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Regulatory sequences in the promoter of the *Dictyostelium* Actin 6 gene

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The promoter region of the developmentally regulated Actin 6 gene of *Dictyostelium* has been dissected by a series of deletions. Functional analysis of the deletions in *Dictyostelium* transformants revealed two short regulatory sequences: a positive upstream element (PUE) between −599 and −572 which increases transcription by a factor of 10 but does not affect the developmental pattern of expression and an upstream activator sequence (UAS) between −249 and −215 which is essential for transcription and proper developmental regulation. The UAS partially coincides with a conserved sequence with dyad symmetry found upstream of several *Dictyostelium* actin genes (Romans and Firtel, 1985a).

Key words: Actin 6 gene/*Dictyostelium*/promoter region/deletion analysis

Introduction

The cellular slime mold *Dictyostelium* provides a simple system to study development and cell differentiation (for review see Loomis, 1975, 1982). A large number of differentially expressed genes have been isolated and characterized (Rowekamp and Firtel, 1980; Mehdy *et al.*, 1983; Barklis and Lodish, 1983; Gerisch *et al.*, 1985; Kimmel and Firtel, 1982; Dowds and Loomis, 1984; Reymond *et al.*, 1984; Datta *et al.*, 1986b; Williams *et al.*, 1985; Noegel *et al.*, 1986). The 17–20 member actin multi-gene family in *Dictyostelium* has been extensively analyzed (Kindle and Firtel, 1978; McKeown *et al.*, 1978; Firtel *et al.*, 1979; McKeown and Firtel, 1981a,b; Romans and Firtel, 1985a,b; Knecht *et al.*, 1986). It has been shown that the actin genes, most of which are almost identical in their coding region, are differentially expressed during development (McKeown and Firtel, 1981a; Romans *et al.*, 1985). In addition, actin mRNA preferentially accumulates in pre-stalk but not pre-spore cells in later development (Tsang *et al.*, 1982; Mehdy *et al.*, 1983). Comparison of 5′ flanking regions of a number of *Dictyostelium* actin genes revealed several short conserved sequences with dyad symmetry which were proposed to be involved in the regulation of gene expression (Romans and Firtel, 1985a).

With the establishment of a transformation system for *Dictyostelium* (Nellen *et al.*, 1984a,b; Nellen and Firtel, 1985), we can now analyze the *cis*-acting sequences necessary for proper developmental regulation of cloned genes in *Dictyostelium*. In several cases it has been shown that 5′ flanking regions of several hundred nucleotides are sufficient for regulated expression of genes re-introduced into *Dictyostelium* cells (Nellen *et al.*, 1984a,b; Nellen and Firtel, 1985; Reymond *et al.*, 1985; Crowley *et al.*, 1985; Datta *et al.*, 1986a,b; Knecht *et al.*, 1986; S.Datta and R.A.Firtel, in press).

The *Dictyostelium* transformation vector pB10SX contains the bacterial *Neo*^{R} gene controlled by the Actin 6 (*Act6*) promoter. We have shown previously that the differential expression of the *Act6*–*Neo*^{R} gene fusion in *Dictyostelium* transformants parallels that of the endogenous Actin 6 gene (Nellen *et al.*, 1984a,b). *Act6* mRNA levels are high in vegetative cells and then drop to ~1/10 of this level in 10 h developing cells (McKeown and Firtel, 1981a; Romans *et al.*, 1985). In order to analyze the *Act6* promoter and to identify sequences essential for transcription in *Dictyostelium*, we have dissected a 719 bp 5′ flanking fragment of the *Act6* gene by deletion analysis. We report the identification of two *cis*-acting regions, a positive upstream element which increases the level of expression but does not alter its temporal pattern and an element that acts as an upstream activator sequence.

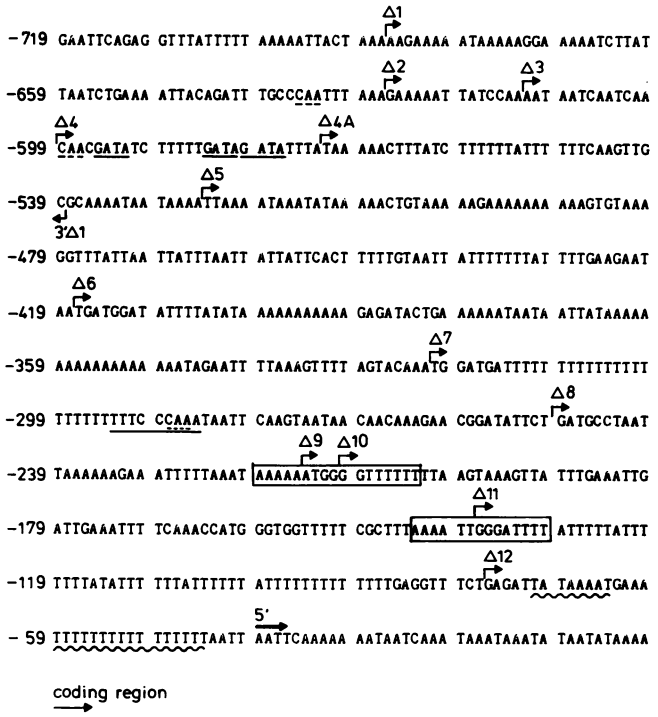

Fig. 1. Sequence of the Actin 6 5′ flanking region. The position of the 5′ deletions and 3′Δ1, the 3′ deletion, are indicated. Regions homologous to Box 4 (Romans and Firtel, 1985a) are boxed, and other sequences mentioned in the text are underlined. The TATA box and oligo(dT) stretch found between the TATA box and the Cap site of *Dictyostelium* RNA polymerase II genes (Kimmel and Firtel, 1982, 1983) is marked with a squiggly line. [Note: because of a very high degree of conservation within the coding regions, the number system for actin genes starts at +1 for the A of the Met initiation codon. For *Act6*, the major Cap site is at −39 (McKeown and Firtel, 1981a).].

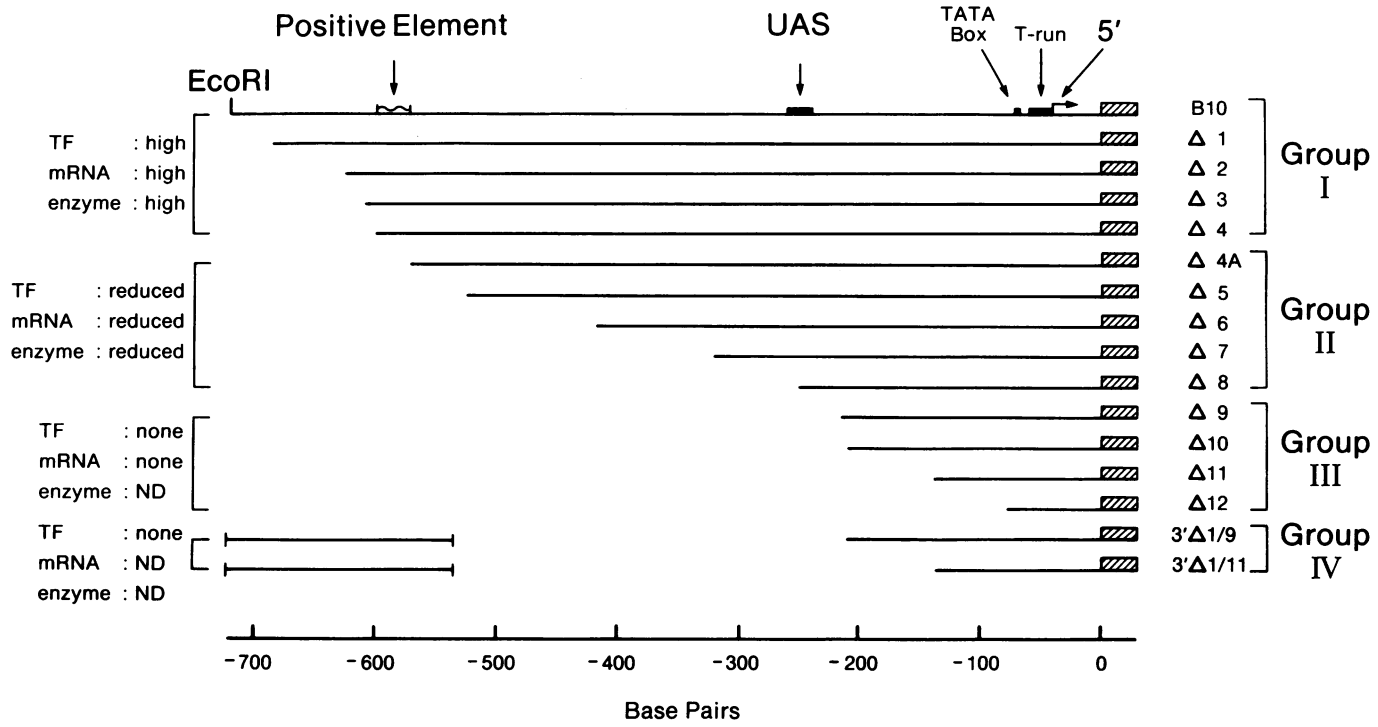

Fig. 2. Regulatory regions in the actin 5′ flanking sequence. Cartoon of deletion constructions of pB10SX and their expression in transformants. The top row shows the 710 bp 5′ promoter region of pB10SX followed by 5′ deletions Δ1−Δ12. Below that the internal deletions 3′Δ1/Δ9 and 3′Δ1/Δ11 are shown. A gap is left between the two parts of the constructs to indicate the missing internal fragment and to show the appropriate position of the fragments. On the right, deletions falling into the same group are bracketed. On the left properties of deletion vectors are indicated: TF = transformation frequency; enzyme = APH II enzyme activity as determined by the lysate assay; mRNA = transcription levels; ND = not determined.

Results

Deletions in the 5′ flanking region of Act6

In order to examine the *cis*-acting regulatory regions, 5′ and 3′ deletions were made in the 719 bp upstream region of the *Act6* cloned into pUC plasmid vectors or M13 phage vectors (see Materials and methods). DNA sequencing of the 5′ flanking region, which contains very few restriction sites, was accomplished by sequencing a series of overlapping deletions. The sequence of this region and the positions of the 5′ deletion used in the subsequent studies are shown in Figure 1. In addition, a series of 3′ end deletions were made and used to construct internal deletions (see Materials and methods). A graphic representation of the deletions used for subsequent analysis is shown in Figure 2.

Transformation efficiency of deletion vectors

To examine the effect of the deletions, the vectors were transformed into *Dictyostelium* strain KAx-3 as described previously (Nellen *et al.*, 1984a,b; Nellen *et al.*, 1986). No differences in transformation properties were observed for a given deletion whether phage M13- or pUC-based vectors were used. This enabled us to construct deletion vectors in M13 and at the same time isolate single-stranded DNA for sequencing and double-stranded replicative form DNA for *Dictyostelium* transformation. Transformation efficiency was examined by comparing the number of plaques formed by different transformants on black filters (Nellen *et al.*, 1984a). pB10SX, the undeleted vector, and pBR322 (negative control) were included in all experiments. The results indicated that deletions fall into three groups: Group I consists of deletions Δ1−Δ4 and all of these had a transformation efficiency similar to pB10SX (~10^3 transformants/plate; Nellen and Firtel, 1985); Group II (Δ4−Δ8) transformed with a significnatly (~10-fold) lower efficiency while Groups III (Δ9−Δ12) and IV (the internal deletions 3′Δ1−9 and 3′Δ1−11)) did not generate any G418-resistant colonies (see Figure 2).

Stable transformants of Groups I and II were selected by increasing the drug concentration in the growth medium to 5 μg/ml (Nellen and Firtel, 1985). Once the cells were growing under these conditions no difference in growth properties was detected between Group I and II transformants. Transformation efficiencies were confirmed by at least three independent transformations with each vector. Deletion Δ10, a representative of Group III, which did not transform by itself, was co-transformed into *Dictyostelium* cells with vector pNEoI-T which contains the *Neo*^{R} gene from Tn*903* fused to the Actin 15 promoter (Knecht *et al.*, 1986). This gene fusion does not share any sequence homology with the *Act6*−Tn*5* fusion although it also encodes a kanamycin phosphotransferase (APH I) and transforms *Dictyostelium* cells with a similar efficiency to pB10SX. Stable co-transformants carrying both vectors in high copy number were recovered.

5′ deletions do not affect copy number in transformants

It was possible that the differences in the transformation frequencies were due to upstream sequences associated with integration of the vector DNA and/or amplification and not involved in gene regulation. We have isolated DNA from all transformants and examined the copy number of the vector DNA by DNA blot analysis. DNA from representative transformants of each group is shown in Figure 3. Group I and Group II transformants contain an estimated 100 copies of the deletion vector. This was determined by comparing the intensity of the hybridization signal with a known amount of DNA in a marker lane and the relative amount of DNA from *Dictyostelium* mini-preps loaded in each lane determined by the relative amount of rDNA (data not shown). The Group III co-transformant contains ~150 copies

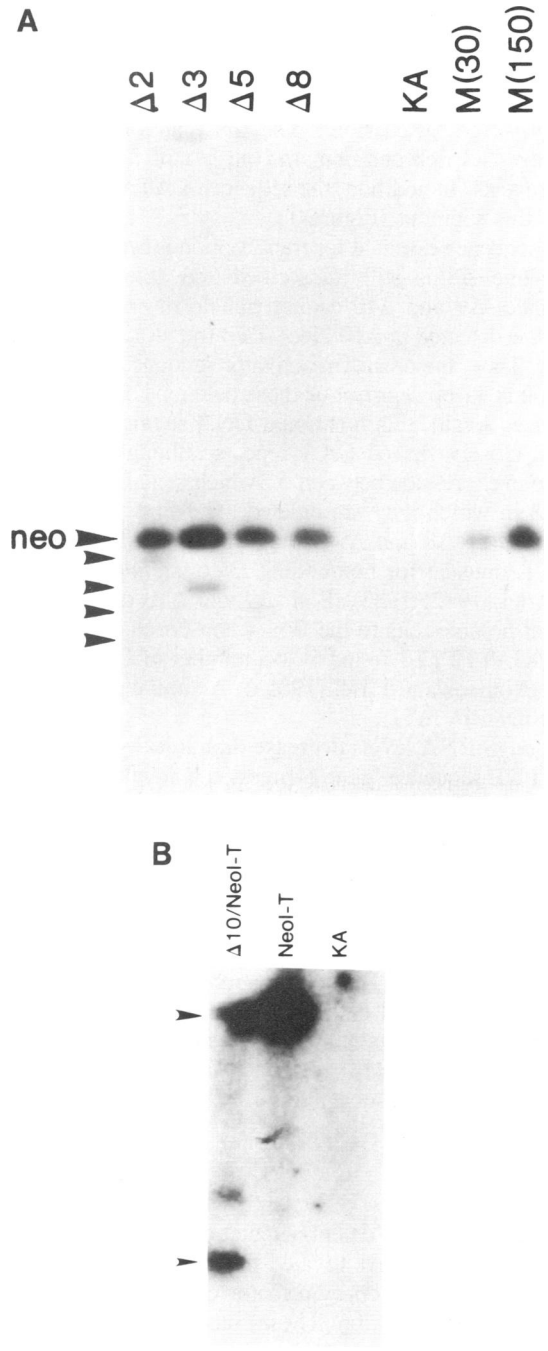

Fig. 3. Vector DNA in transformants. Approximately 1 μg of mini-prep DNA cut with *Pst*I was separated on a 1% horizontal agarose gel and blotted to nitrocellulose or Biodyne membrane. Hybridization was performed with nick-translated B10SX DNA. KA = DNA from untransformed KAx-3 cells. M(30) and M(150) is pB10SX marker DNA at 30 and 150 copy equivalents/genome. To determine the actual copy number as discussed in the text, the amount of DNA was normalized to the amount of rDNA as seen by visible ethidium bromide staining bands since one cannot directly quantitate nuclear DNA in mini-preps. (**A**) Upper arrow shows the *Neo*^{R} gene. Lower arrows show the position of 5′ fragment from gene fusion. Not all of the autoradiogram is shown. (**B**) The NeoI-T co-transformants are shown. Upper arrow is NeoI-T vector and lower arrow is the *Neo*^{R} gene from pB10SX-Δ10. Only part of the autoradiogram is shown.

of the NeoI-T vector, and ~50 copies of the deletion vector. This is somewhat different from our previous observations (Nellen and Firtel, 1985) where both vectors were found in roughly equal copy number in co-transformants but similar to

Table I.

Strain	Enzyme activity (units/mg)
KAx-3	background
pB10SX	1.3×10^5
Δ5	4.6×10^4
Δ6	5.9×10^4
Δ8	3.2×10^4

1 unit is defined as the amount of enzyme which will catalyse the formation of 1 pmol of phosphorylated kanamycin in 1 min (M.Weinstein and W.F.Loomis, unpublished observation).

observations with other constructs (S.Datta and R.A.Firtel, in press). These data show that low transformation frequency in the deletion vectors is not the result of low copy number or the failure of the plasmids to become established in transformed cells.

Expression of the Act6−Neo^R *gene fusion*

We have examined the level of expression of the *Act6−Neo*^{R} gene fusion in transformants by assaying kanamycin phosphotransferase activity and mRNA levels. The Tn*5*-encoded kanamycin phosphotransferase (APH II) can be easily assayed on a native gel (Fregien and Davidson, 1985; Nellen *et al.*, 1984a) or in a cell lysate (M.Weinstein and W.F.Loomis, unpublished observation). The second method was used to measure enzyme activities in transformants from Groups I and II. As shown in Table I enzyme activities are reduced ~10-fold in Group II transformants.

It has been previously shown by *in vivo* pulse labeling studies and nuclear run-on studies that total actin mRNA expression is regulated at the level of transcription (Kindle *et al.*, 1977; Kindle, 1978; W.McCarron and A.Jacobson, personal communication; U.Saur and W.Nellen, unpublished observation). The kinetics of appearance of mRNA expressed from 15 of the 17−20 actin genes, including Actin 6, has been quantitated throughout growth and development (McKeown and Firtel, 1981a; Romans *et al.*, 1985; Knecht *et al.*, 1986). Actin 6 mRNA is present at moderate levels during vegetative growth, increases during the first hours of development and then decreases thereafter. We have previously shown that the *Act6−Neo*^{R} gene fusion has a regulation similar to the endogenous Actin 6 gene in transformants.

To examine transcription of *Act6−Neo*^{R} genes directed by the deleted promoters, RNA was isolated from vegetative cells and from cells developed for 10 h in shaking culture (Mehdy *et al.*, 1983) and probed for the expression of *Act6−Neo*^{R} gene transcripts and endogenous actin mRNAs as an internal control (see Figure 4). In all Group I transformants, *Neo*^{R} mRNA levels were indistinguishable from those in pB10SX transformants: in vegetative cells expression is high and decreases to ~1/10 by 10 h of development. An example of this is shown in Figure 4 (data from other Group I deletions not shown). In contrast, Group II transformants had significantly reduced mRNA levels in vegetative cells, ~1/10 of that in Group I transformants or roughly equal to RNA levels in 10 h cells of Group I transformants. After 10 h of development, *Act6−Neo*^{R} RNA was still detectable but the level was ~10-fold lower than in vegetative cells (see example in Figure 4 and data not shown) showing that the developmental down-regulation of expression is still present in Group II deletions. No difference was detected in mRNA levels of any of the Group II transformants (data not shown). In pB10SX-Δ10/NeoI-T co-transformants, no mRNA transcribed from the *Act6−Neo*^{R} fusion could be detected (Figure 4B)

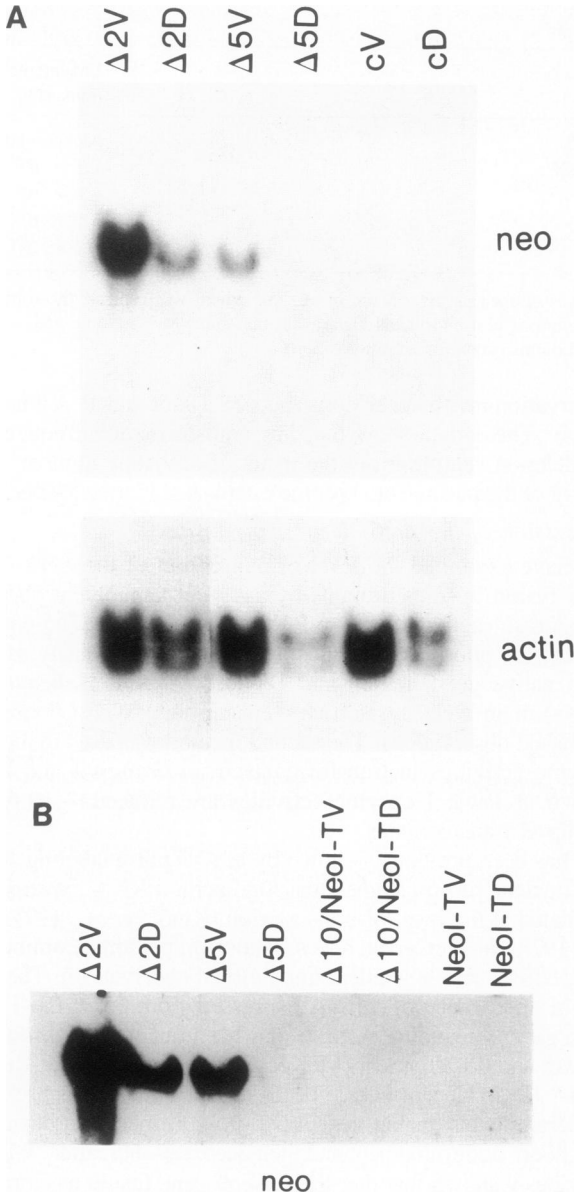

Fig. 4. Transcription of the *Act6*−*Neo*^{R} gene fusions. 5 μg of total RNA were loaded in each lane of 1.2 or 1.3% vertical agarose gels containing formaldehyde. Hybridization was performed with either a nick-translated purified *Neo*^{R} gene fragment insert or a nick-translated actin gene insert on parallel filters. (**A**) shows the expression of Δ2, a group I, and Δ5, a Group II deletion. c = RNA from untransformed cells; V = vegetative cells, D = 10 h developed cells. **Upper panel**: *Neo*^{R} gene probe. **Lower panel**: actin gene probe. The two different sized actin mRNAs are seen (see McKeown *et al.*, 1978). (**B**) shows an over-exposure of a blot containing RNA from Δ2 and Δ5 plus RNA from Δ10 and NeoI-T co-transformants and NeoI-T transformants to show the absence of *Act6*−*Neo*^{R} transcripts from Δ10 in the co-transformants. As expected no *Act6*−*Neo*^{R} transcripts are seen in NeoI-T transformants. Tn*903* *Neo*^{R} gene does not cross-hybridize to the Tn*5* *Neo*^{R} gene probe used to detect RNA from pB10SX.

though the level and developmental control of endogenous actin mRNAs were identical to the control untransformed cells or other transformants (see Figure 4A and data not shown).

Sequence analysis of the Actin 6 5′ flanking region

Sequences involved in the regulation of transcription could be specified by comparing transcription levels of different deletion vectors. The sequence responsible for the ~10-fold increase in mRNA levels is bracketed by deletions Δ4 and Δ4A or is interrupted by the Δ4A break point; Δ4 shows high levels of expression while decreased mRNA levels are first observed in Δ4A (data not shown). Thus part or all of the positive upstream element (PUE) is located within the 27 bp between deletions Δ4 and Δ4A (see Discussion). The first half of this sequence is relatively GC rich and contains the last of five closely spaced CAA repeats. In addition, the sequence GATA is repeated twice within this segment (Figure 1).

The sequence essential for transcription is bracketed by Δ8 and Δ9. While Δ8 is still transcribed and able to confer G418 resistance, Δ9 and Δ10 cannot transform and no *Act6*−*Neo*^{R} mRNA is detected in Δ10/NeoI-T co-transformants (Δ9 was not tested). Thus, the upstream activator sequence (UAS) is located within this 35 bp segment or these deletions disrupt an essential sequence. Again, this functional DNA segment is preceded by several closely spaced CAA repeats although there is no difference in expression between Δ7 which contains these sequences and Δ8 in which they are deleted. The first part of the region delineated by Δ8 and Δ9 containing the UAS is also GC rich which is unusual for non-coding *Dictyostelium* DNA (Kimmel and Firtel, 1982, 1983). Both Δ9 and Δ10 disrupt a sequence element homologous to the Box 4 consensus sequence AAAAATGGGATTTTTT found 5′ to a number of *Dictyostelium* actin genes (Romans and Firtel, 1985a). A similar sequence element is disrupted by Δ11.

Although RNA levels decrease dramatically after elimination of the PUE sequence, gene expression is regulated indistinguishably from the endogenous *Act6* gene in all further deletions which can still promote transcription. It cannot be excluded that two functional sequences, a UAS and a promoter with developmental specificity, are located in the 35 bp fragment between Δ8 and Δ9. Moreover, sequences further 3′ between the end point of the Δ9 and the TATA box could also be necessary for differential gene expression. The combination of 3′ Δ1 with Δ9 and Δ11 generates an internal deletion of 324 and 401 bp respectively. Both constructs contain the PUE sequence but are unable to transform cells to G418 resistance, indicating that the PUE sequence alone cannot substitute for the UAS element and promote transcription.

Discussion

Functional assays of deletion mutations within the 5′ flanking sequences of *Act6* have revaled two functional regions: a PUE increasing transcription by a factor of 10 and a UAS which is essential for transcription. These constructs containing the UAS exhibit proper developmental regulation suggesting the possibility that those sequences essential for promoter function may also confer the pattern of expression during development. Sequences responsible for the PUE function reside in a 27 bp fragment between Δ4 and Δ4A or are disrupted by Δ4A. Four closely spaced CAA repeats proceeding this segment and three GATA repeats within this segment are unusual features (see Figure 1). However, these sequences are also found elsewhere in the 719 bp *Act6* 5′ flanking region and we do not know whether these repeats are involved in the increase of transcription. At present, we also cannot discount the possibility that the PUE could lie 5′ to the end of Δ4 and that enhancing function of the element is repressed by the close proximity of M13 or pUC vector sequences.

The UAS lies between Δ8 and Δ9 or is dissected and inactivated in Δ9. The 5′ end of this region is GC region. We have noted that Δ9 disrupts the sequence AAAAATGGGGTTTTT homologous to the consensus sequence AAAAATGGGATTTTT

found upstream in a number of actin genes and denoted Box 4 in Romans and Firtel (1985b). Since the differential expression of the *Act6* gene is regulated at the level of transcription and the 719 bp fragment is sufficient to generate the same expression pattern in a gene fusion, regulatory sequences have to be present in this fragment. Because transcription is lost in consecutive deletions before changes in differential regulation can be observed, the regulatory regions have not been identified directly in this experimental approach. It is striking, however, that Cohen *et al.* (in press) also find a loss of transcriptional activity before detecting changes in the differential expression pattern in deletions of the Actin 15 promoter. Analogous to our findings with the Actin 6 promoter, they find no more transcription after deleting the oligo (A) tract of Box 4. Moreover, internal and linker-scanner deletion analysis of a 35 bp region containing the transcription control elements of a *Dictyostelium* pre-stalk-specific gene *pst-cath* shows a similar pattern to that seen with *Act6*; i.e. an internal deletion removing part of this short element results in the loss of promoter function as well as no longer showing the proper temporal and spatial pattern of expression (S.Datta and R.A.Firtel, in press). In both Actin 6 and 15 genes there is a CCC with a short oligo(dA) stretch on one side and short oligo(dT) stretch on the other side. Though this homology is striking, the *Act6* deletion series clearly shows that loss of the CCC motif at −289 in itself does not affect transcription. It is important to note, however, that the sequence organization of the CCC-containing region is different to the complement to Box 4 (see Figure 1). The similarities between Actin 6 and Actin 15 suggest that the destruction of Box 4 could be responsible for the loss of transcription activity. It should be noted that the 5′ flanking region of the Δ9 construct still contains a region (see boxed sequence at −136 in Figure 1) which is homologous to Box 4 but contains two rather than one dT residues preceding the GGG. If Box 4 is the *cis*-acting regulatory region for Actin 6, it would suggest that this extra dT residue destroys the function of the sequence or that more than one copy of Box 4 is required. It is possible that the lack of transcription of the Δ9 construct could result from the juxtaposition of plasmid sequences next to the deletion end point. However, this does not appear to be the case since construct 4′Δ1/9, which contains 151 nucleotides of 5′ Actin 6 sequence added 5′ to Δ9, is also not expressed.

The combination of 3′Δ1 with Δ9 and Δ11 generates an internal deletion of 324 and 401 bp respectively. Both constructs contain the PUE sequence but are unable to transform cells to G418 resistance. This demonstrates that the PUE sequence alone cannot substitute the UAS element and promote transcription. We do not feel that it is likely that there are effects of sequences adjacent to the 5′ end of the deletions on transcriptional activity since the constructs were cloned into two different vectors, M13 and pUC, with and without *Hin*dIII linkers. In addition, the addition of the ~180 nucleotides of 3′Δ1 *Dictyostelium* sequence 5′ to deletions Δ9 and Δ11 does not restore promoter function in the 3′Δ1/Δ9 and 3′Δ1/Δ11 constructs even though these sequences are very AT rich in contrast to the M13 or pUC vector sequences. At the present time, we do not know whether the PUE has a similar enhancing activity on other actin genes or other genes expressed at different developmental stages.

In our previous transformation experiments stable transformants always contained a high copy number of the transformation vector and the *Act6*−*Neo*^{R} gene fusion is transcribed at high levels. This suggested that high levels of *Act6*−*Neo*^{R} gene mRNAs were necessary to confer G418 resistance. Our present data indicate that this is not the case since Group II transformants have only 10% the fusion gene mRNA and 25−50% of APH II activity as pB10SX transformants and contain the same number of copies of the vector. However, since the transformation frequency is reduced 10-fold, we cannot discount that the site of integration may be important in controlling the number of productive integrations and transformants. It should be noted that the difference between mRNA and protein levels can be accounted for by the recent observations (W.Thienen, H.MacWilliams and W.Nellen, unpublished observation) that the APH II enzyme is very stable and is accumulated. Therefore, the high copy number integration of the vector is not directly related to the production of high levels of APH II.

Materials and methods

Construction and mapping of deletions

Deletions were made from the single *Eco*RI site lying 719 bp 5′ to the *Act6* translation start site in pB10SX using *Bal*31 exonuclease (see Figure 1). Plasmid B10SX (Nellen and Firtel, 1985) was linearized at the *Eco*RI site 5′ to the Actin 6 5′ flanking sequences and digested with *Bal*31 and the ends repaired with the large fragment of DNA polymerase I. After resection, the *Act6*−*Neo*^{R} gene carrying the *Act8* 3′ termination/polyadenylation region was excised with *Bam*HI (Figure 2) and ligated into a plasmid three different ways: for deletions Δ1, Δ2, Δ3, Δ4, Δ4A and Δ5 the fragment was cloned into the *Bam*HI/*Hin*cII sites of pUC18 after repairing the end product by exonuclease *Bal*31 with the Klenow fragment of DNA polymerase I. For deletions Δ9 and Δ11 the end produced after *Bal*31 end was also blunt-ended, *Hin*dIII linkers were added, the fragment was excised with *Bam*HI and cloned into the *Hin*dIII/*Bam*HI sites of pUC18. For deletions Δ6, Δ7, Δ8, Δ10 and Δ12 the end produced by digestion with *Bal*31 was repaired, the *Bal*31/*Bam*HI fragment was isolated and ligated into the *Bam*HI/*Hin*cII sites of M13mp19.

*Bal*31 deletions from the 3′ end of the *Act6* 5′ flanking region were made starting at the *Hin*dIII site lying at nucleotide +23 in the *Act6* coding region from vector pA6T5, the original *Act6*−*Neo*^{R} fusion gene (Nellen *et al.*, 1984). The ends were repaired and religated using *Hin*dIII linkers. A single 3′ deletion, 3′ Δ1 was used to make internal deletions by fusing it to 5′ deletions Δ9 and Δ11 at the *Hin*dIII sites at the end of the respective deletions (3′ Δ1−Δ9 and 3′ Δ1−Δ11).

The location of the deletions was mapped by dideoxy sequencing (Sanger *et al.*, 1977) after cloning the respective fragment into M13 vectors. For the deletions Δ6, Δ7, Δ8, Δ10 and Δ12 subcloning was not necessary. Deletion mapping extended the 5′ flanking sequence of the *Act6* gene (Romans and Firtel, 1985a) to position −719 (Figure 3).

Deletion vectors were constructed using standard procedures (Maniatis *et al.*, 1982). DNA sequence determination was carried out by the dideoxy chain termination method (Sanger *et al.*, 1977).

Dictyostelium transformation

Dictyostelium transformants were obtained as described (Nellen *et al.*, 1984; Nellen and Firtel, 1985; Nellen *et al.*, 1986) using the KAx-3 strain of *Dictyostelium discoideum* (Poole and Firtel, 1984).

Other methods

Mini-pre DNA isolation from *D. discoideum* and Southern blots were carried out as described previously (Nellen and Firtel, 1985; Nellen *et al.*, 1986). Large-scale plasmid DNA preparations were performed by a modification of the Birnboim and Doly (1979) protocol: the crude preparation was treated with RNase A, phenol extracted twice, ethanol precipitated and redissolved. DNA was then precipitated with 6.5% polyethylene glycol followed by another ethanol precipitation. RNA isolation and Northern blots were performed as described by Mehdy *et al.* (1983).

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