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Functional Analysis of Elements Affecting Expression of the β-Actin Gene of Carp†

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Regulatory regions of the β-actin gene of the common carp (*Cyprinus carpio*) have been examined by linking upstream, 5′-flanking sequences and regions of the first intron to a bacterial chloramphenicol acetyltransferase (CAT) reporter gene. By analysis of the mRNA products and encoded CAT activity, we have identified four putative regions that influence expression: (i) a negative regulatory region 2,300 to 1,100 base pairs (bp) ahead of the gene; (ii) a proximal promoter element, containing the highly conserved CCAAT, CC(A/T)$_6$GG, and TATA boxes, that is within the first 204 bp upstream of the initiation site; (iii) a negative element of 426 bp in the 5′ region of the first intron; and (iv) a positive 304-bp element near the end of the first intron that contains highly conserved sequences found in all characterized β-actin genes. The positive intron element is not a classical enhancer; it is position and orientation dependent, as has been observed in other housekeeping genes in vertebrates. Depending on the elements joined together, CAT gene expression can be modulated more than 500-fold in transfected mouse cells.

Like most eucaryotic genes, β-actin genes are regulated at the transcriptional level by *trans*-acting protein factors which bind to *cis*-acting DNA sequences in the neighborhood of the promoter. Although the actin gene family is expressed in all tissues, individual actin genes show tissue and developmental specificity in their expression. The β-actin gene is expressed in certain cell types, presumably because of differential binding of transcriptional factors to the regulatory elements of the gene. Transcriptional control elements commonly consist of a proximal or basic promoter, often including CAAT and TATA boxes located within 100 nucleotides (nt) upstream of the transcriptional start site, and other modulating elements that enhance or reduce (silence) expression. Except for genes transcribed by RNA polymerase III (3, 15, 47), the basic promoter is generally immediately in front of the gene and the enhancers and silencers are ahead (5′) or behind (3′) the gene (1, 12, 13, 29, 41, 54) or inside the gene, often in introns (1, 16). The modulating elements generally operate in an orientation- and position-independent manner, and they direct the appropriate timing, tissue specificity, and quantitative levels of expression of the gene throughout development (16, 21, 22, 25, 30, 36, 39, 44–46, 52).

The β-actin promoters contain a typical CAAT box, a TATA box, and an evolutionary conserved CC(A/T)$_6$GG unit, termed the CArG motif (31, 34, 37; Z. Liu, Z. Zhu, K. Roberg, A. Faras, K. Guise, A. Kapuscinski, and P. B. Hackett, Sequence, in press). This motif has also been found in cardiac and skeletal α-actin genes and a number of other actin genes (31). The CArG sequence motif was reported to be required for muscle-specific expression of the cardiac α-actin gene (33) and to be a tissue-specific enhancer for the human skeletal α-actin gene (36). A CArG-binding factor, CBF, has been found to regulate the human skeletal and cardiac α-actin (18, 32, 33, 35) and human β-actin (14) genes. CBF has been shown to be nearly indistinguishable from the c-*fos* serum response factor (8, 19, 38, 53) and is subject to serum induction (38, 53).

Many aspects of β-actin regulation are not understood. Several reports characterizing regulatory elements of the human β-actin gene are inconsistent. For instance, an enhancer element was identified in the first intron of the β-actin gene (23), but the same region was found by Frederickson et al. (14) to have a minor effect on transcription and by Ng et al. (38) to have an inhibitory transcriptional effect. Moreover, putative protein factors that bind to this region were different; Frederickson et al. (14) showed that the protein factor CBF was competed for by a serum response factor, but this was not observed by Kawamoto et al. (23).

We have isolated and sequenced the β-actin gene from two species of carp, the common carp (*Cyprinus carpio*) (26; Liu et al., in press) and the grass carp (*Cnopharyngodon idella*) (27). The two carp genes have identical sequences in regions implicated for regulation of β-actin expression. Consequently, we have dissected putative transcriptional control elements of the *C. carpio* gene in order to construct expression vectors that can be used for gene transfer into fish. Transgenic fish are extremely useful for studying the genetics of development because of the easy collection and injection of fertilized eggs, the early development of eggs in a transparent sac outside the mother, and the ability to induce parthenogenesis for production of homozygous offspring. Mouse L cells, which have been shown to express heterologous β-actin genes accurately and efficiently (49), were used as a host system. Here, we report that the carp β-actin gene is controlled by multiple regulatory elements. The proximal promoter element directs a fairly high level of expression. An upstream distal element negatively modulates this expression, and an internal regulatory element in the first intron enhances expression in an orientation- and position-dependent manner.

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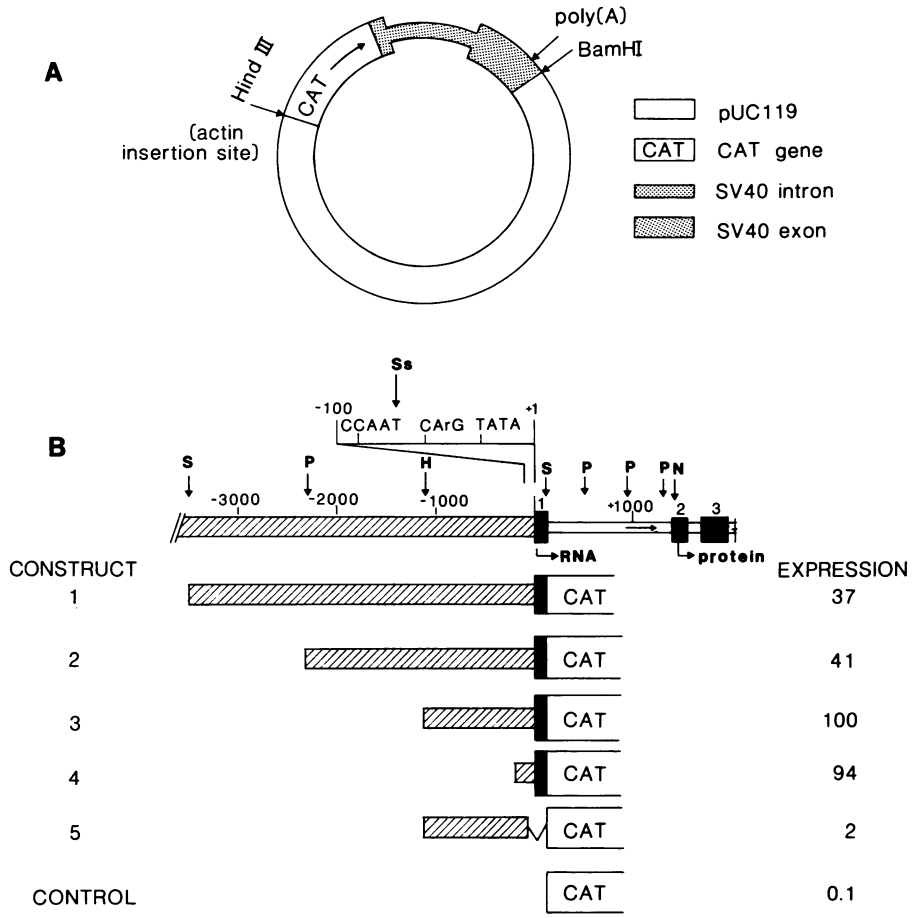

FIG. 1. (A) Initial cloning vector for all of the carp-CAT constructs. The CAT gene with accompanying SV40 early gene intron and poly(A) sequence was moved from the RSV-CAT clone of Gorman et al. (18) into pUC119. All subsequent cloning was done as described in Materials and Methods by adding carp β-actin gene sequences into the *Hin*dIII site. (B) Schematic diagram of constructs 1 to 5, showing the various 5′-flanking sequences plus the first 68 bp of exon 1 of the carp β-actin gene. The first 100 bp of the 5′-flanking element (−100 to +1) are amplified, showing the CCAAT, CArG, and TATA boxes. The relative levels of CAT gene expression, from averages of four or more determinations each (see Fig. 2), are given on the right. Symbols; ▨, 5′-flanking sequence; ■, exon; □, intron. The polarity of intron 1 is designated with an arrow. The ligated CAT gene is designated by the CAT box. The initiation sites for transcription and translation are marked with bent arrows for RNA and protein, respectively. Restriction enzyme sites: S, *Sal*I; P, *Pst*I; H, *Hin*dIII; Ss, *Sst*I; N, *Nco*I.

MATERIALS AND METHODS

Construction of plasmids. Test plasmids were constructed by standard molecular techniques unless otherwise indicated. All test constructs were verified either by restriction endonuclease mapping or by dideoxy sequencing (48). The CA16 clone contains the complete carp β-actin gene plus 3,500 base pairs (bp) of 5′-flanking sequences (Liu et al., in press). All of the test constructs except pCAT contain portions of the carp β-actin gene ligated to the bacterial chloramphenicol acetyltransferase (CAT) gene (17). The plasmid constructs are designated numerically in the figures for convenience and description of their construction. The nucleotide coordinates are with respect to the transcriptional initiation site at nt +1.

The vector pRSVCAT (18) was the source for the CAT gene. The cloning vector was plasmid pUC118 or pUC119 (51), depending on the desired orientation. Construct 1 (pSal/SalCAT) was made by positioning the 3.5-kilobase-pair (kb) *Sal*I-*Sal*I β-actin upstream sequence 5′ to the CAT gene. Similarly, constructs 2 (pPst/SalCAT), 3 (pHin/Sal CAT), and 4 (pRC6CAT) were assembled by inserting the 2.3-kb *Pst*I-*Sal*I upstream sequence, the 1.1-kb *Hin*dIII-*Sal*I upstream fragment, and a 204-bp upstream sequence, respectively, 5′ to the CAT gene. The 204-bp upstream sequence in construct 4 was derived from the reverse cloning procedure (26); the fragment begins 204 nt in front of the transcription initiation site and extends to the *Sal*I site, 68 nt inside exon 1. Construct 5 (pHin/SstCAT) was constructed by cloning the *Hin*dIII-*Sst*I fragment (−1100 to −71) containing the CAAT box but not the TATA and CArG boxes ahead of the CAT gene. The CAT gene without any eucaryotic promoter sequences (pCAT) was used as a control for basal expression (Fig. 1B).

Constructs 6 to 24 were assembled to test effects of conserved sequences in intron 1 on transcription. Construct 6 (pHin/NcoCAT) contains 1,100 bp of 5′-flanking sequence plus exon 1 and intron 1 and 2 bp of exon 2. The restriction enzyme *Nco*I, whose recognition sequence includes the β-actin AUG initiation codon, cleaves between the first and second bases of the second exon. To avoid creation of a fusion protein with the β-actin amino-terminal sequences added to the CAT gene, the β-actin gene was first digested with *Nco*I, partially filled in with the *Escherichia coli* DNA polymerase (Klenow fragment; Bethesda Research Labora-

tories, Inc. [BRL]) in the presence of only dCTP, treated with mung bean nuclease (BRL), redigested with *Sal*I (which digested in the first exon of the gene), and then force cloned into the *Sal*I-*Sma*I sites of pUC119. The plasmid was sequenced to ensure that the proper clone was constructed. The resulting promoterless construct 19 (pSal/NcoCAT) contained part of the first exon, the first intron, the 3'-splice junction of intron 1 (without the β-actin AUG codon), and the CAT gene. The 1.1-kb *Hin*dIII-*Hin*dIII upstream β-actin fragment was then inserted into the *Hin*dIII site of construct 19, generating construct 6. For convenience, in the following constructs, the 413-bp *Pst*I (533)-*Pst*I (946) fragment is designated Pst2 and the 304-bp *Pst*I (946)-*Pst*I (1250) fragment is designated Pst1; Δ indicates a deletion; R designates a fragment in reversed orientation; and 2× or 3× designates double or triple copies of the sequence. Constructs 7 (pHin/NcoΔ[Pst1 &2]), 8 (pHin/NcoΔ[Pst2]), 9 (pHin/NcoΔ[Pst2] Pst1R), 10 (pHin/NcoΔ[Pst1]), 11 (pHin/NcoΔ[Pst1] Pst2R), 12 (pHin/NcoΔ[Pst2] 2×Pst1), 13 (pHin/NcoΔ[Pst2] 2× Pst1R), and 14 (pHin/NcoΔ[Pst2] 3×Pst1) were made by deletions, from construct 6, or either or both of the *Pst*I fragments of the intron and, in some cases, reinsertion of one or more *Pst* fragments in the normal or reversed orientation. Construct 15 was made in similarly to construct 12. For constructs 16 to 18, construct 19 (pSal/NcoCAT) was digested with *Eco*RV, ligated to *Hin*dIII linkers, digested with *Hin*dIII, and religated. Construct 16 (pHin/NcoΔ[Hin-RV] CAT) was obtained by insertion of the 1,100-bp upstream *Hin*dIII fragment into the *Hin*dIII site of the plasmid. Construct 17 (pHin/NcoΔ[Hin-RV Pst1]CAT) was made by deletion of the 304-bp *Pst*I fragment in the intron out of construct 16; construct 18 (pHin/NcoΔ[Hin-RV] Pst1R CAT) was made by reinsertion of the 304-bp *Pst*I fragment back into construct 17 but with an inverted orientation. The insertion of the 3.5-kb upstream *Sal*I fragment into construct 19 resulted in construct 20 ($pSal_2$/NcoCAT). Constructs 21 and 22 were made by inserting the 304-bp *Pst*I fragment into the *Pst*I site of construct 4 downstream of the CAT gene in both orientations. Constructs 23 and 24 were made by inserting the 304-bp *Pst*I fragment at the *Pst*I site 2,300 bp upstream of the promoter in construct 2.

Transfection and CAT assay. DNA transfection of mouse L cells was accomplished by the DEAE-dextran method (28). CAT activities were assayed by the procedure described by Gorman et al. (17). Briefly, nearly half-confluent dishes of mouse L cells were transfected with 16 to 32 μg of DNA per 100-mm-diameter dish. Sixty hours after transfection, extracts were prepared and incubated with [^{14}C]chloramphenicol (Amersham Corp.) and 0.5 mM acetyl coenzyme A for 1 h at 37°C. For a given analysis, samples containing a constant amount of extract (9) were chromatographed on silica gel thin-layer plates. After autoradiography, spots on the gel were cut out and counted. The percentage of conversion of chloramphenicol to acetylated derivatives for each construct was determined from the average of at least four transfections. The relative CAT activity of each construct was normalized against that of either construct 3 or construct 4, which contained the proximal promoter and had similar activities. A 50-μg sample of cellular extract from cells transfected with construct 3 or 4 gave approximately 0.02 U of CAT activity when measured against commercial (Sigma Chemical Co.) preparations of CAT analyzed under identical conditions (e.g., Fig. 6).

RNA isolation and S1 nuclease analysis. Total cellular RNA was prepared from cultured mouse cells 48 h after transfection (10). Transfected cells from five 100-mm-diameter plates were harvested and combined for RNA isolation and analysis.

For examination of the steady-state transcription levels as well as the 3' splice site of intron 1, S1 nuclease mapping was performed (2). A probe of 1,520 bp spanning from the *Hin*dIII site 5' of the first intron to the *Eco*RI site in the CAT gene was used. The probe was continuously labeled from an initial single-stranded M13 clone, using the Klenow fragment of *E. coli* DNA polymerase (BRL) and [^{32}P]dATP (3,000 Ci/mmol; Amersham). Approximately 10^6 cpm of probe was hybridized to 50 μg of total cellular RNA, the hybrid was digested with 1,000 U of nuclease S1 (BRL) at 30°C for 1 h, and the reaction products were ethanol precipitated and analyzed on an 8% polyacrylamide gel containing 8 M urea.

RESULTS

Analysis of the β-actin proximal promoter. Our previous studies mapped the transcriptional initiation site and identified putative promoter elements on the basis of sequence conservation with other vertebrate β-actin genes (27; Liu et al., in press). The putative proximal promoter includes the CAAT, CArG, and TATA boxes, which begin at −88, −59, and −27, respectively. To find additional, *cis*-acting regulatory regions, we made several constructs containing various regions of the β-actin gene fused to the CAT gene (17) (Fig. 1A). Each construct was subsequently transfected into mouse L cells. We first made a simple construct containing the first 1,100 bp of 5'-flanking sequences plus the first 68 bp of exon 1 (construct 3), which was used as a standard (Fig. 1B). We made construct 4, which contained only 204 bp of the β-actin 5' upstream sequence, by the reverse cloning procedure (26). CAT activities of constructs 3 and 4 indicated that these constructs contained a functional promoter of nearly equal strength when assayed in mouse fibroblasts (Fig. 2). For all of the CAT analyses shown, the assays were repeated at least four times with independent transfections. The results usually varied by less than 10%. We made construct 5, which contained 1.1 kb of β-actin upstream sequence with its 3' end at the *Sst*I site at −69; it lacked both the CArG and TATA boxes (Fig. 1B). In contrast to constructs 3 and 4, construct 5 showed almost no CAT activity (Fig. 2). These results showed that the functional proximal promoter is located within a 204-bp upstream region and that the TATA and CArG boxes are required for expression.

The β-actin gene contains an upstream regulatory sequence. The results presented above defined the proximal promoter. We extended the 5' upstream sequence from 1.1 kb to either 3.5 or 2.3 kb (constructs 1 and 2) to determine whether further upstream sequences altered gene activity. Both of these constructs gave about 40% the activity of construct 3 (Fig. 1 and 2). Since our assays were reproducible to within 10%, these data suggest that presence of a negative regulatory element between 1.1 and 2.3 kb upstream of the transcriptional start site.

The carp β-actin gene contains a unidirectional regulatory sequence in its first intron. In addition to the upstream transcriptional domains, we tested for possible downstream regulatory domains, since evolutionarily conserved sequences exist in the first intron (31; Liu et al., in press), especially in the 304-bp *Pst*I fragment (Pst1), which contains a 10-bp CArG box identical to that in the proximal promoter. We made a construct which contained 1.1 kb of upstream sequence plus exon 1 (which is not translated) and intron 1. Construct 6 was made such that the 3' splice junction of intron 1 was retained while the AUG initiation codon for the

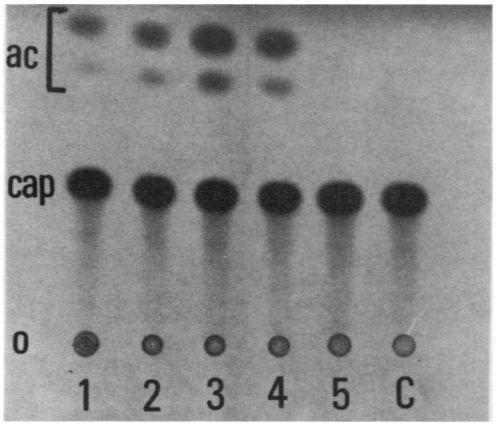

FIG. 2. Typical CAT assays for constructs 1 to 5. Acetylated derivatives of [^{14}C]chloramphenicol were produced during 1-h incubations with equal amounts (9) of extracts from transiently infected mouse L cells, separated on thin-layer plates, and visualized by autoradiography. After exposure to film, the spots were cut out of the plates and quantified by liquid scintillation counting. The average expression level of CAT from each construct, from four or more determinations, is presented in Fig. 1B. Lanes: 1 to 5, constructs 1 to 5, respectively; C, promoterless CAT control. o, Origin; cap, [^{14}C]chloramphenicol; ac, acetylated forms of [^{14}C]chloramphenicol.

β-actin polypeptide in exon 2 was removed to avoid the production of a fusion protein. In the presence of upstream proximal promoter domains, addition of these sequences resulted in fivefold-higher CAT activity (Fig. 3 and 4; compare constructs 3 and 6). We did extensive deletion studies to locate the regulatory domains within the intron. When an internal 717-bp fragment of the first intron was removed from construct 6 (construct 7), the CAT activity dropped to 10% that given by the proximal promoter alone (see below). This deletion left 96 bp of the 3′ end of the intron, including the branch site and 3′ junction sequences important for accurate splicing. Insertion of the 413-bp *PstI* fragment of the larger 717-bp fragment back into construct 7, in both the original and inverted orientations (generating constructs 10 and 11, respectively), did not raise CAT activity. However, when the 304-bp *PstI* fragment of the 717-bp fragment was put back into construct 7 in its original orientation (construct 8), the CAT activity returned to the level of construct 6. Since classical enhancers work in an orientation-independent manner, we inverted the orientation of the 304-bp fragment (construct 9) but did not recover any CAT activity. The results (Fig. 3 and 4) clearly show that the carp β-actin gene contains a unidirectional domain in its first intron that is located in the 304-bp *PstI* fragment 108 bp upstream from the 3′ splice junction; inversion or removal of this element nearly abolished expression.

The unidirectional element in intron 1 is copy number dependent. In addition to performing the orientation test, we examined the effects of copy number of the intron domains on CAT expression (Fig. 3). The results were more complex. Two tandem copies of the 304-bp *PstI* intron fragment in its original orientation produced expression similar to that produced by the single copy (Fig. 3 and 4; compare constructs 8 and 12). However, two copies of the same fragment in an inverted orientation showed an intermediate level of expression (construct 13), about 50-fold higher than with a single copy inverted (construct 9) and about twice that of the basal promoter level (construct 3). When the same fragment

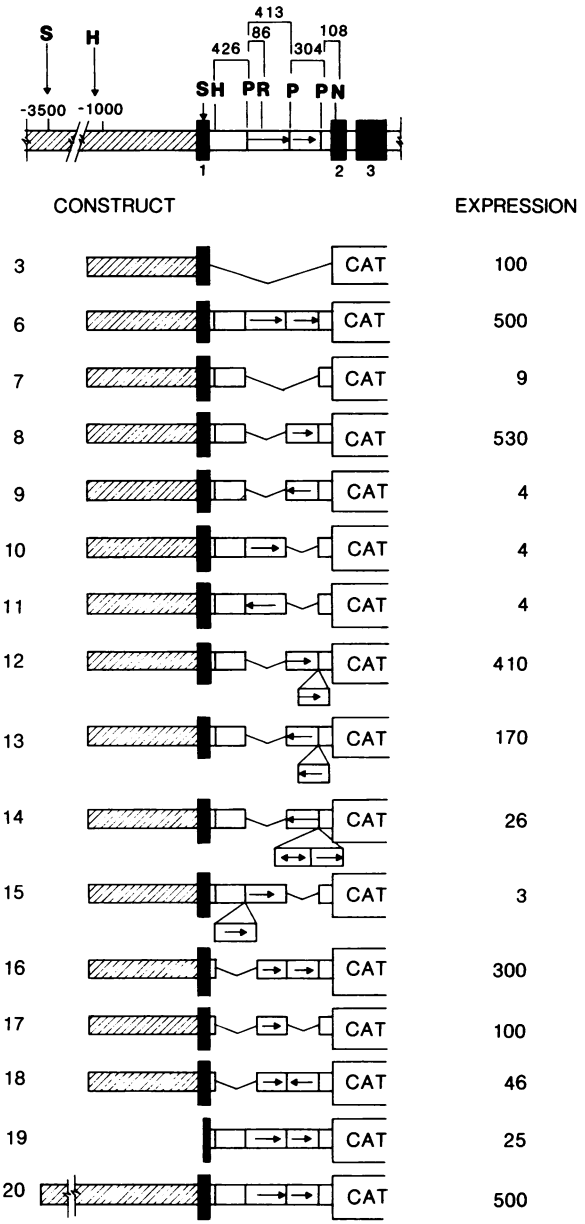

FIG. 3. Schematic diagram of constructs 3 to 20, made as described in Materials and Methods. The arrows in the subregions of intron 1 designate polarity. Sizes (in nucleotides) of the subregions are given at the top. The level of expression for each of the constructs is an average of four determinations from data similar to those shown in Fig. 4. Restriction enzyme sites: S, *SalI*; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; R, *Eco*RV. Symbols: ▨, 5′-flanking sequence; ▮, exon (numbered at the bottom); □, intron. The ligated CAT gene is designated by the CAT box.

was present in three copies, with two copies in opposite orientations and the orientation of the middle copy not determined, CAT activity was only about 5% that of the single copy in its original orientation (Fig. 3 and 4; compare constructs 8 and 14). These results suggested the possibility that some kind of interaction occurred either between these multicopy intron elements or between the intron sequences and the identical CArG region in the proximal promoter element. In contrast to the copy number effects of the 304-bp fragment, there were no changes in the CAT levels when the

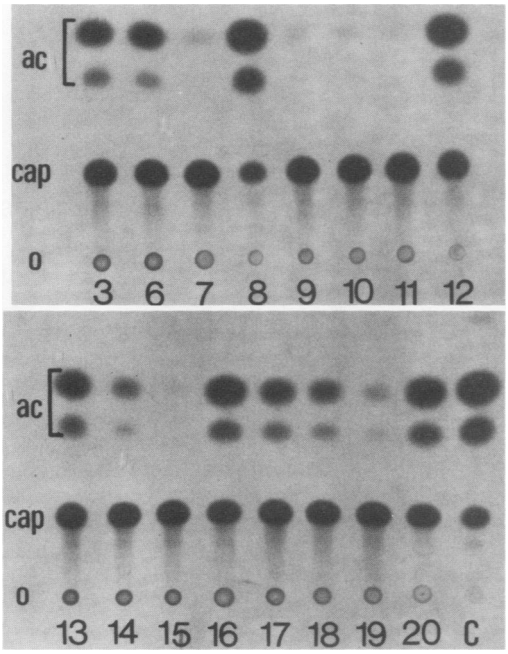

FIG. 4. Typical CAT assays for constructs 6 to 20. The procedure was the same as that for Fig. 2. Lanes 3 and 6 to 20 represent the corresponding constructs of Fig. 3; lane C, control with purified enzyme. Only one-fifth amount of cell extract was used for construct 6.

413-bp *Pst*I fragment was inverted or present in two copies; the CAT activity was very low (Fig. 3 and 4; compare constructs 10, 11, and 15).

Intron 1 contains more than one domain that affects expression. Although the clones with deletions in intron 1 still contained the proximal promoter sequence (construct 3) and therefore were expected to give the basal level of activity, deletion of either 717 bp (construct 7) or 304 bp (construct 10) of the first intron gave virtually no CAT activity. One possibility was that other sequences in this intron could reduce mRNA formation in absence of the positive, unidirectional domain in the 304-bp *Pst*I fragment. To test this possibility, we made deletions in the 5′-proximal portion of the intron. We deleted 512 bp, 14 bp downstream of the 5′ splice junction, by using construct 6 to generate construct 16. Construct 16 had a fairly high CAT activity, about three times that of the basal promoter and close to that of construct 6 with the complete intron. Further deletion of the 304-bp *Pst*I fragment from construct 16, generating construct 17, produced a level of expression comparable to that obtained with just the proximal promoter (construct 3) but 30-fold higher than that from constructs 7, 10, and 11. These results indicated that the 5′ region of the intron could repress expression when the unidirectional domain further downstream was removed. Interestingly, insertion of the 304-bp fragment back into construct 17 in the opposite orientation, generating construct 18, decreased expression about twofold, consistent with potential interactions between downstream sequences or the proximal promoter elements. As an initial attempt to examine whether intron 1 activates transcription from the proximal promoter or contains a stronger alternative promoter to direct additional fourfold CAT activity, we tested a promoterless clone, construct 19, with the 3′ third of exon 1 plus all of intron 1. The construct had only 25% residual activity in the absence of a natural proximal promoter (Fig. 3 and 4). The transcriptional initiation site of this construct was not examined. The presence of the entire intron also abolished the effects of the upstream negative regulatory element (−1100 → −2300), as shown by construct 20, which had the same activity as construct 6, which lacked the upstream negative region. In summary, these results suggest that intron 1 of the carp β-actin gene has several domains which affect expression of various degrees, depending on orientation and the presence or absence of other loci.

The unidirectional element shows position dependence. Because the positive domain in the intron was functional in only one direction, we tested whether this region could act like an enhancer when moved 5′ or 3′ to the gene. Accordingly, we made constructs 21 and 22 (Fig. 5), consisting of the proximal promoter element of construct 4 plus the 304-bp *Pst*I intron 1 fragment that had positive effects on expression (construct 8; Fig. 3) 3′ of the gene. Constructs with this element in both orientations were produced. Since only the 5′ portion of the noncoding exon 1 were included, there should not have been any complications from either splicing or creation of a fusion protein. Both constructs 21 and 22 showed slightly lowered levels of CAT activity, about 75% that of the proximal promoter (Fig. 5 and 6). We further tested the enhancing effect of the 304-bp intron element by positioning it upstream, in both orientations, of the extended β-actin promoter, which has negative as well as positive elements (construct 2; Fig. 1 and 2), to produce constructs 23 and 24 (Fig. 5). In either orientation, the levels of CAT activity were similar to that of construct 2, which lacked the intron fragment, and those of constructs 21 and 22, in which the intron element was downstream of the CAT gene in either orientation (Fig. 5 and 6). Apparently, the positive domain in intron 1 was functional only when situated in the intron.

The regulatory elements do not affect RNA splice site selection. Since the elements identified above are located in the intron, we examined whether the observed effects were due to changes in RNA processing. We examined transcripts by S1 nuclease mapping from the critical constructs 3 (proximal promoter only), 6 (promoter plus complete intron 1), 8 (promoter plus positive intron 1 element), 9 (promoter plus inverted positive intron 1 element), 10 (promoter plus specific deletion of positive intron 1 element), 16 (promoter plus intron 1 lacking the 512 *Hin*dIII-*Eco*RV sequence), and 19 (lacking the promoter) (Fig. 1 and 3). All of the other constructs are variations on these constructs.

The results of S1 nuclease mapping are shown in Fig. 7. Approximately equal amounts of total RNA, quantified by A_{260}, were used for each assay. For all constructs, the 270-nt product expected from correct splicing at the 3′ end of intron 1 was obtained (Fig. 7, top arrow). The relative intensity of the protected 270-nt band for each construct corresponded with the associated CAT activity; the only exception was the RNA from construct 16, which was almost twice that expected from the CAT assays. Constructs 9 and 10 did not yield any protected products, including either large products that might have been derived from unspliced or misspliced precursor RNAs or RNAs initiated at unusual initiation sites inside intron 1. Constructs 3, 6, 8, 16, and 19 yielded bands of lesser intensities at 190 and 80 nt (Fig. 7, bottom two arrows) which probably were derived from spurious S1 nuclease cleavage after melting of the 270-nt protected fragment at a sequence containing eight consecutive A · T base pairs. Likewise, other sets of bands of even lesser intensities are consistent with melting at other A/T-rich regions of the 270-nt protected sequence. Only with con-

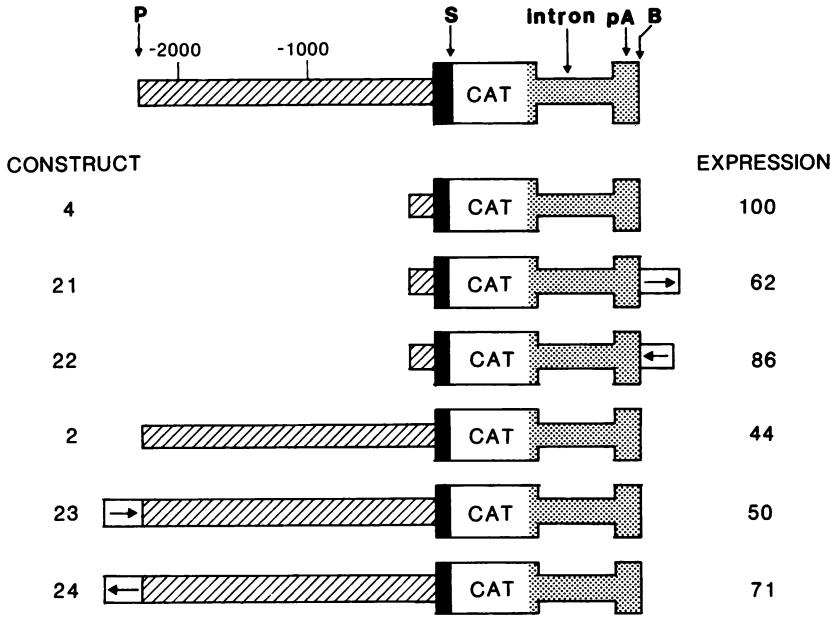

FIG. 5. Schematic diagram of constructs 21 to 24, made as described in Materials and Methods. Symbols; ▨, 5'-flanking region of the carp β-actin gene; ▮, exon 1. The CAT gene is designated by the CAT box, and SV40 sequences are stippled, with the intron being narrower than the SV40 early exon. Boxes with arrows are the 304-bp *Pst*I fragment from intron 1 in normal or opposite polarity, as designated by the direction of the arrows. pA, poly(A) cleavage-addition signal. Restriction enzyme sites: B, *Bam*HI, S, *Sal*I; P, *Pst*I.

struct 8 did we detect any anomalous RNA product, a band of approximately 360 nt that might be the result of cryptic promoter activity approximately 90 nt upstream of the junction of intron 1 with exon 2. This product was less than 10% of the 270-nt product.

Overall, the results from the S1 nuclease mapping confirm those of the CAT assays, (summarized in Fig. 1 and 3) and indicate that the intron elements did not affect selection of RNA splicing sites.

DISCUSSION

We have analyzed several elements that appear to regulate expression of the carp β-actin gene. The CAT assays were used for quantitative measurements because of the wide range of activity over which the assay is valid and the ease of running a large number of repeats of each assay to reduce the overall uncertainty of expression level to less than 10%. Our results from the S1 nuclease mapping generally supported those from the CAT assays. We concluded that expression of the carp β-actin is affected by at least five elements, four of which have been investigated in this study. Two putative control regions are upstream of the gene, a negative locus between −2300 and −1100 and the positive proximal promoter element consisting of the CAAT, CArG, and TATA boxes; two regions are in the first intron that separates the noncoding first exon from the second exon with the AUG initiation codon; and a fifth locus is near the polyadenylation site that is responsible for down regulation of β-actin in muscle cells (12), which we have not examined (Fig. 8).

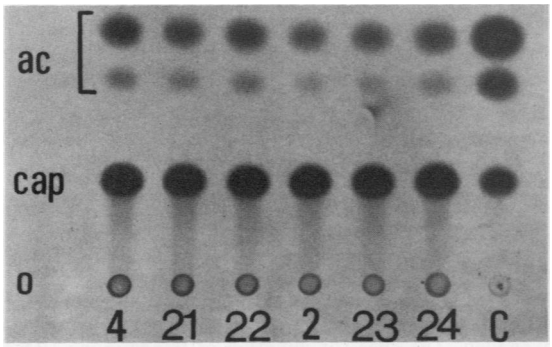

FIG. 6. Typical CAT assays of constructs 21 to 24 conducted as described for Fig. 2. Lanes 4 and 21 to 24 represent the corresponding constructs shown in Fig. 5; lane C, control with purified enzyme.

The β-actin proximal promoter contains classical CAAT and TATA boxes plus the ubiquitous CArG sequence found in most actin proximal promoters (31). The CAAT box was shown previously to be required for the promoter activity (14), and we have shown here that the CArG and TATA boxes are also required (construct 5; Fig. 1 and 2). The carp β-actin proximal promoter by itself is relatively strong, equivalent to that of a Rous sarcoma virus (RSV)-CAT construct (Z. Liu et al., unpublished data) which has the enhancer-promoter complex of RSV (18) and is itself a strong promoter of transcription (18, 28). The upstream negative regulatory element has not been precisely mapped within the 1.2-kb *Pst*I (−2300)-*Hin*dIII (−1100) fragment. In the human β-actin gene, the CArG motif is repeated in this region (−1433 to −1418; 34).

The most striking observation we have made is the complexity of the domains in the first intron that affect expression in mouse L cells. We have examined the contributions of three regions of the first intron with respect to their abilities to modulate expression. First, the 304-bp *Pst*I fragment, containing the highly conserved CArG box and other sequences (31, 34; Liu et al., in press), is a positive regulatory element that has both orientation and position dependence (constructs 8, 9, and 21 to 24). Second, the exon 1 proximal 512-bp *Hin*dIII (106)-*Eco*RV (618) intron element negatively influences expression (constructs 7 and 16 to 18)

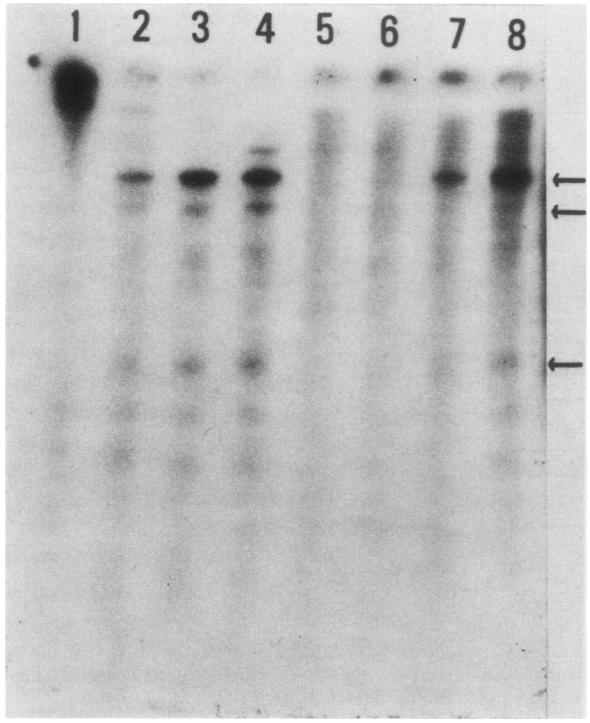

FIG. 7. Nuclease S1 mapping of CAT transcripts. Total cellular RNA (50 μg) was hybridized to a probe of 1,520 bp, from the *Hin*dIII site in intron 1 (Fig. 3) to the *Eco*RI site in the CAT gene 270 bp downstream from the splice site. The mRNA-probe hybrid was digested with 1,000 U of S1 nuclease and incubated at 30°C for 1 h. The reaction products were analyzed on a sequencing gel. Lanes: 1, probe; 2, construct 3; 3, construct 6; 4, construct 8; 5, construct 9; 6, construct 10; 7, construct 19; 8, construct 16. Top arrow, 270-nt protected band expected from correctly spliced mRNAs; bottom two arrows, 190- and 80-nt bands, respectively, presumably derived from the digestion at an A+T-rich sequence. Sizes were determined by *Hin*fI-digested pBR322 markers (not shown).

in the absence of the 304-bp *Pst*I element. Third, the middle 413-bp *Pst*I fragment that separates the other two elements apparently has no function, except perhaps as a spacing element. There is an 86-bp overlap between the 512- and 413-bp elements, leading us to presume that the negative activity in intron 1 resides in the 426-bp region between the *Hin*dIII (106) and *Pst*I (532) sites. In many of the constructs that we have made, we have necessarily altered the geometries and separations of various regions that could have an effect on expression. These studies are our initial foray into mapping control sites within the first intron of the carp β-actin gene.

The 304-bp *Pst*I element is especially interesting. The presence of this element largely abolishes the negative activities of the other intron regions (constructs 6 and 8) as well as of the upstream negative element (construct 20). However, the 304-bp element will not increase expression when placed either behind the CAT gene (which, with the simian virus 40 [SV40] intron and polyadenylation signal, is 1.6 kb) or 2.2 kb ahead of the proximal promoter (constructs 21 to 24). Thus, in terms of its orientation and position dependence, the 304-bp intron-activating element is not a classical enhancer.

In contrast to our findings with the carp β-actin gene, Kawamoto et al. (23) showed that the human β-actin intron 1 regulatory element acted in an SV40 promoter-CAT construct like an enhancer, independent of orientation and position. Frederickson et al. (14), using a human β-actin promoter-intron element combination with the CAT gene, obtained a twofold enhancement of expression; they did not report any effect of intron orientation. In contrast, Ng et al. (38) used a similar human β-actin gene construct and showed that the presence of the intron sequence inhibited the basal proximal promoter activity. They also linked the intron control element to the SV40 promoter and found that it enhanced expression, but an orientation effect was not reported.

Orientation- and position-dependent 5′ regulatory elements have been observed in several vertebrate genes (7, 21,

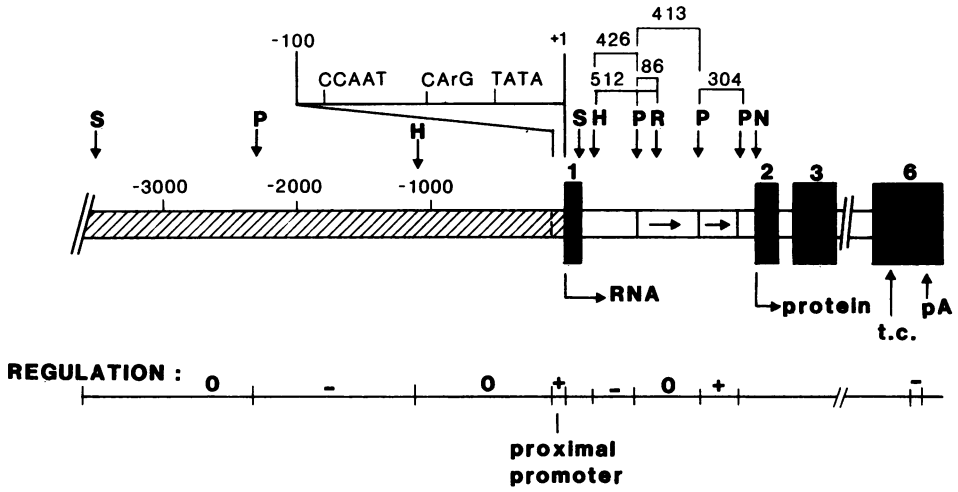

FIG. 8. Summary of the carp β-actin gene regulatory regions. The map shows the β-actin gene with the portion between exons 3 and 6 deleted. The restriction enzyme sites used to produce various clones are designated at the top, along with the sizes (in nucleotides) of the fragments. The first 100 bp of the proximal promoter element have been expanded. The sites for initiation of transcription (RNA) and translation (protein), termination of translation (t.c.), and polyadenylation (pA) are designated. Restriction enzyme sites: H, *Hin*dIII; P, *Pst*I; R, *Eco*RV; S, *Sal*I. The regulatory loci identified in this report are designated by + if positive, − if negative, and 0 if neutral. The region in exon 6 was investigated by Deponti-Zilli et al. (12) with the chicken β-actin gene.

24). The troponin I gene (55), the mouse ribosomal protein L32 gene (11), and the collagen genes (4–6, 20, 40, 42, 43, 50) appear to have regulatory elements arranged similar to those we find for the carp β-actin gene. In the human collagen α 1(I) gene, a 274-bp intron 1 fragment strongly inhibited expression when placed in an inverted orientation but not in its natural orientation (4–6). DNA-DNA looping interactions between the promoter and the first intron have been proposed to explain the orientation dependence of transcriptional regulation (6). Our data may support a similar set of complex interactions for the carp β-actin gene and, by inference from the similarity of regulatory sequences, for other vertebrate actin genes as well. Putative intron control sequences have been conserved evolutionarily in vertebrate β-actin genes (Liu et al., in press). Our data from constructs composed of multiple copy numbers of the intron elements are consistent with the hypothesis of interactions between the upstream and intron elements. Although direct tests may be difficult since the conserved sequences in the proximal promoter are probably essential and the multiplicity of control elements may be differentially responsive to various cell lines with a variety of available *trans*-acting protein factors, further analysis of control regions should reveal the intricacies of the regulation of β-actin gene expression.

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