Positive and Negative Regulatory Elements Mediating Transcription from the *Drosophila melanogaster* Actin 5C Distal Promoter

YANG-TSUNG CHUNG AND ELIZABETH B. KELLER*

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

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The major cytoskeletal actin gene of *Drosophila melanogaster*, the actin 5C gene, has two promoters, the distal one of which controls synthesis of actin in a tissue- and developmental stage-specific manner. This very strong promoter has widely been used for expression of heterologous genes in cultured cells. To locate functional regulatory elements in this distal promoter, mutants of the promoter were fused to the bacterial chloramphenicol acetyltransferase gene and assayed for transient expression activity in cultured *Drosophila* embryonic Schneider line 2 cells. The results showed that the upstream end of the promoter extends to 522 bp from the transcription start site. In addition, there are two remote activating regions about 2 kb upstream. Between -522 and -379 are two regions that exert a strong negative effect. Downstream from these negative regions are at least six positive regions and a TATA element. The strongest positive determinant of the promoter was identified at -320 as AAAATGTG by footprinting and by a replacement experiment. When the relevant region was replaced by a synthetic sequence containing this element in a random context, the transient expression activity was restored. The sequence TGTATG located at -355 was also identified as a positive element by a similar replacement approach. Apparently the very high activity of this promoter is the result of the combined activities of multiple factors.

The actin 5C gene is the major cytoskeletal actin gene in *Drosophila melanogaster* (14-16). It has two distinct promoters (4, 5) (Fig. 1A), one closer to the coding region, called the proximal promoter, which provides a uniform level of transcripts in different tissues, and a distal promoter, which is strongly activated in specific tissues and is developmentally regulated (7, 30). The distal promoter is of special interest because it is the strongest promoter so far tested in transient expression assays in cultured *Drosophila* embryonic cells (18) and therefore is often used in expression vectors.

In our study of these two promoters, we first completed a survey of the proximal promoter (8). This constitutive promoter has a distinctive strong positive element, A5CE-1 (AAGTTGAGTTG), at -320 from the transcription start site, an essential element at -35, and five GAGA elements, some of which are essential. It has no recognizable TATA box but has an initiator sequence (20). It also has the sequence CCATATATGG, which in vertebrates is a serum response element (SRE) (24), but no function has been ascribed to this sequence in the actin 5C proximal promoter (8).

To initiate the study of the distal promoter, it was necessary to complete the sequencing of the 5'-flanking region of the gene. When the two promoters were compared, no sequence homology was found except for the 10-bp SRE-like sequence present in both. In contrast to the proximal promoter, the distal promoter has a canonical TATA element that binds a protein factor in a *Drosophila* nuclear extract fraction (25). The two actin 5C promoters are therefore governed by entirely different sets of transcriptional elements. This study was undertaken to identify the regulatory elements of the distal promoter. We were surprised to find that the activity of this strong promoter could be increased another threefold by the deletion of two upstream promoter

regions. These regions presumably contain negative elements. At least six positive regulatory regions were found downstream from the negative regions. The essential positive elements in two of these regions were identified. In each case, the region was replaced by a synthetic sequence containing the element in a random context, and this operation restored the activity. Expression from the distal promoter was doubled by the presence of two remote activating regions about 2 kb upstream. Thus, the very high transcriptional activity of the actin 5C distal promoter in the cultured embryonic cells is due to a large number of positive-acting factors that are strong enough to far outweigh the influence of some negative-acting factors.

MATERIALS AND METHODS

Source and construction of plasmids. A genomic DNA clone that contains the entire *Drosophila* actin 5C gene, pDmA2 (14), was a gift from E. A. Fyrberg. Expression plasmids containing mutants of the actin 5C distal promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene in the vector pUC^{PL}-CAT (5) were provided by B. Bond-Matthews. These plasmids have 5' deletions at -2364, -1200, -247, and -90 and were used as the starting material for making the following additional 5' deletion mutants by using available restriction sites: -2071, -1984, -1866, -1395, -772, -594, -522, -361, -278, -219, and -43, all fused to the CAT gene.

To make fine 5' deletion mutants with nucleotide endpoints downstream from -522, the -772-CAT fusion plasmid was cleaved with *Spe*I at position -522 and treated with BAL 31 exonuclease. The ends were made blunt with Klenow fragment and four deoxynucleoside triphosphates, attached to synthetic *Xho*I linkers, and then ligated with T4 DNA ligase. The selected clones were further digested with *Xho*I and self-ligated to remove the upstream sequences between the *Xho*I linker and the *Xho*I site in the polylinker. The resulting 5' deletion mutants with deletion endpoints at

* Corresponding author.

FIG. 1. Regulatory regions of the distal promoter of the actin 5C gene. (A) Schematic diagram of the actin 5C gene. The filled box in exon 3 is the coding region. Dashed lines show how either exon 1 or exon 2 is spliced to exon 3 (4). (B) Nucleotide sequence of the coding strand of the distal promoter from -540 to the start of exon 1. See Materials and Methods for sequencing. Nucleotides are numbered with respect to the start site of exon 1 at +1 (4). Several sequences homologous to the binding sites of known *Drosophila* transcription factors are marked: a caudal-binding site (consensus, TTTATG or CATAAA) (9), a dAP1-binding site (consensus, TGACTCA) (26), three *myb*-binding sites [consensus, (C/T)AAC(G/T)G or C(C/A)GTT(G/A)] (2), the TATA element (25), and the cap site sequence [consensus, TCA(G/T)TC] (20). An SRE-like sequence, CCATATATGG (5, 24), is also indicated. Elements identified in this report are also marked: A5CE-2, A5CE-3, and a sequence complementary to A5CE-3 labeled cA5CE-3.

-464, -449, -440, -431, -379, -348, -330, and -140 were verified by DNA sequencing.

A series of internal deletion mutants of the actin 5C distal promoter, ID1 to ID12, was generated by first subcloning a fragment encompassing the region to be deleted into pBluescriptSK(+) (Stratagene), making the deletion between available unique restriction sites, religating, and then reinserting the fragment into its original location in the promoter-CAT fusion plasmid.

Several mutants were prepared by replacing a deleted region containing a possible regulatory element by a synthetic sequence containing the element in a random context. Two synthetic oligonucleotides, 5'-TCGAGCGAAAATGT GTCCG-3' and 5'-TCGACGGACACATTTTCGC-3', were annealed to give the duplex B3 with *Xho*I cohesive ends. B3 was inserted into the *Xho*I site of the -278-CAT fusion plasmid to give pB3(-278). Two synthetic oligonucleotides, 5'-TCGAGAGCGTGTATGCTGG-3' and 5'-TCGACCAG CATAACGCTC-3', were annealed to give B12 and then inserted into the *Xho*I site of the -330-CAT fusion plasmid to give pB12(-330). Plasmids pB12(-348) and pB21(-348) were made by inserting B12 in opposite orientations into the -348-CAT fusion plasmid. All constructs were tested for the presence and orientation of the inserted oligonucleotide by DNA sequencing.

Plasmid pCD15 has the actin 5C distal promoter fragment from -361 to -278 inserted into the *Eco*RV site of pBluescriptSK(+). It was used to make probes for band shift

and footprint assays. Plasmid pGapdh2(-937)/lacZ was obtained from X. H. Sun (29).

Transient expression assays. *Drosophila* embryonic Schneider line 2 (S-2) cells (28) were grown at 23°C in Schneider medium (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal bovine serum. For transfection, cells were seeded into 3 ml of fresh medium in a Falcon 25-cm² T flask to give a density of approximately 10⁶ cells per ml. Samples (10 µg) of supercoiled plasmid DNAs purified in CsCl gradients, which included 1 µg of test plasmid, 2 µg of internal control plasmid pGapdh2(-937)/lacZ (29), and 7 µg of carrier plasmid pUC18, were introduced into cells 24 h later by the calcium phosphate coprecipitation method (10). Forty-eight hours after transfection, the cells were harvested, washed with phosphate-buffered saline, suspended in 200 µl of 0.25 M Tris hydrochloride (pH 7.8), and disrupted by three freeze-thaw cycles. Subsequently, the cell debris was pelleted by centrifugation and the supernatant was assayed for β-galactosidase and CAT activities as previously described (8). Each test plasmid was assayed independently three or four times, and each assay was done in duplicate.

Band shift assay. A crude nuclear extract of *Drosophila* embryonic Kc cells (12) was prepared as described by Parker and Topol (25) and purified by stepwise elution through a heparin-agarose column with buffers containing 0.1, 0.25, 0.4, and 0.6 M KCl (8). A DNA probe containing the promoter region from -361 to -278 was prepared by ³²P end

labeling the *Hind*III-*Sac*II fragment from pCD15 with Klenow fragment. The band shift assay (13, 17) was done as previously described (8) except that the binding reaction mixture included 1 μ g of poly(dI-dC) · poly(dI-dC) in a final volume of 10 μ l.

MPE footprinting. Two-stage methidiumpropyl-EDTA (MPE) footprint analysis (19) was performed as previously described (8). Coding- and noncoding-strand probes containing the promoter region from -361 to -278 were cut from pCD15 with *Hind*III-*Sac*II and *Kpn*I-*Xba*I, respectively. Each was labeled by a 3'-end-filling reaction, using Klenow fragment, [α - 32 P]dCTP, and the other three deoxynucleoside triphosphates. The DNA-protein binding reaction as in the band shift assay was scaled up to 50 μ l. After binding was complete, the mixture was treated with MPE-Fe(II), and the bound and free DNAs were separated on a native gel. Subsequently, the DNAs from the bound and free bands were isolated and run on a sequencing gel together with an A+G sequencing ladder of the probe (23).

DNA sequencing. The *Eco*RI-*Sac*II fragment derived from pDmA2 (14), which encompasses the 2.5-kb 5'-flanking region of the actin 5C gene, was digested with appropriate restriction enzymes, and the resultant fragments were subcloned in opposite orientations into the replicative forms of bacteriophage M13mp18 and -mp19. The single-stranded templates prepared from these M13 clones were sequenced by the dideoxy-chain termination method (27).

Nucleotide sequence accession number. The entire 2,483 bp of the 5'-flanking sequence has been entered into the GenBank/EMBL data library under accession number X15730.

RESULTS

Nucleotide sequence of the actin 5C gene 5'-flanking region. The previous DNA transfection assays of Bond-Matthews and Davidson showed that optimal production of transcripts originating from exon 1 requires the following four 5'-flanking regions: -2.4 to -1.7 kb, -1.2 to -0.25 kb, -247 to -90 bp, and downstream from -90 bp (5). The 5'-flanking region containing these required sequences had previously been sequenced only to -217 bp (4, 30). We therefore determined the nucleotide sequence of this region to -2,483 bp in preparation for identifying essential regulatory elements. Sequencing was done on both strands, using the dideoxy-chain termination method (27).

The sequence of the distal promoter to -540 is shown in Fig. 1B. Evidence is presented below that the promoter extends only to -522. The sequence of the upstream activating region which extends from -2071 to -1866 (see below) is not given here since the regulatory elements were not identified in this study. The distal promoter shows no apparent homology with that of the proximal promoter associated with exon 2 except for the presence of a 10-bp palindromic sequence, CCATATATGG (5, 8). The divergence of the two actin 5C promoter sequences is consistent with the finding that the two promoters are regulated differently during development and in different tissues (7, 30).

The distal promoter was searched for the consensus sequences of known *Drosophila* regulatory elements. These are indicated on the sequence in Fig. 1B together with the new elements identified in this study.

Effect of the remote upstream region on the actin 5C distal promoter activity. Bond-Matthews and Davidson reported that deletion of the 5'-flanking region from around -2,400 to -1,700 bp was associated with a twofold drop in the actin 5C

distal promoter activity (5), suggesting that essential regulatory elements lie in this remote region. Since the region is so far upstream from the start site of exon 1, it might be an enhancer that can act over a large distance. To delineate this remote upstream region more precisely, we constructed a series of 5' deletion mutants of the actin 5C flanking region (nucleotides -2364 to +88) fused to the CAT gene and tested them in transient expression assays in cultured *Drosophila* embryonic S-2 cells (Fig. 2A).

The loss of promoter activity caused by deletion of the sequence between -2364 and -2071 is too slight to be of significance. Deletion of the sequence from -2071 to -1984 and then to -1866 resulted in a stepwise decrease in activity to about 60% of the level obtained with the full flanking region. Further deletion of the sequences extending from -1866 to -772 had no effect on the expression level. Taken together, these results suggest that the 206-bp region between -2071 and -1866 contains two domains with positive elements that bind factors acting at a long distance. Identification of these remote activating elements was not pursued.

Interestingly, these remote upstream elements lost their activity when either the sequence between -1288 and -772 or the sequence between -772 and -43 was deleted (Fig. 2B; ID1 and ID2). The activity of the remote region appears to depend on the presence of both of these downstream regions.

Identification of five essential regulatory regions in the actin 5C distal promoter. We then analyzed the 5'-flanking region that is closer to the start of transcription. In Fig. 3A, the promoter activities of 5' deletion mutants are compared with that of the D-772 construct, which includes the whole promoter. Deletion of the sequence between -772 and -522 had no significant effect on promoter activity. Deletion of the sequence between -522 and -361 caused CAT activity to increase threefold, indicating the presence of negative regulatory elements. On the contrary, deletion of the sequence between -361 and -278 drastically reduced the expression level by about sixfold, revealing that strong positive elements reside within this 84-bp region. Further deletion to -247 did not affect the expression level. Deletion from -247 to -219 caused an additional twofold drop in activity, suggesting the presence of another positive element. Further deletion to -90 had no discernible effect on promoter activity even though the sequence removed includes an SRE-like sequence (5, 24) at -110. Deletion from -90 to -43 resulted in a further twofold loss of the activity.

Altogether, these results show that the distal promoter extends to about -522 from the start of exon 1, so this is the sequence given in Fig. 1B. They also reveal four separate regulatory regions in the actin 5C distal promoter determining the level of expression in embryonic S-2 cells. These are labeled in Fig. 3. Region A represses transcription, whereas regions B, C, and E make positive contributions to promoter activity. Of these regulatory regions, B is the primary positive determinant of promoter activity, since the greatest reduction in activity was seen upon its removal.

To confirm the presence of the regulatory regions identified by 5' deletion analysis, we evaluated a series of internal deletions (Fig. 3B). ID3 to ID6 confirmed that region A has a negative regulatory effect and that regions B and C have strong independent positive effects in the context of the whole promoter. That the activity of B is independent of its distance from the start site is shown by ID6 to ID9. The positive effect of C is also independent of distance from the start site, as shown by ID10 to ID12.

FIG. 2. Effect of the remote upstream region on the activity of the actin 5C distal promoter. The various 5' deletion mutants (A) and internal deletion mutants (B) of the actin 5C 5'-flanking region fused to the CAT gene were assayed for transient expression activity in *Drosophila* S-2 cells (see Materials and Methods). Boundaries given are relative to the start of transcription (4). Each measured CAT activity was corrected by the internal control β -galactosidase activity and is presented relative to the value obtained with the D-2364 construct, which was taken as 100%. Each value represents the average of four independent experiments and is followed by the standard deviation.

A surprising result of the internal deletion analysis was the finding that deletion of the sequence between -219 and -140 caused a drop in promoter activity from 304% (D-361) to 108% (ID10) (Fig. 3). This region is therefore denoted positive region D in Fig. 3. The drop in activity is also seen in comparing ID6 (140%) and ID7 (75%). That the D region had a positive effect was surprising, since this was not seen in the 5' deletion assays (Fig. 3A; D-219 and D-140).

Regions A and B of the actin 5C distal promoter contain multiple regulatory elements. Because the regulatory regions A and B defined by the studies described above are quite wide, we analyzed each further, using fine 5' deletion mutants generated by exonuclease BAL 31. The results of the transient expression assays are diagrammed as a CAT activity profile in Fig. 4. Deletion of the sequence between -522 and -464 increased promoter activity from 104 to 180%, whereas deletion to -431 caused very little, if any, change in activity. Further deletion to -379 resulted in an additional increase in activity from 200 to 295%. These data suggest that negative elements are located in two subregions of A: A1 (-522 to -464) and A2 (-431 to -379). In contrast, sequential deletions of subregions B1 (-361 to -348), B2 (-348 to -330), and B3 (-330 to -278) progressively decreased promoter activity to one-sixth of the level obtained with the D-361 construct. The stepwise reduction in activity suggests that region B contains at least three positive elements.

Identification of two positive elements in region B, the major determinant of the actin 5C distal promoter activity in S-2 cells. To detect specific interactions of nuclear proteins with region B (-361 to -278), we carried out the band shift assay (13, 17), using a crude nuclear extract prepared from cultured *Drosophila* embryonic Kc cells and fractions from

heparin-agarose chromatography of the extract. Inclusion of the crude extract in the binding mixture resulted in only a single shifted band (Fig. 5A, lane 6) even though all three subregions of B were present. The protein responsible for this specific DNA-binding activity eluted between 0.25 and 0.4 M KCl (the 0.4 M KCl fraction) (lane 4). No binding activity was observed in any other fraction (lanes 2, 3, and 5).

To locate the protein-binding site within region B precisely, we next performed a two-stage MPE footprinting (8, 19), using a partially purified 0.4 M KCl fraction and a DNA fragment corresponding to region B. Probes for both the coding and noncoding strands were radiolabeled at either end. Protein-DNA complexes were radiolabeled, treated with the MPE-Fe(II) mixture, and fractionated on a native polyacrylamide gel. The shifted DNA band and the free DNA band were eluted from the gel, denatured, and electrophoresed on a standard sequencing gel. Distinct regions of almost complete protection were seen on each strand (Fig. 5B). Each footprint is 8 nucleotides in length, spanning from -324 to -317, and has the sequence motif AAAATGTG (Fig. 5C). We have provisionally designated this protein-binding site in subregion B3 as the actin 5C element 2 (A5CE-2).

To determine whether this binding site is responsible for the activity of subregion B3, we constructed plasmid pB3(-278), in which a synthetic oligomer containing the A5CE-2 sequence in a random context was inserted into the D-278 construct (Fig. 6C). The result of the transient expression assay showed that the presence of the synthetic element increased the promoter activity from 50% (D-278) to 139%, a value close to the 148% obtained with the genomic B3 sequence present (D-330) (Fig. 6C). Therefore, A5CE-2, which was identified by the binding of a Kc cell protein

FIG. 3. Transient expression analysis of deletion mutants that define five discrete regulatory regions of the actin 5C distal promoter. (A) 5' deletion mutants; (B) internal deletion mutants. The analysis in *Drosophila* S-2 cells was done as for Fig. 2 except that the promoter activities of the mutants are given relative to that of the D-772 construct, which includes just the promoter. Each CAT value represents the average of four independent experiments and is followed by the standard deviation. Brackets above the diagram mark the locations of the five essential regulatory regions defined by this analysis, A to E. Region A is negative, indicated by the - sign; regions B to E are positive, indicated by + signs.

factor, is responsible for the strong promoter activity of region B3 in S-2 cells, and the strong induction due to A5CE-2 in S-2 cells must result from the presence of an A5CE-2-binding protein in S-2 cells.

Turning to subregions B1 and B2, we noticed that B1, which is only 14 bp long, contains the sequence TGTATG at -355 and that the identical sequence is present in reverse orientation just 6 bp downstream in B2 (Fig. 6). The TGTATG and CATACA could be a regulatory pair functioning as an inverted repeat. To determine whether TGTATG is the active element of B1, we introduced a synthetic oligomer containing TGTATG in a random context in place of B1 [Fig. 6B; B12(-348)]. The presence of the synthetic element raised the transient expression activity from 215% (D-348) to

291%, a value close to the 305% obtained with the genomic B1 present (D-361) (Fig. 6B). We conclude that TGTATG is the active element of B1 and that its exact position is not critical. However, when the synthetic element was inserted in the same place but in the opposite orientation [Fig. 6B; B21(-348)], there was no increase in activity, which shows that the orientation of the TGTATG at -355 is critical. We do not know whether the other member of the inverted repeat, the reverse sequence CATACA in B2, is the active element of the B2 region. If it is, its orientation is also critical, since it could not be replaced by a synthetic oligomer containing TGTATG [Fig. 6B; B12(-330)]. In Fig. 1B, the TGTATG is marked as A5CE-3 and its complementary sequence CATACA is marked as cA5CE-3.

FIG. 4. Transient expression analysis of the fine 5' deletion mutants of the actin 5C distal promoter region from -522 to -278. Deletion plasmids were generated from the D-772 construct by using exonuclease BAL 31 as described in Materials and Methods. The analysis in *Drosophila* S-2 cells was done as for Fig. 2. Numbers on the abscissa represent the 5' deletion endpoint of each mutant. CAT values are given relative to that of D-772, which includes the whole promoter, and is taken as 100%. Each value represents the average of three independent experiments. The error bar indicates the standard deviation.

DISCUSSION

In the previous study of the *Drosophila* actin 5C distal promoter by Bond-Matthews and Davidson (5), inclusion of the remote 5'-flanking region located roughly between -2400 and -1700 increased the level of transient expression about twofold, raising the possibility that the region includes an enhancer. After completing the sequencing of the 5'-flanking region to -2483 in this study, we assayed some more 5' deletions in this remote region. We identified two activating segments, one between -2071 and -1984 and the other between -1984 and -1866 (Fig. 2A). Although these two segments have only a 1.7-fold effect on promoter activity, the effect seems to be significant, considering that the actin 5C distal promoter is such an extremely strong promoter. But curiously, these remote activating segments had no effect when fused to the homologous core promoter at -43 just upstream from the TATA box (Fig. 2B; ID2). Also, they had no effect when the sequence between -1288 and -772 was deleted (Fig. 2B; ID1). The 1.7-fold effect of the remote activating region appears to depend on downstream elements. A number of examples of activating factors that function only in concert like this have been published (e.g., reference 6).

A 5' deletion analysis of the 5'-flanking region was used to establish that the full promoter extends to about -522 (Fig. 3A). Then a series of 5' deletions was used to locate individual promoter regions (Fig. 3A and 4). The first deletions produced the most surprising result obtained in this study, the finding of two strong negative regions at the upstream end of this very powerful promoter. After deletion

of both negative regions, expression was three times that of the full promoter (Fig. 3A). Since the negative segments are upstream from the positive elements of the promoter, factors binding to these elements cannot act simply by interposing between positive factors and their target sites. In fact, when the downstream positive region B (-361 to -278) or C (-247 to -219) was deleted, the negative region still exerted its inhibition on the residual activity (Fig. 3), suggesting that the inhibitory factors directly target the transcription initiation complex. A number of examples of direct negative action on initiation complexes have recently been reported (3, 11). Since this actin 5C distal promoter is used in expression vectors for its high activity (18), a construct with the negative region deleted to give even higher expression might be useful.

The 5' deletion analyses delineated at least five positive regions between the negative region and the TATA box (Fig. 3A and 4). Internal deletion analysis confirmed these five and added a sixth (Fig. 3B). The active element of the most distal of these regions, -361 to -348, is a TGTATG. When the distal region was replaced with a synthetic double-stranded oligonucleotide, including the TGTATG in a random context, the activity of the region was restored (Fig. 6B), but the oligonucleotide inserted in the reverse orientation was inactive. Therefore, the TGTATG in the distal region functions only in its normal orientation. The next positive region, -348 to -330, contains CATACA, the sequence complementary to TGTATG, just 6 bp downstream from TGTATG. These two may form a functional inverted repeat. In a search of 42 *Drosophila* promoters, the single sequence TGTATG was found only in one case, at -184 in the yolk protein 1 promoter (21). The TGTATG, like the A5CE-1 in the proximal promoter (8), is definitely not a general regulatory element.

The third positive region, -330 to -278, includes the A5CE-2 element AAAATGTG, which was identified by MPE footprinting (Fig. 5). When this third positive region was replaced with a synthetic double-stranded oligonucleotide including the A5CE-2 in a random context, the activity of the region was regained (Fig. 6C). Thus, A5CE-2 is the only active element in this 53-bp region. A putative caudal-binding site, CATAAA (9), present in this third region at -290, is apparently inactive in S-2 cells. The A5CE-2 contributes a threefold stimulating effect to expression, the strongest effect caused by any positive element in this powerful promoter. In searching many *Drosophila* genes, we have found the exact A5CE-2 sequence in the promoters of a number of genes. It is therefore possible that the protein factor which binds to A5CE-2 also participates in the transcriptional regulation of other genes.

The fourth positive region, -247 to -219, contains two putative binding sites of transcriptional factors: dAP1 (26) and myb (2) (Fig. 1B). We have detected a *Drosophila* Kc nuclear protein bound to this region in a band shift assay (unpublished data), but the exact site has not yet been identified by footprint analysis.

These first four positive regions from -361 to -219 comprise a very strong position-independent activating unit. When directly linked to the basal promoter just upstream from the TATA element, they induced promoter activity eightfold (Fig. 3; D-43 and ID12). This result suggests that the inducing effect of the factors binding to these four regions is exerted on the transcription initiation complex. We have found that this positive segment (-361 to -219) displays a TATA element-dependent behavior (Y. T. Chung and E. B. Keller, unpublished data). When placed upstream

FIG. 5. Identification of a protein-binding site at -320 in the actin 5C distal promoter. (A) Band shift assay (see Materials and Methods). The end-labeled DNA probe was the distal promoter region from -361 to -278. It was incubated without nuclear extract (lane 1) or with 5 μ g of different heparin-agarose column fractions of the Kc cell nuclear proteins (lanes 2 to 6): 0.1 M KCl fraction (lane 2); 0.25 M KCl fraction (lane 3); 0.4 M KCl fraction (lane 4); 0.6 M KCl fraction (lane 5); and crude extract (lane 6). (B) Two-stage MPE footprint analysis (see Materials and Methods). The autoradiograph of the sequencing gel shows the ladder from the free DNA band (F), from the protein-bound DNA band (B), and from a Maxam-Gilbert G+A reaction (G/A) (23) as a marker. The regions protected from MPE-Fe(II) cleavage are bracketed. (C) Summary of the MPE footprint analyses. The footprint regions are marked by brackets on the DNA sequence of the actin 5C distal promoter from -350 to -300.

of the *Drosophila hsp70* promoter (22) or the *Drosophila Adh* distal promoter (1), it effectively enhanced the strength of these two heterologous promoters that contain different TATA sequence motifs. But this distal positive segment was inactive when linked to the TATA-less actin 5C proximal promoter.

A fifth positive regulatory region between -219 and -140 was detected by internal deletion analysis (Fig. 3B; ID10). In this analysis, its presence in the D-361 construct increased promoter activity almost threefold over that of the deletion

mutant. However, when the same region was deleted in the sequential 5' deletion analysis, there was no drop in activity (Fig. 3A; D-219 and D-140). This discrepancy could mean that the action of this fifth region depends on an upstream element. This fifth region contains a putative *myb*-binding site (2) (Fig. 1B). The weak effect of the sixth positive region that lies between -90 and -43 was detected in the 5' deletion analysis (Fig. 3A).

In this study, we have identified 10 regions or elements mediating the expression of the actin 5C distal promoter in

FIG. 6. Assay of potential regulatory elements located in the actin 5C distal promoter region B between -361 and -278 by replacing potential elements with synthetic elements and assaying transient expression. (A) Map indicating region B and subregions B1, B2, and B3. (B) Sequences of 5' deletion mutants D-361, D-348, and D-330 and of the constructs B12(-348), B21(-348), and B12(-330) derived from them by insertion in different orientations of a synthetic oligomer (underlined) containing the potential element TGTATG (overlined with arrow) in a random context, as described in Materials and Methods. The transfection analysis was done as for Fig. 2 except that the activities of the mutants are given relative to that of the D-278 construct, which is taken as 50% (see, Fig. 3A). Each value represents the average of four independent experiments and is followed by the standard deviation. (C) Sequences of D-278 and the B3(-278) construct made from it by insertion of a synthetic oligomer (underlined) containing the element ASCE-2 (overlined) in a random context; assayed as for panel B.

embryonic S-2 cells. If we add the sequences homologous to the binding sites of known *Drosophila* transcription factors that are present in the promoter, the total number of regulatory elements would be more than 15 in addition to the TATA box and the initiator sequence. Studies with germline transformation will be required to determine which elements regulate the expression of this distal promoter in different tissues at different stages of development.

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