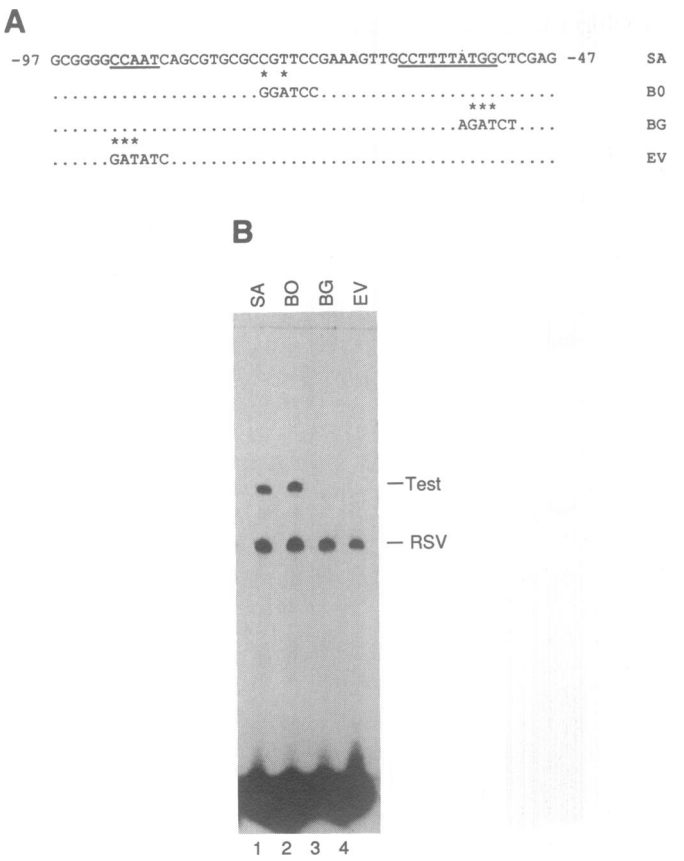

Figure 5. Transcriptional activity of human β-actin promoter point mutants in transfected HeLa cells. (A) Nucleotide sequences of the wild-type and point mutant β-actin proximal promoter regions. The top line shows the wild-type sequence present in pβAGβ.SA (SA) from −97 to -47, encompassing the CCAAT box and proximal CCArGG box (both underlined). Nucleotides altered by oligo-directed, site-specific mutagenesis generating point mutants pβAGβ.B0 (B0), pβAGβ.BG (BG), and pβAGβ.EV (EV) are indicated by asterisks. (B) Primer extension analysis of total cellular RNA isolated from HeLa cells transiently transfected with the constructs depicted in (A). Test and RSV are as described in Fig. 1.

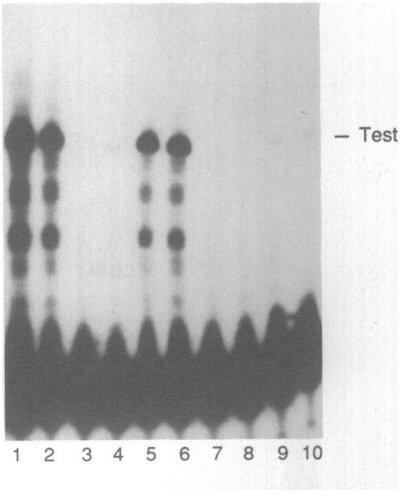

Figure 6. Trans-activation of a heterologous promoter by human β-actin proximal promoter fragments in transfected HeLa cells. Primer extension analysis of total cellular RNA isolated from HeLa cells transiently transfected with constructs pMG$_0$β (lanes 1 and 2), pMG$_1$β, (lanes 3 and 4), pMG$_1$β.SX (lanes 5 and 6), pMG$_1$β.EV (lanes 7 and 8), and pMG$_1$β.BG (lanes 9 and 10). pMG$_0$β, derived from pMG$_0$ (23) by removal of the internal control β-globin gene, contains the SV40 enhancer upstream of the Ad2MLP. The other constructs are described in Materials and Methods. Test indicates the position of the 125 bases primer extension product, which corresponds to the rabbit β-globin transcripts initiated from the Ad2MLP. The primer extension product generated from the heterologous promoter constructs is similar in size to that generated from the RSV LTR thereby precluding the use of pRGβ as an internal control. Three separate transfection experiments yielded the same pattern of transcriptional activities.

A particular spatial alignment between the CCAAT box and proximal CCArGG box is required for trans-activation

A prediction of the hypothesis that the CCAAT and proximal CCArGG box elements of the β-actin proximal promoter function together as a unit is that proper spatial alignment between the two cis-acting elements is required for trans-activation. Therefore, mutants containing insertions or deletions at the BamHI restriction site between the two elements (in pβAGβ.B0) were created (Fig. 7A). Transient expression assays in HeLa cells revealed that insertion of 2 (pβAGβ.B2) or 4bps (pβAGβ.B4) reduced or abolished transcriptional activity, whereas insertion of 10bps (pβAGβ.B10) restored transcriptional activity near to the level of pβAGβ.B0 and its parent, pβAGβ.SA (Fig. 7B, compare lanes 3, 4 and 6 with lanes 2 and 1). Interestingly, insertion of 4bps between the TATA box and proximal CCArGG box (pβAGβ.X4) did not significantly alter transcriptional activity from this promoter (Fig. 7B, lane 5). To control for the possibility of inserting a new cis-acting element with the XmaIII adapter in pβAGβ.B10, this same insertion was introduced in the CCAAT box mutant pβAGβ.EV, generating pβAGβ.EB10. This construct was incapable of stimulating transcription (Fig. 7B, lane 7). Deletion of 4 (pβAGβ.BL4) or 10bps (pβAGβ.BL10) between the CCAAT and proximal CCArGG boxes (Fig. 7B, lanes 8 and 9, respectively) showed a periodicity in transcriptional activity similar to that observed for the insertion mutants. The above results demonstrate that proper alignment of the CCAAT and proximal CCArGG boxes, but not between these upstream elements and the TATA box, is critical for β-actin promoter activity *in vivo*. No significant differences have been observed in the *in vitro* binding activities of either NF-Y or p67^{SRF} to radiolabelled SmaI-NarI restriction fragments (containing the β-actin promoter from −246 to −36) from any of the insertion or deletion mutants (49).

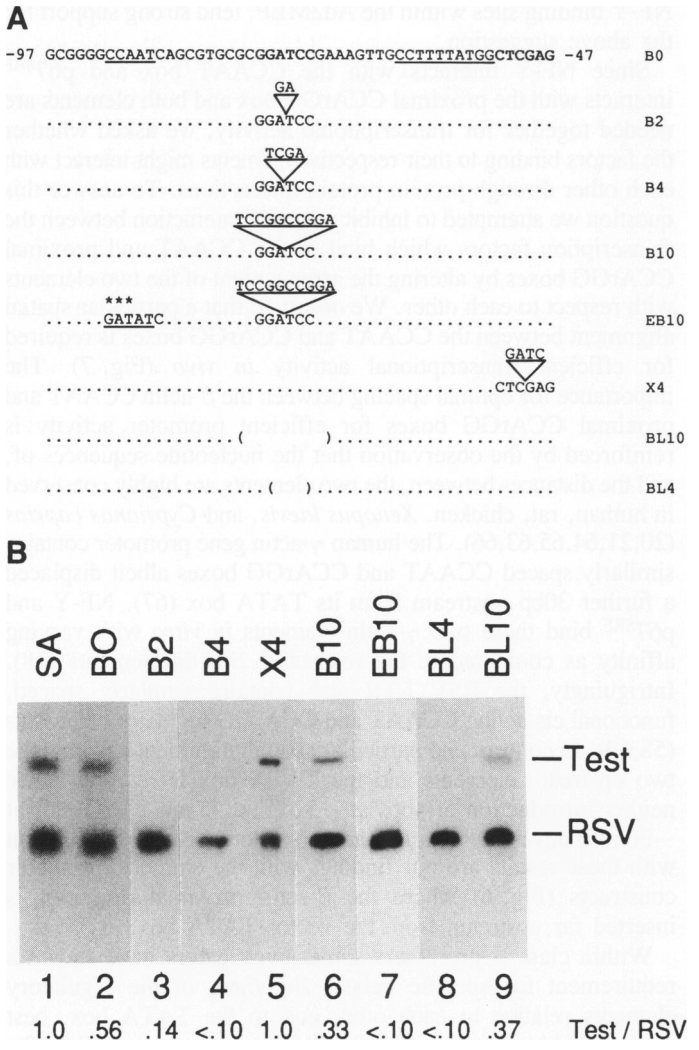

Figure 7. Spatial alignment requirements for transcriptional activity of the β-actin proximal promoter in transfected HeLa cells. (A) Nucleotide sequences of the insertion and deletion mutant β-actin proximal promoter regions. The top line shows the sequence of the BamHI point mutant pβAGβ.B0 (B0) from −97 to −47, encompassing the CCAAT box and proximal CCArGG box (both underlined). Insertions or deletions at the BamHI and XhoI restriction sites were constructed as described in Materials and Methods. Nucleotide additions are indicated above the point of insertion, while deletions are denoted by blank regions bordered by brackets. The resultant constructs are pβAGβ.B2 (B2), pβAGβ.B4 (B4), pβAGβ.B10 (B10), pβAGβ.EB10 (EB10), pβAGβ.X4 (X4), pβAGβ.BL10 (BL10), and pβAGβ.BL4 (BL4). The asterisks above three nucleotides in EB10 indicate the same CCAAT box point mutation described in Fig. 4. (B) Primer extension analysis of total cellular RNA isolated from HeLa cells transiently transfected with the constructs depicted in (A). Test and RSV are as described in Fig. 1. Test/RSV: the average ratio of primer extension products generated from the test constructs to the internal control plasmid pRGβ (normalized to 1.0 for pβAGβ.SA) as determined by densitometric scanning of autoradiograms from at least three separate transfection experiments.

DISCUSSION

The human β-actin promoter displays strong transcriptional activity in transiently transfected HeLa cells (16,23,32,33,51). A substantial amount of this activity resides within the proximal promoter region which consists of three cis-acting regulatory elements, the CCAAT (−91 to −87), CCArGG (−62 to −53), and TATA (−29 to −23) box consensus sequences. This report describes the detailed characterization of the CCAAT and proximal CCArGG boxes, and the trans-acting factors that interact with them.

Based on experiments described herein, we conclude that the β-actin CCAAT binding factor is the heterodimeric transcription factor NF-Y first reported as the protein that binds to the Y-box of murine MHC class II genes (38). Recently, the cDNA cloning of the two subunits of murine NF-Y has been published (45). Several criteria, including its DNA binding and chromatographic properties, size, and subunit composition, indicate that NF-Y is certainly the same factor as CP1 and CBF, originally identified by their ability to bind to the CCAAT elements of the Ad2ML and α2(I) collagen promoters, respectively (41,52). Other promoters that likely contain binding sites for NF-Y include: rat albumin; RSV and murine sarcoma virus LTR; human and HSV TK; α-, β- and γ-globin; MHC class I H-2^{x}; human hsp70; and sea urchin sperm histone H2B (38,41,45,53,54,55,56,57,58,59). Thus, NF-Y appears to be involved in regulating transcription of a wide variety of genes, including those that are constitutively expressed, cell cycle dependent, developmentally regulated and tissue-specific. It is not surprising, therefore, that NF-Y binding activity is detected in nuclear extracts from every mammalian cell type we have examined (44).

Previously, we showed that restriction fragments spanning each of the β-actin CCArGG boxes interact with a HeLa nuclear factor corresponding to p67^{SRF}, as judged by GMS assays using the wild-type and non-p67^{SRF} binding c-*fos* dyad symmetry element (DSE) as competitors (23). In contrast, others have reported little

or no competition by the c-*fos* DSE for a HeLa factor(s) binding to probes spanning the β-actin proximal and intron CCArGG boxes (32,47). GMS assays utilizing anti-p67^{SRF} antibodies (Fig. 4B) or *in vitro* translated recombinant p67^{SRF} (49) establish that p67^{SRF} interacts directly with the proximal CCArGG box. Recent studies (29,30) have shown that another protein present in HeLa nuclei, p62^{TCF}, binds to the c-*fos* promoter CCArGG box region through interaction with p67^{SRF}. Thus, two p67^{SRF}-DNA complexes are observed in GMS assays, containing either p67^{SRF} alone (CI) or together with p62^{TCF} (CII). Through comparison of the protein-DNA complexes observed in GMS assays using crude HeLa nuclear extract- or reticulocyte lysate-derived p67^{SRF}, it is very likely that p62^{TCF} also interacts with the β-actin proximal CCArGG element *in vitro*. Thus, the β-actin proximal CCArGG element may function similarly to the c-*fos* DSE, which mediates multi-faceted regulation of c-*fos* transcription through complex interactions with p67^{SRF}, p62^{TCF}, p62^{DBF}, FOS, and possibly ETS (26,27,28,29,30,43,60,61,62).

We and others have shown that β-actin promoter activity *in vivo* is retained, albeit reduced, in constructs containing the CCAAT and proximal CCArGG boxes but lacking the distal and intron CCArGG boxes (23,32,33). Our 5′ deletion and point mutational analyses (Figs. 1 and 5) confirm the work of Kawamoto *et al.* (32), who introduced linker-scanner mutations into the CCAAT and proximal CCArGG boxes and found loss in promoter function. We extended this observation by showing that point mutation of the CCAAT and proximal CCArGG boxes completely block the ability of NF-Y and p67^{SRF} to bind these elements *in vitro* (Figs. 2 and 4). Taken together with previous demonstrations by us (23) and others (31) that deletion of the CCAAT box results in nearly complete loss of promoter function both *in vivo* and *in vitro*, we conclude that NF-Y plays a central role in regulating β-actin transcription.

Since the β-actin promoter contains two other CCArGG boxes that are strong binding sites for p67^{SRF} and exhibit transcription stimulatory activity (23), the contribution of the interaction of p67^{SRF} with the proximal CCArGG element to overall transcription of the β-actin gene is uncertain. Our results (Fig. 5) indicate that the proximal CCArGG element is essential for β-actin promoter activity in the absence of the two other CCArGG elements. Recent studies have indicated that DNA fragments spanning the intron CCArGG box contain enhancer-like activity (23,47) and confer inducibility to either serum or fibroblast growth factor (FGF) (33,47). In contrast, the proximal CCArGG box appears to mediate induction of transcription by serum following 10 hours exposure, but not by FGF (33), suggesting that the mechanisms by which the proximal CCArGG element confers responsiveness to external stimuli is different from that for the intron CCArGG element. Inducible transcriptional activity through the distal CCArGG box has not been reported. We propose that the functional differences between the CCArGG elements arise through formation of multi-protein complexes, comprised of the CCArGG box-bound p67^{SRF} and various combinations of p67^{SRF}-interactive proteins, in a fashion similar to that described for the c-*fos* DSE. Further work will involve the identification of nuclear proteins that interact with the distal and intron CCArGG elements *in vitro* and examination of constitutive and growth factor inducible transcription *in vivo* using the pβAGβ.SA and pβAGβ.BG vectors with inserted distal and intron CCArGG elements.

The inability to detect any transcriptional activity *in vivo* by β-actin promoters with CCAAT or proximal CCArGG box mutations suggests that these elements function together as a unit. Alternatively, β-actin transcription might simply require more than one functional cis-acting element upstream of the TATA box. However, the c-*fos* DSE has been shown to be sufficient for trans-activation from several truncated promoters containing only the TATA element (28,48,63). Results obtained here (Fig. 6), using chimeric promoter constructs containing the wild-type or point mutated human β-actin promoter CCAAT and proximal CCArGG boxes inserted upstream of the MLTF and NF-Y binding sites within the Ad2MLP, lend strong support for the above suggestion.

Since NF-Y interacts with the CCAAT box and p67^{SRF} interacts with the proximal CCArGG box and both elements are needed together for transcriptional activity, we asked whether the factors binding to their respective elements might interact with each other through protein-protein interactions. To answer this question we attempted to inhibit potential interaction between the transcription factors which bind to the CCAAT and proximal CCArGG boxes by altering the arrangement of the two elements with respect to each other. We observed that a particular spatial alignment between the CCAAT and CCArGG boxes is required for efficient transcriptional activity *in vivo* (Fig. 7). The importance for optimal spacing between the β-actin CCAAT and proximal CCArGG boxes for efficient promoter activity is reinforced by the observation that the nucleotide sequences of, and the distances between, the two elements are highly conserved in human, rat, chicken, *Xenopus laevis*, and *Cyprianus caprios* (20,21,64,65,63,66). The human γ-actin gene promoter contains similarly spaced CCAAT and CCArGG boxes albeit displaced a further 30bp upstream from its TATA box (67). NF-Y and p67^{SRF} bind these two γ-actin elements *in vitro* with varying affinity as compared to the respective β-actin elements (68). Intriguingly, the RSV LTR also contains similarly spaced, functional cis-acting CCAAT and CCArGG regulatory elements (58,69). In contrast, no particular spatial alignment between the two upstream elements and the TATA box is required since neither introduction of 4bps at −52 (Fig. 7) nor 2 or 10bps at −36 (49) adversely affects β-actin promoter activity. Consistent with these results are our findings with the chimeric promoter constructs (Fig. 6) where the β-actin proximal promoter is inserted far upstream from the vector TATA box.

Within class II promoters some investigators have shown a requirement for specific helical alignment of the regulatory elements relative to each other and to the TATA box, best exemplified by the studies on the SV40 early promoter (8,9,10). It can be envisioned that this requirement arises through a physical impediment of interaction between transcription factors bound at cis-acting regulatory elements and, in certain cases, with general transcription factors present in the pre-initation or initiation complex. However, insertion of variable lengths of nucleotides between certain upstream elements and TATA boxes do not necessarily vary transcription in a periodic manner (11,12,13,14). Although these studies appear to contradict the model described above, they suggest that periodicity in activity or spatial alignment may be more complicated than originally thought and may perhaps be dependent on the nature of the cis-acting elements and their respective trans-acting factors. For example, a recent study of the α-actin promoter found that half-helical insertions between cis-acting elements increased

transcriptional activity, leading to the suggestion that activity is achieved in the wild-type promoter through protein-induced torsional deformation (70). Interestingly, the β-actin proximal CCArGG box is near a site of intrinsic DNA bending (32) and it has been shown that protein-induced bending occurs through binding of $p67^{SRF}$ to DNA (71). The above data, taken together with our own observations, indicate that the β-actin promoter will provide a very useful model to investigate the intricacies of protein-protein and protein-DNA interactions involved in trans-activation in mammalian cells.

Further substantiation of the notion that NF-Y bound at the CCAAT box and $p67^{SRF}$ bound at the proximal CCArGG box regulate transcription through protein-protein interactions will require visualization of such a ternary complex. Such a multicomponent protein-DNA complex has not been detected by *in vitro* binding assays using probes spanning the β-actin proximal promoter region. However, GMS assays comparing the binding activity of $p67^{SRF}$ to the β- and γ-actin proximal CCArGG boxes and the c-*fos* DSE demonstrate that the γ-actin proximal CCArGG box represents a very strong $p67^{SRF}$ binding site; a higher order protein-DNA complex has been detected using a chimeric template consisting of the β-actin CCAAT box and γ-actin proximal CCArGG box (68). Further studies will involve the elucidation, at a molecular level, of the protein-protein interactions that occur between proteins bound at the CCAAT and proximal CCArGG boxes.

ACKNOWLEDGEMENTS

We thank R.M. Gronostajski and S. Leung for critical reading of the manuscript, N. Chung for technical assistance, J. Knox for nuclear extracts and the FIB2.6 oligo, R. Treisman for pT7ΔATG and pT7Δ926, D. Mathis and C. Benoist for MHC class II Eα, human α-globin, HSV TK oligos, and anti-NF-Y antibodies, and R. Prywes for anti-$p67^{SRF}$ antibodies. This work was supported by a grant to N.G.M. from the National Cancer Institute of Canada (NCIC). N.G.M. is a Research Scientist of the NCIC. R.M.F. and C.Y.T. hold Medical Research Council of Canada Studentships.

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