**The β Actin Promoter**

**HIGH LEVELS OF TRANSCRIPTION DEPEND UPON A CCAAT BINDING FACTOR**

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Although β actin mRNA is down-regulated during myogenesis, the β actin promoter confers constitutive expression when joined to heterologous genes trans-}

ferred into a variety of different cell backgrounds, including differentiated muscle. Normal promoter activity is dependent upon the binding of a ubiquitous factor to the CCAAT-box element. Loss or reduction in factor binding correlates with a major reduction in promoter activity both in vitro and in vitro. The binding domain covers approximately 23 base pairs as determined by DNase footprinting. Methylation of A and G residues in and adjacent to the CCAAT box results in the loss of factor binding. Mutations across the binding domain indicate that the sequence GCCAATCAG within the domain is sufficient as a recognition sequence for factor binding. This binding is not competed by the α cardiac actin CCAAT sequence. Bandshift experiments demonstrate a predominant single band of similar mobility in nuclear extracts from various cells and tissues, with the exception of HeLa cells. The prevalence of the factor and its recognition sequence in a variety of promoters suggests that this factor has a common role in the transcriptional activation of several eukaryotic promoters.

Actin is an abundant cytoskeletal protein in eukaryotic cells and at least six major isoforms have been identified in vertebrates (Vandekerckhove and Weber, 1978). These isoforms can be subdivided into muscle and nonmuscle types. The actin isoforms vary little in amino acid sequence but are differentially regulated during development (DePonti-Zilli et al., 1988; Hayward and Schwartz, 1986; Paterson and Eldridge, 1984; Vandekerckhove and Weber, 1979). β actin is the major nonmuscle or cytoplasmic actin isoform and it is expressed in most eukaryotic nonmuscle cells, as well as in undifferentiated myoblasts. During myogenesis, the β actin isoform is down-regulated and is eventually replaced by the muscle-specific c isoforms (Hayward and Schwartz, 1986; Paterson and Eldridge, 1984). The up-regulation of the muscle-specific c actins appears to be largely mediated by cis-acting elements located in the promoter region (Carroll et al., 1988; Minty and Kedes, 1986; Miwa et al., 1987). The element responsible for the down-regulation of β actin transcription is localized in the 3'-untranslated domain of the gene (DePonti-Zilli et al., 1988). The β actin promoter in the absence of this 3'-sequence element is constitutive in all cell types examined to date, including muscle (Seiler-Tuyns et al., 1984).

Thus, the factors responsible for β actin expression are present in the muscle background even after down-regulation of the endogenous β actin gene has taken place. Here we have analyzed the chicken β actin promoter region and identified a 5'-cis sequence element that forms a DNA-protein complex which is essential for full promoter activity. The binding activity is widely distributed in a variety of cells and tissues and may play a role in the transcriptional activation of numerous promoters containing the factor recognition sequence.

**MATERIALS AND METHODS**

**Cell Cultures and Transfection**—Culture conditions for chicken primary myoblasts and skin fibroblasts were as described elsewhere (Paterson and Stromman, 1972). Briefly, tissues were removed from 12-day-old chicken embryos, trypsinized, and plated on collagen-coated 25-cm² flasks in Dulbecco’s modified Eagle’s medium containing 2% chicken embryo extract, 10% horse serum, and supplemented with glutamine and gentamycin. Twelve to 18 h after plating, the cells were transfected by the calcium phosphate-DNA coprecipitation method as described by Gorman (1982). Cultures were cotransfected with the chloramphenicol acetyltransferase (CAT) and the luciferase genes. The CAT gene served as the reporter gene and was activated by the various promoter constructs. The luciferase gene, driven by an enhancerless SV-40 promoter, was introduced as an internal standard for normalization purposes (De Wet et al., 1987). Each flask was exposed to a total of 20 µg of plasmid and the ratio of luciferase to CAT plasmid in the transfection mixture was 1:10. As a source for mouse nuclear extracts the myogenic cell line C2C12 was grown as previously described (Seiler-Tuyns et al., 1984). HeLa and L cells served as sources of nuclear extract from cell lines of human and mouse origin, respectively.

**Plasmid**—The structure of the construct carrying the luciferase gene has been described in detail elsewhere (De Wet et al., 1987). The β actin promoter region was excised from the gene as a HinfI fragment 339 base pairs long. This fragment was modified with Sau3A linkers and cloned into p8 CAT, a derivative of the pEMBL plasmid (Dente et al., 1985). The β actin promoter deletions were obtained by cutting with XhoI at position -272 and subsequently digesting with nucleases Bal31. The digests were flush ended with Klenow, and ligated with BamHI linkers.

**Expression Assays**—CAT assays and luciferase assays were performed as described elsewhere (Billette et al., 1988; De Wet et al., 1987). For the CAT and luciferase assays 0.1–1% and 5%, respectively, of the cell extract from a 25-cm² plate was used for each assay. Variations due to handling and processing were minimized.

**In vitro transcription** was performed as described by Cao et al. (1987) with mouse L cell nuclear extracts prepared as described by Heberlein and Tjian (1988). Briefly, assays were performed in 20–30 µl with 200 ng to 1 µg of supercoiled template. Transcripts were analyzed by primer extension assay using MMLV reverse transcriptase and a chloromphenicol acetyltransferase primer for position 4921–4944 in PSV2-CAT (Gorman, 1985) as described (Cao et al., 1987).

**Bandshift Assays**—Kinased oligonucleotide (Maniatis et al., 1982) or restriction fragments (10,000–40,000 cpm/reaction) were incubated for 10 min at room temperature, with 10 µg of protein from nuclear
extracts in binding buffer (75 mM NaCl, 0.1 mM EGTA, 15 mM Tris, pH 7.5, 0.5 mM DTT, 5% glycerol) supplemented with 1 μg of poly(dI-dC), 10 μg of RNA in a total volume of 20–30 μl. The incubation mixture was electrophoresed in 1% agarose, 0.5 X TBE (Mariat et al., 1982), at 30 mAmp for 2.5 h. Gels were dried and exposed for 4–12 h at −70 °C.

DNase Footprinting and Methylation Interference—DNase footprinting (Galas and Schmitz, 1978) was carried out on a fragment derived from DEL2, which is 220 base pairs long. This fragment was incubated as described above and subsequently 10 units of DNase I was added for 2 min. The reaction was stopped by the addition of EDTA to a final concentration of 20 mM. After electrophoresis in 1% agarose, 0.5 X TBE, the content of the gel was transferred to DEAE paper. The bound and the free fragments were eluted in 1.5 M NaCl overnight, extracted with phenol/chloroform and chloroform, and precipitated with ethanol. The DNase I-digested bound and free fragments were electrophoresed in an 8% sequencing gel.

Methylation interference was assayed by partially methylation the fragment (Maxam and Gilbert, 1981) with dimethyl sulfate prior to bandshift electrophoresis. After isolating bound and free fragments, as described above for DNase footprinting, the methylated fragments were cleaved with piperdine and electrophoresed in an 8% sequencing gel.

Oligonucleotides and Fragments—Oligonucleotides were synthesized by M. Brownstein (Laboratory of Cell Biology, National Institutes of Mental Health, Bethesda, MD) on an Applied Biosystems DNA synthesizer. They were deblocked and gel-purified prior to labeling and hybridization. After hybridization the double-stranded oligonucleotide was purified on nondenaturing 10–15% acrylamide gels run in 1.5 X TBE.

The following double-stranded oligonucleotides were used as competitors or probes:

1) 50-mer, β actin (-106 to -56) included CCAAT box: 5’-CGGGGACGAGCCGGCGGCGGCCTTAGCAGTCATGCTTCTTTTGATGCGG

2) 39-mer, β actin (3’-untranscribed regulatory region, DePonti-Zilli et al., 1988): 5’-GGCTACCTGCTGATCACTGGAATAAGACGATTCT AACAT AAA

3) 50-mer, α cardiac actin with CArG2 element, -157 to -97 (Eldridge et al., 1982), at 30 mAmp for 2.5 h. Gels were dried and exposed for 2 min. The reaction was stopped by the addition of EDTA to a final concentration of 0.4 M NaCl. Stirring was continued for 20 min after which the suspension was centrifuged at 100,000 X g for 60 min in a swinging bucket rotor. The nuclear pellet was recovered and suspended in solution I. The suspension was mixed with a magnetic stirrer while the CCAAT box was added for 60 min. The reaction was stopped by the addition of EDTA to a final concentration of 20 mM. After electrophoresis in 1% agarose, 0.5 X TBE, the content of the gel was transferred to DEAE paper. The bound and the free fragments were eluted in 1.5 M NaCl overnight, extracted with phenol/chloroform and chloroform, and precipitated with ethanol. The DNase I-digested bound and free fragments were electrophoresed in an 8% sequencing gel.

Restriction fragments from the respective β actin deletions were obtained by partial digestion with the restriction enzymes BamHI and SalI. The promoter inserts were isolated by gel electrophoresis in 1% agarose and 0.5 X TBE.

The following restriction fragments were used as competitors as:

1) α cardiac actin (−100 to +15) with CCAAT-like and TATA elements, Eldridge et al., 1985): 5’-CGCGACGCGCGAACATAGGAGGAGGGTGCCTGCGCCGCGGACACGGGCGGGGCTGATATAAGGGCACGTTCTGGCAGGCGGGCCAGCGGCGCTCCTCGGT

2) αβ hybrid (the portion of the construct which is derived from chicken α cardiac actin position −100 to −60 (Eldridge et al., 1985) is bracketed, and the remaining part is derived from chicken β actin), position −53 to +1: 5’-CGCGACGCGCGAACATAGGAGGAGGGTGCCTGCGCCGCGGACACGGGCGGGGCTGATATAAGGGCACGTTCTGGCAGGCGGGCCAGCGGCGCTCCTCGGT

A fragment containing the β actin region upstream of the CCAAT box extended from position −95 to position −339 (see Fig. 1).

**RESULTS**

**The Structure of the β Actin Promoter Deletions**—The β actin promoter (Kost et al., 1983) contains a CCAAT box at position −89, a TATA box at position −89, and numerous spl-like sequences (Dyman and Tjian, 1985), all of which have been suggested to play a role in promoter activity (Fig. 1). In addition, a sequence element which is reminiscent of a second CCAAT box can be found in the upstream region, at position

- CTCGAG

**Fig. 1. The sequence of the β actin promoter region.** Indicated in the sequence are relevant restriction sites and consensus sequences, which may act as binding sites for factors, which regulate promoter activity. Positions of deletions and important structural elements are expressed in relation to the start of transcription, which is indicated as [+1]. WT stands for the original promoter construct which gains the two HinfI sites. The deletions are named DEL1-DEL8 in order of decreasing size. The core consensus sequences for the spl binding site and the CARe box have been described in detail elsewhere (Dyman and Tjian, 1985; Minty and Kedes, 1986).
−220. Another putative regulatory element, termed the CArG box (Minty and Kedes, 1986), has been implicated in the function of the β actin promoters and is located between the CCAAT and TATA boxes at position −60. Deletions of the β actin promoter were constructed to estimate the contribution of these various sequence elements to the activity of the promoter and are displayed in Fig. 1.

Deleting the CCAAT Box at Position −89 Impairs Promoter Activity in Vivo and in Vitro—Promoter activity was determined by transfecting primary cultures with the various constructs shown in Fig. 1. The deletions were numbered DEL1−DEL8 in order of decreasing size. Wild type (WT) designates the original promoter construct which is flanked by the two HinfI sites (Fig. 1). All CAT constructs were cotransfected with a plasmid containing the luciferase gene driven by an enhancerless SV-40 promoter element. Extracts were adjusted to identical luciferase activities in order to normalize for variations in transfection and extraction efficiency. Two cell backgrounds were selected as targets for the transient transfection assays: embryonic chick myoblasts, which were allowed to fuse, and embryonic skin fibroblasts. The cultures were harvested at 72 h after transfection because at this time myoblast fusion is essentially complete and fibroblast cultures are confluent.

When normalized to luciferase activity, CAT activity was slightly higher in fused myotubes than in skin fibroblasts. The difference in expression of normalized CAT activity in the two cell backgrounds did not exceed 25%. A comparison of the different deletions within each cell background revealed that CAT activity remained relatively constant at wild type levels up through DEL4 (Fig. 2). DEL4 is 106 base pairs long and extends 15 base pairs upstream of the CCAAT box at position −89. Removing this CCAAT box caused a drop in activity to about 30% of the wild type value (DEL5, Fig. 2). Further deletions 3′ to this CCAAT box resulted in additional but less pronounced decreases in CAT activity (DEL6 and DEL7). After eliminating the TATA element (DEL8), promoter activity declined to background levels. Thus, the region in or adjacent to the CCAAT element is important for full promoter activity. In contrast, sequence elements upstream of position −106 do not appear crucial and may be deleted without significant loss of promoter function. Consequently, the numerous putative sp1 core binding sites, as well as the second CCAAT-like element at position −220 in the upstream region, have little, if any, influence on promoter activity in vivo. The role of the CARG element at position −60 is unclear since practically all of the promoter activity is lost when sequences 5′ to −60 are deleted.

In vitro transcription of the various deletions in L cell nuclear extracts (Fig. 3) resulted in a sharp decline in promoter activity in constructs carrying deletions of the CCAAT element at position −89 (DEL5 and DEL6). The promoter continued to function at a very low level until the TATA element was removed (DEL8). Without the TATA element, the promoter was inactive. In contrast to the in vivo situation, however, the promoter appeared to be more dependent on sequence elements upstream of the CCAAT box at position −89 for full activity in vitro. Promoter activity equal to or above wild type levels was observed only with DEL1 and DEL2. The activity declined to 80 and 70% of the wild type value in DEL3 and DEL4. This may suggest a dependence, in vitro, on upstream sequence elements whose effects are not easily detected in vivo. Two of the sp1 core consensus sequences were removed between DEL2 (−220) and DEL3 (−131).

The Binding of a Factor to the Promoter Region Is Correlated with Promoter Activity—In order to identify trans-acting factors that interact with the promoter region, bandshift assays were performed with the various promoter deletions (Fig. 4). Since the β actin promoter is active in most cell backgrounds, nuclear extracts from different sources were first tested for binding activity. For this purpose the promoter fragment from DEL3 was used as a labeled probe because this fragment extends about 40 base pairs upstream of the CCAAT box at position −89 and displayed full promoter activity in vivo (Figs. 1 and 2). Nuclear extracts were obtained from embryonic brain, heart, and breast muscle and from fused myotube cultures. The extracts were incubated with end-labeled probe and assayed for binding by bandshift during agarose gel electrophoresis. A prominent bandshift was observed with all extracts examined and the shifted band migrated to approximately the same position in every case (Fig. 4A). This suggested that the DNA-protein complex had a similar size in all extracts and was consistent with the observation that the β actin promoter was active in all cell backgrounds tested. For practical reasons, all further analyses of factor binding were performed with nuclear extracts from embryonic chicken brain.

In order to correlate factor binding with promoter activity in vivo and in vitro, labeled promoter fragments of different lengths were tested for their ability to generate a bandshift
the bandshift complex was directly correlated with full deletions described in Fig. 1 by cutting with the restriction extracts from different sources. Bound used as a labeled probe. The probe was incubated with nuclear was observed with the region upstream of the smallest fragment which still exhibits binding activity with the promoter deletion. E, the activities in vivo and in vitro, formation of the promoter size indicated by competition with the fragment derived from DEL4 containing the CCAAT element at position -89. B, no competition was observed with the chicken \( \alpha \) cardiac actin promoter (-100 to +15), the sequence elements upstream of the \( \beta \) actin CCAAT box (-332 to -95), or with a hybrid construct containing the \( \alpha \) cardiac CCAAT-like element fused with the \( \beta \) actin TATA region (see "Materials and Methods"). C and D, instead of a restriction fragment, an oligonucleotide, which spans 50 base pairs of the \( \beta \) actin CCAAT region (-106 to -56), was used for bandshift and competition. C, the results indicate specific competition and were obtained by competing with the unlabeled oligonucleotide itself. D, no competition was observed with unrelated oligonucleotides, one derived from the \( 3' \)-untranslated region of the \( \beta \) actin gene (40-mer, DePonte-Zilli et al., 1988), the other from a region upstream of the \( \alpha \) cardiac actin CCAAT-like element (50-mer, see "Materials and Methods"). Fig. 5. Bandshift competition. In A and B the fragment derived from DEL4 was used as a labeled probe and incubated with brain extract. Competitors were mixed with the labeled fragment prior to the addition of extract. Competitors were added at 10-, 20-, and 50-fold molar excesses (10X, 20X, 50X). Lanes without competitor are indicated by OX, A, the result indicates specific competition obtained by competition with the fragment derived from DEL4 containing the CCAAT element at position -89. B, no competition was observed with the chicken \( \alpha \) cardiac actin promoter (-100 to +15), the sequence elements upstream of the \( \beta \) actin CCAAT box (-332 to -95), or with a hybrid construct containing the \( \alpha \) cardiac CCAAT-like element fused with the \( \beta \) actin TATA region (see "Materials and Methods"). C and D, instead of a restriction fragment, an oligonucleotide, which spans 50 base pairs of the \( \beta \) actin CCAAT region (-106 to -56), was used for bandshift and competition. C, the results indicate specific competition and were obtained by competing with the unlabeled oligonucleotide itself. D, no competition was observed with unrelated oligonucleotides, one derived from the \( 3' \)-untranslated region of the \( \beta \) actin gene (40-mer, DePonte-Zilli et al., 1988), the other from a region upstream of the \( \alpha \) cardiac actin CCAAT-like element (50-mer, see "Materials and Methods").

Factor Binding Is Specific for the CCAAT Region—The specificity of the binding was assessed by competing the bandshift shown in Fig. 4 with related and unrelated DNA fragments. A fragment derived from DEL4 was initially used as a labeled probe in the competition assays because this is the smallest fragment which still exhibits binding activity (Fig. 4B). Binding to the DEL4 fragment could be competed with the unlabeled fragment itself (Fig. 5A). No competition was observed with the region upstream of the \( \beta \) actin CCAAT box (position -220 to -106) or with the \( \alpha \) cardiac actin promoter region (position -100 to +15) which contained different CCAAT and TATA elements (Fig. 5B). Thus, the \( \alpha \) cardiac actin promoter does not appear to bind the same CCAAT or TATA factor as the \( \beta \) actin promoter, as judged by this assay. A hybrid construct containing the \( \alpha \) cardiac actin CCAAT region and the \( \beta \) actin TATA region (see "Materials and Methods") did not compete for binding to the probe derived from DEL4. This suggested that the factor binding domain in the \( \beta \) actin promoter was located between positions -106 and -53. Therefore, a double-stranded oligonucleotide was synthesized which contained the 50 base pairs downstream of position -106, a domain which also included the \( \beta \) actin CCAAT box. This oligonucleotide displayed a specific bandshift when incubated with brain extract and this shift was completely eliminated when unlabeled oligonucleotide was added at a 100-fold molar excess (Fig. 5C). No competition was observed with unrelated oligonucleotides from either the untranslated \( 3' \)-end of the \( \beta \) actin gene or from the \( \alpha \) cardiac actin promoter (Fig. 5D). These competition experiments confirm that the binding activity in the \( \beta \) actin promoter CCAAT element at position -89 was distinct from both the \( \alpha \) actin CCAAT element and the \( \beta \) actin upstream CCAAT-like elements.

Nuclear Factors Bind to a Region of the Promoter That Defines the CCAAT Element—In order to map the binding domain more precisely, the promoter fragment derived from DEL2 was analyzed by DNase footprinting and methylation interference. DNase footprinting revealed a protected region covering about 23 base pairs (-97 to -74) in both coding and
noncoding strands. The CCAAT box (−89) was included in the protected binding domain (Fig. 6A).

Binding interference due to G and A methylation was observed in the noncoding strand with the methylation of the two G residues and the single A residue within the GGTTA element complementary to the CCAAT box. Methylated A residues on the noncoding strand were consistently observed to interfere with binding and were located within the CCAATCA sequence of the CCAAT box (Fig. 6B).

Promoter Activity during in Vitro Transcription Is Eliminated by Specific Competition—An in vitro transcription competition assay was used to determine if the factor which binds over the CCAAT element is responsible for promoter function. In vitro transcription was monitored on the wild type promoter construct as a function of increasing amounts of various competitor DNAs. The specific competitor was the same 50-base pair oligonucleotide used as competitor in the bandshift assays described in the preceding section (Fig. 5 and D). This oligonucleotide covers the DNase protected domain of the CCAAT binding factor. Consequently, any hypothetical factor that might interact with the TATA element is unaffected by the competition. The results show that the promoter activity was eliminated at a 100-fold molar excess of specific competitor. The effective competition, in terms of molar excess, occurred over the same range in both the in vitro transcription and bandshift assays. No comparable competition was observed with an oligonucleotide from the 3' end of the β actin gene or with an oligonucleotide from the α cardiac actin promoter (position −100 to +15) containing different CCAAT and TATA elements (Fig. 7). These results imply that the factor which binds to the CCAAT box confers substantial transcriptional activity to the β actin promoter. In the absence of factor binding, β actin promoter activity declines to a level similar to that observed with the promoter deletions that do not contain the CCAAT box (Fig. 3).

Interspecies Comparison of β Actin Promoter Sequences Reveals Similarities in the CCAAT Region—The β actin promoter is active in a variety of cell backgrounds from different species in both stable and transient transfections (Billeter et al., 1988; DePonti-Zilli et al., 1988; Seiler-Tuyns et al., 1984). Consequently, it is likely that the factor-complex which is responsible for β actin expression involves a sequence element in the promoter, which is conserved in different species. The three published sequences of human (Nakajima-Iljima et al., 1985), chicken (Kost et al., 1983), and rat (Nudel et al., 1983) β actin promoters were compared for similarities within the CCAAT binding domain (Fig. 8A). Positions with identical sequences in all three promoters, when aligned to the CCAAT box, are underlined. The longest stretch of sequence similarity between the three species occurred in a region of 9 base pairs, which included the CCAAT box at −89.

In view of this limited sequence similarity between the different β actin promoters, the ability of factors in nuclear extracts derived from tissues taken from different species to bind to the chicken β actin promoter was investigated. For this purpose the 50-base pair oligonucleotide described in Fig. 5 was used as a labeled probe. This oligonucleotide contains the 23-base pair binding domain of the chicken β actin promoter and additional flanking sequences. The results demonstrated that nuclear extracts from chicken brain, rat liver, and mouse L cells gave almost identical bandshift patterns (Fig. 8B). Although the complex formed with nuclear extracts from different species suggests factors of similar size, we have not done the competition footprinting or methylation interference assays to determine the precise relationship in these band shift patterns. In contrast, the bandshift pattern with nuclear extracts from HeLa cells consisted of at least four bands. This array was reproducible with independent prepa-

![Fig. 6. DNase footprinting and methylation interference.](image)

For both assays a fragment derived from DEL2 (+1 to −220) was used as a labeled probe. A, DNase footprinting of coding (C) and noncoding (N) strands. Free (F) and bound (B) were separated by bandshift after digestion with DNase. Brackets indicate the approximate binding domain. B, methylation interference. The methylated residues that interfere with binding are indicated by arrows. The lower panel summarizes the results from DNase footprinting and methylation interference. Brackets indicate the binding domain delineated by DNaseI protection, and arrows indicate methylated bases which block factor binding.

![Fig. 7. Competition of in vitro transcription from the wild type β actin promoter.](image)

A, competition with 50-mer β actin CCAAT domain (see Fig. 5). The oligonucleotide was added to the transcription reaction at 10-, 50-, 70-, and 100-fold molar excess relative to the concentration of the same sequence element in the wild type plasmid. B, competition with a nonspecific competitor, in this case the α cardiac actin sequence upstream of the CCAAT-like element (50-mer, see "Materials and Methods").
rations of HeLa cell nuclear extract and most likely represented the binding of multiple factors to the promoter region. A minor band migrated to the same position as the prominent band observed with the other extracts (Fig. 8, bracket; Fig. 8B, H, bracket). However, competition and bandshift experiments have not been carried out to determine the relationship of these multiple bands to the factor binding domain seen with brain nuclear extracts. It is not clear whether the bandshift pattern observed with the HeLa cell nuclear extracts is representative of extracts for all human cells or reflects a specific difference associated with this particular cell line.

**Factor Binding Is Associated with the Hexamer GCCAAT—**

In order to establish to what extent the nucleotides adjacent to the β actin CCAAT box (Fig. 8) were important for factor binding, oligonucleotides were synthesized, which contained replacements of specific sequence elements. Initially, the 9-base pairs, GCCAATCAG, which constitute the core segment that is similar between the different β actin promoters, were placed in the α cardiac actin promoter, which itself does not bind the β actin factor (Fig. 9A). We have previously shown that this replacement still allows developmentally regulated expression of the α cardiac actin promoter (Quitschke et al., 1988). This oligonucleotide eliminates the conserved sequence elements adjacent to the β actin CCAAT binding domain. Factor binding to this hybrid oligonucleotide was diminished only marginally since the bandshift pattern was essentially unchanged and competition, compared to the wild type construct, was similar (Fig. 9, A and B, lanes NC, HY). Consequently, the peripheral homology between β actin promoters in the region adjacent to the CCAAT box plays only a minor role in factor binding to the CCAAT element.

Next, the 9-base pairs, GCCAATCAG, were analyzed using transverse mutations in the wild type promoter. The 9-base pair sequence was modified so that three consecutive base pairs were replaced by the appropriate nucleotide transversions. This generates three different sequence replacements: PM1 (TAAAATCAG), PM2 (GCCGCCGCAG), and PM3 (GCCAATACT) (Fig. 9A). The ability of the various replacements to shift or compete for binding to the wild type construct was determined (Fig. 9, A and B). PM1 and PM2 were unable to compete for binding and did not shift when incubated with extract, indicating that replacing the first three or the middle three nucleotides of the 9-base pair domain with the transversions completely abolished factor binding. Replacing the last 3 base pairs did not eliminate competition or shift altogether. However, the competition and shift with PM3 were not as effective as with the wild type (WT) or the hybrid (HY) oligonucleotides. This was taken to indicate that the three base pairs mutated in PM3 have some influence on factor binding but that it is considerably less pronounced than the mutations in PM1 and PM2 (Fig. 9A). This may reflect the fact that only a single contact on the coding strand involving an A residue, as defined by methylation interference assays (Fig. 6), is affected by PM3, whereas in PM1 and PM2 methylation alters two and three adjacent contact points, respectively, on both strands.

**FIG. 8. Interspecies comparison of binding domains and bandshift patterns.** A, comparison of promoter sequences within the CCAAT binding domain of chicken, human, and rat. Regions of sequence identity between the three species are underlined. B, comparison of bandshift patterns between nuclear extracts of mouse (M) (C2C12, muscle cell line), chicken (C) (brain), rat (R) (liver), and human (H) (HeLa cell line) origin.

**FIG. 9. Effects of sequence mutations on the bandshift pattern.** Various sequence modifications of oligonucleotides containing the CCAAT binding domain were tested for their ability to bind and compete with the CCAAT binding factor from chicken brain nuclear extract. A, WT indicates the wild type sequence within the CCAAT binding domain. In sequence HY the nucleotide peripheral to the 9-base pair GCCAATCAG have been replaced by the corresponding sequence from the α cardiac actin promoter, thus eliminating most of the sequence identity peripheral to the 9-base pair core. PM1, PM2, and PM3 are a series of mutations in 3-base pair increments within the GCCAATCAG domain of the β promoter. The underlined bases are unmodified. B, bandshift assay with the various oligonucleotides described in A. C, competition assay with the various oligonucleotides described in A against the native promoter CCAAT oligonucleotide (WT).
DISCUSSION

The β actin promoter region contains several sequence elements that have been implicated in the function of cellular and viral promoters. Among these are five putative spl elements (Dyman and Tjian, 1983), a TATA box (−29), a CCAAT (−89), a second CCAAT box-like element at position (−220), and a putative regulatory element termed a CARG box (Minty and Redes, 1986) at (−60). The deletion experiments indicate that the spl core consensus sequences at the upstream elements that have been implicated in the function of cellular promoters of different nuclear extracts bind to the promoter and this binding activity in so many different cell types and tissues. The binding activity in so many different cell types and tissues explains the prevalence of this cis-acting sequence required for full activity. The mechanism by which this sequence element is responsible for the high level of expression that is common to other tissues. The control of the H2B-1 histone gene is mediated by a sequence-specific negative regulatory element that prevents the activation of the CCAAT binding protein with the promoter (Barberis et al., 1987).

A different example is seen within the β actin gene, which is actively expressed in most vertebrate cell backgrounds at all developmental stages and is not repressed in muscle tissue where it is replaced by the respective muscle-specific actin isoforms. In contrast to the sea urchin H2B-1 histone gene, the β actin gene is not down-regulated by the interaction of a factor or factors with a negative regulatory element within the promoter. The β actin promoter displays undiminished activity in a muscle background, when it is isolated from the gene (Billeter et al., 1988; Seiler-Tuyns et al., 1984). This cis-acting sequence element required for down-regulation has been defined to 40 base pairs near the polyadenylation signal in the untranslated 3′-end region of the β actin gene (De Ponti-Zilli et al., 1988). In the absence of this sequence element, the β actin promoter depends primarily on a CP-1-like CCAAT binding factor for full activity. The mechanism by which this 3′ regulatory sequence controls β actin transcription is under investigation.

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