

# Identification and characterization of multiple A/T-rich *cis*-acting elements that control expression from *Dictyostelium actin* promoters: the *Dictyostelium actin* upstream activating sequence confers growth phase expression and has enhancer-like properties

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## ABSTRACT

The promoter elements in the *Dictyostelium actin 15* and *actin 6* genes required for full growth phase expression were identified by assaying promoter/luciferase reporter constructs. We find that these promoters contain common *cis*-acting elements, an actin upstream activating sequence (UAS) and sequences proximal to the transcription start site that overlap with a poly(dT) region. The *actin 15* promoter has two additional *cis*-acting elements not present in the *actin 6* promoter that may account for the higher level of expression from the *actin 15* promoter. All of the identified promoter elements are unusual for *Dictyostelium* in that they are all A/T-rich. Two *cis*-acting elements, the actin UAS and the poly(dT) domain were studied in greater detail. The actin UAS was tested on a heterologous promoter from the prespore-specific gene *SP60* and shown to have the ability to confer growth phase expression. The actin UAS also exhibited the ability to function in a distance- and orientation-independent manner and activate expression synergistically when present in two copies. The poly(dT) domain of the *actin 15* promoter was studied in greater detail by using a genetic selection scheme to define parameters that effect the strength of this element. This element is comprised of 45 consecutive dT residues immediately upstream of the putative TATA box. We show that the length of the homopolymer dT region correlates with the expression level of the promoter. The poly(dT) element is also shown to function to promote wild-type levels of expression with small deviations in the sequence, indicating that the element is not required to be homopolymeric to function.

## INTRODUCTION

*Dictyostelium discoideum*'s asexual life cycle can be divided into vegetative growth and developmental phases (1). *Dictyostelium* cells grow vegetatively as unicellular amoebae when nutrients are plentiful. Depletion of nutrients initiates the developmental phase, which takes ~24 h and culminates in the formation of a fruiting body, consisting of spore cells supported by a slender stalk.

The relatively simple life cycle of *Dictyostelium discoideum* facilitates the study of gene expression in a eukaryote during the growth and development of the complete organism. Recent studies on gene expression in this system have concentrated on determining upstream elements that control transcriptional activation during the developmental phase and have identified a number of elements, including those that control expression mediated through cell surface cAMP receptors (2–4). In contrast, comparatively little is known about transcriptional regulatory elements controlling growth phase gene expression or the essential characteristics of promoters transcribed by RNA polymerase II in this system. *actin* promoters, which direct expression at high levels during vegetative growth, can be used as an experimental system for studying these questions.

The 20 member *actin* gene family of *Dictyostelium* has been previously characterized (5,6 and references within). Actin RNA constitutes 2–3% of the RNA synthesized during the vegetative phase (7), increases ~3-fold during early development, then decreases rapidly to ~10% of its vegetative level as multicellular development proceeds. The rate of total actin protein synthesis shows a similar pattern during growth and development (8). While most individual members of the *actin* gene family that have been examined have a similar pattern of expression, differences in the expression level and developmental kinetics of individual genes have been reported (9,10). *actin 6* (*Act6*) and *actin 15*

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(*Act15*) are members of this gene family that have expression patterns similar to the expression pattern of total actin (11,12,13).

Specific promoter regions that may be sufficient to direct growth phase expression can be identified by examining *actin* promoter structure and thus lead to a better understanding of potential regulatory mechanisms controlling gene expression during this phase of the life cycle. An understanding of *actin* promoters will also facilitate the interpretation of heterologous promoter studies that define the function of cell type-specific regulatory elements using deleted *actin* promoters (3,4). The *Act15* promoter is a good candidate for such a detailed analysis of growth phase *Dictyostelium* promoters because a short fragment containing ~270 bp upstream from the transcription initiation sites has been shown to confer the same quantitative and qualitative expression pattern as 2.7 kb of upstream sequences (11). Since the *Act6* and *Act15* genes are both expressed during growth phase, analysis of these two promoters should allow us to compare the two promoters and to identify common elements responsible for generating this expression pattern and to further characterize these common elements.

In this study, we have used internal and 5' deletion mutants to examine the structure of both the *Act15* and *Act6* promoters. The analysis reveals four different *cis*-acting elements within the *Act15* promoter and three *cis*-acting elements within the *Act6* promoter. These promoter elements are all A/T-rich, which is unusual for essential *Dictyostelium* promoter elements described to date. The results of this study identify a common promoter element, designated the actin UAS, that has not been found in other promoters to date. We show that the actin UAS can confer growth phase expression on a gene not normally expressed during this phase of development and show that it has some properties of an enhancer, such as the ability to activate transcription synergistically and in an orientation- and distance-independent manner. Another common promoter element found in both promoters is a homopolymeric stretch of dT residues, which we have named the poly(dT) element. We performed a functional analysis of the *Act15* poly(dT) element that examined the relationship between promoter strength and the length and sequence composition of the homopolymer dT region. The analysis included a scheme that selects for oligonucleotides that can replace the dT residues of the homopolymer dT element and reconstitute a wild-type or increased level of promoter activity.

## MATERIALS AND METHODS

### *Dictyostelium* growth and transformation

The axenic strains KAx-3, a wild-type strain (14), and JH010, a thymidine auxotroph (15), were grown and transformed by electroporation as previously described (16,17). Constructs containing the *Thy1* gene as a selectable marker were transformed into JH010 cells and stable transformants selected in the absence of exogenous thymidine (15,16). Whole transformed populations were used in the analyses and were maintained as logarithmically dividing cultures. The copy number of the transformed DNA containing the *thy1* marker was determined by Southern hybridization analysis (18) on genomic *Dictyostelium* DNA and shown to be uniformly ~5–10 copies/cell (data not shown).

The axenic strain RH120 was derived from JH010 by transforming pRH120 (see below) into JH010 and selecting for a clonal thymidine prototroph (15). RH120 expresses the *Dictyostelium discoideum* plasmid (*Ddp*) 2 *trans* factor which allows plasmids containing the *Ddp2 cis* element to replicate

extrachromosomally (19). Constructs containing the various *Act15* promoter–neomycin phosphotransferase I (NPTI)–*Act15* 3' terminator fusions (see below) were transformed into RH120 cells and stable transformants were selected in the presence of various concentrations of G418 (20).

For the transient expression assays, constructs lacking a *Dictyostelium* selectable marker were electroporated into KAx-3 cells (16,17) and then harvested for transient expression assays 12–24 h later (see below).

### Luciferase assays

Luciferase assays were done as described previously (17) on vegetative whole cell extracts made from logarithmically growing cells.

### 5' and internal deletion constructs of the *Act15* and *Act6* promoters

The *Bam*HI fragment from VIIIuc containing the *Act15* promoter–luciferase–2H3 3' terminator gene fusion (17) was cloned into the *Bam*HI sites of pAT153L (21) and pGEM7Zf(+) (Promega) producing pAct15  $\Delta$ -299 and pRH6, respectively. 5' and 3' deletions were generated by unidirectional exonuclease III digestions of pAct15  $\Delta$ -299 and pRH6, respectively, followed by sequential treatment with S1 nuclease and T4 DNA polymerase (see Figure 1A for deletion end points). The *Bg*III linker, 5' GAAGATCTTC, was added to the end point of each deletion. The internal deletions were constructed by inserting the *Bam*HI–*Bg*III fragment from the 3' deletion into the *Bg*III site of the 5' deletion or the *Aar*II–*Bg*III fragment from the 3' deletion into the *Aar*II and *Bg*III sites of the 5' deletion (see Figure 2). In both cases, the *Bg*III linker, 5' GAAGATCTTC, is introduced at the fusion site of the internal deletions. (Note: when the deletion constructs are referred to by name, they will be designated pAct15  $\Delta$ -# or  $\Delta$ -\*/-#, where # indicates the nucleotide at the 5' deletion end point and \* indicates the nucleotide at the 3' deletion end point.)

The ~750 bp *Eco*RI–*Hind*III fragment containing the 5' non-coding region and the first eight amino acids of *Act6* (6) was cloned into the *Eco*RI and *Hind*III sites of pGEM7Zf(+) (Promega) producing pRH2. pRH2 was digested with *Hind*III and the 1.8 kb *Hind*III fragment from VIIIuc (17) containing the luciferase coding region was cloned into the *Hind*III site producing pRH3. pRH3 was digested with *Cla*I, which digests in the luciferase coding region, and *Bam*HI. The *Cla*I–*Bam*HI fragment containing the luciferase–2H3 3' terminator fusion fragment from VIIIuc was inserted to produce pRH5. The *Act6*–luciferase–2H3 3' terminator fusion fragment from pRH5 was isolated as a *Bam*HI fragment and cloned into the *Bam*HI site of pAT153L (21) to produce pAct6–722. 5' and 3' deletions were generated by unidirectional exonuclease III digestions of pAct6–722 and pRH5, respectively, followed by sequential treatment with S1 nuclease and T4 DNA polymerase. *Bg*III linkers, GAAGATCTTC, were added to the end points of each deletion. The internal deletions were constructed by inserting the *Bam*HI–*Bg*III fragment from the 3' deletion into the *Bg*III site of the 5' deletion (see Figure 2). (Note: the 5' deletions will be designated pAct6–# and the internal deletions pAct6–\*/-#, where # denotes the nucleotide at the 5' deletion end point and \* denotes the nucleotide at the 3' deletion end point, respectively.)

The constructs for selecting stable transformants were made by inserting a ~3 kb *Thy1 Sal*I fragment from thy-IP 42 (16) into the unique *Sal*I site of the reporter constructs (see Figure 2).

The *Thy1* fragment encodes a protein that can complement JH010, a thymidine auxotroph (see above). The orientation of the *Thy1* fragment was determined by using an asymmetrically located *NsiI* site in the *Thy1* fragment. Constructs that were used for subsequent experiments were chosen such that the *Thy1* and *Act15* promoters transcribed in the same direction (see Figure 2) to prevent interference between the two promoters.

#### DNA curvature analysis

The ~50 bp *BamHI*–*BglIII* fragment from the *Act15* promoter 3' deletion  $\Delta$ –260 (see Figure 1A) was inserted into the *BglIII* site of pCY4 (22). The construct was digested individually with the following restriction enzymes: *EcoRI*, *HindIII*, *BstNI*, *EcoRV*, *NheI* and *BamHI*. The reactions were subsequently extracted with phenol/chloroform, precipitated with ethanol and assayed by electrophoresis on 8% polyacrylamide gels. The gel was stained with ethidium bromide and a photograph taken on a short-wave UV light box.

#### Site-directed mutagenesis to generate the *Act15* 5' deletion to –123

Site-directed mutagenesis to convert the dA at position –127 to a dG and the dT at position –124 to a dC, thereby creating a *BglIII* site (see Figure 1A), was carried out in the following manner. The ~750 base pair *BglIII*–*EcoRI* fragment from the 5' deletion construct pAct15  $\Delta$ –138 was subcloned into the *BamHI* and *EcoRI* sites of M13mp19. Single-stranded DNA was prepared from this construct to serve as the template for site-directed mutagenesis (18) using the oligonucleotide primer 5' AAAAAAAAAAAGATCTTTGAATCCCGGAAGA. Site-directed mutagenesis was carried out according to the manufacturer's directions using the Amersham Oligonucleotide-Directed *In Vitro* Mutagenesis Kit, Version 2. The resultant clones were screened for the creation of a *BglIII* site and sequenced to verify the constructs contained the desired mutations. Sequencing of the downstream homopolymer dT region (see Figure 1A) showed that it contained three additional dT residues, possibly due to strand sliding in this region (23). The *BglIII*–*EcoRI* fragment from the site-directed mutant was fused to the luciferase reporter gene by subcloning it back into the *BglIII*–*EcoRI* vector backbone of a 5' deletion construct to generate pAct15  $\Delta$ –123.

#### Generating the *Act15* promoters containing a double point mutation

A fragment containing the sequence from –299 to –128 of the *Act15* promoter was generated by a polymerase chain reaction using the pBR322 *EcoRI*(cw) sequencing primer (Promega) and the oligonucleotide 5' AAAAAAGATCTTTGAATCCCAT as primers directed against pAct15  $\Delta$ –299 (see above). The resulting fragment was digested with *BamHI* and *BglIII*, gel-purified and inserted in the wild-type orientation into the *BglIII* site of pAct15  $\Delta$ –123, generating the parental *Act15* promoter with point mutations at positions –127 and –124 (see Figures 1A and 6). Where indicated, the sequence GATC was deleted from these promoters by digesting with *BglIII* and treating with S1 nuclease.

#### SP60 promoter–luciferase expression vector constructs

SP60 5' $\Delta$ 20luc and SP60 5' $\Delta$ 42luc, two SP60 5' promoter deletions fused to the firefly luciferase reporter gene, both contain

the same polylinker with a *BamHI* site immediately adjacent to the deletion end point (21). Oligonucleotides containing an actin UAS (see Figure 8A) were cloned into the *BamHI* site. The number of oligo inserts was identified by digesting with *HincII/EcoRI* or *XbaI/EcoRI*. The orientation of the inserts was determined by digesting with *HincII/BamHI*.

#### Construction of the *Act15*–NPTI and *Ddp2* trans factor expression vectors

pRH128 is a shuttle vector (see Figure 9A) used to test the ability of various wild-type and mutant *Act15* promoters to confer G418 resistance and was constructed in the following way. pRH105, a plasmid containing the *Ddp2* cis region which allows a plasmid to replicate extrachromosomally in cells expressing the *Ddp2* trans factor (19), was constructed by inserting an ~700 bp *HindIII*–*ClaI* fragment from *Ddp2* into the *HindIII/ClaI* sites of a pUC19 derivative (24). A plasmid containing the neomycin phosphotransferase I (NPTI) gene was digested at the *XhoI* site located at the 5' end of the NPTI gene, filled-in with Klenow and a *BamHI* linker, 5' AGTATGGATCCACT, added to generate pRH119. A 1.3 kb *PvuI*–*EcoRI* fragment from pA15T containing the 3' end of the NPTI gene and the *Act15* 3' terminator (11) and the 0.4 kb *BamHI*–*PvuI* fragment from pRH119 containing the 5' end of the NPTI gene was inserted into the *EcoRI* and *BamHI* sites of pRH105 in a 3-way ligation to generate pRH128.

The final gene fusions were constructed by inserting the desired *Act15* promoters as a *BamHI* or *BglIII*–*BamHI* fragment at the unique *BamHI* site in pRH128 and confirming the proper orientation with *HindIII* and *BamHI*. A *BamHI* site was placed on the 3' end of each *actin* promoter fragment by digesting with *HindIII*, filling-in with Klenow and adding the *BamHI* linker 5' AGTATGGATCCACT. On the 5' end, pAct15  $\Delta$ –299 was digested at the *BamHI* site, pAct15  $\Delta$ –183 and  $\Delta$ –95 were digested with *BglIII* and the *Act15* promoter deleted to –162 had a *BamHI* site placed at that position by digesting with *DraI* (see Figure 1A) and adding the *BamHI* linker, 5' CGGATCCG.

The plasmid pRH120 was used to generate the cell line expressing the *Ddp2* trans factor. The plasmid was constructed as follows. The *BglIII*–*KpnI* fragment encoding the *Ddp2* trans region (19) was inserted into the *BglIII* and *KpnI* sites of pSP72 (Promega) to construct '860'. pRH120 was made by inserting the ~3.0 kb *Thy1* *SalI* fragment from thyIP-42 (16) into the *SalI* site of '860'.

#### Generating constructs with different length homopolymer dT regions

*Act15* promoters containing a homopolymer dT element of either 19 or 11 dT residues were made by digesting at the *BglIII* site of  $\Delta$ –121/–95 and  $\Delta$ –121/–95 truncated to –162 using *DraI* (see above), filling-in with Klenow and inserting the *BspMI* oligonucleotide 5' GCAGGTGCGGCCGACCTGC (see Figure 9B). This oligo contains a *BspMI* site on each end. *BspMI* is a restriction enzyme that digests in the region flanking its recognition sequence. By digesting with *BspMI*, filling-in with Klenow and self-ligating the plasmid, eight nucleotides are removed. Rather than self-ligating the plasmid, another *BspMI* oligo can be inserted and another '*BspMI* cycle' performed to remove another eight nucleotides. With two and three cycles, promoters with only 19 and 11 dT residues in the poly(dT) element were generated, respectively.



the parental level of expression (11). To examine the region required for full expression, additional 5' deletions between -299 and -183 were examined. An *Act15* promoter deleted to -275 showed no decrease in expression level relative to the parental promoter, while promoter deletions to -244, -233, -211 or -183 all had similar 3- to 6-fold lower levels of expression, indicating that a positive-acting region overlaps with the region between -275 and -244 (see Figure 3).

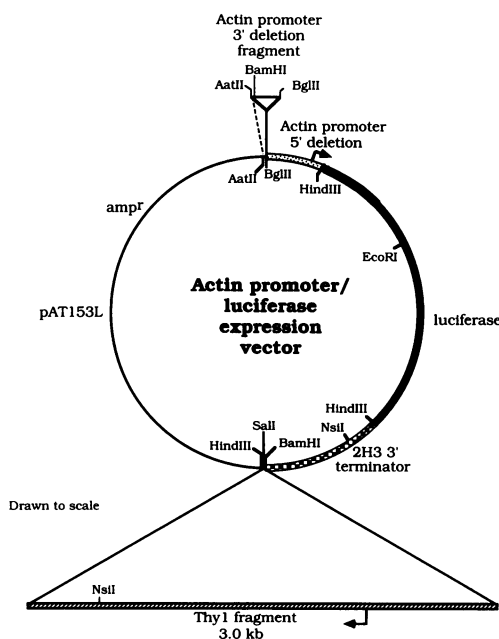
The DNA overlapping with the sequence between -275 and -244 contains two noteworthy characteristics. A 9 bp direct repeat, ATTTATTTT, lies between -279 and -261 (see Figure 1) and three sets of 4 or 5 consecutive dT residues that are spaced one helical turn (10 bp) of DNA apart lie between -284 and -260. The latter region of DNA would be predicted to be curved based on the periodicity of the dT residues (25). This prediction was tested using a circular permutation assay with the plasmid pCY4 (22). The plasmid pCY4 contains a 375 bp tandem repeat around the polylinker. When a fragment is inserted into the pCY4 polylinker and the construct digested with a series of restriction enzymes, a set of fragments in which the insert is circularly permuted with respect to the ends is generated (see Figure 4A). The presence of curved DNA can be detected because a DNA fragment with a curved region near the center migrates more slowly than DNA fragments with curvature at an end (22). A DNA fragment containing the sequences between -299 and -260 was inserted into the pCY4 *Bgl*III site. As shown in Figure 4B, when the insert moves closer to the center of the fragment, there is a decrease in the mobility of the DNA, indicating that the insert DNA does contain an intrinsic bend. On the basis of

this characteristic, this region was designated the 'curved DNA element'.

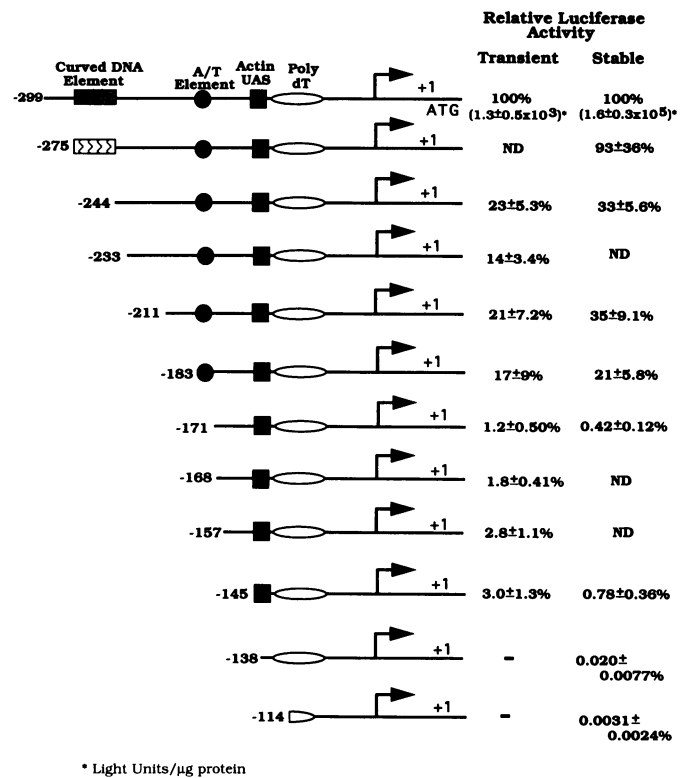
**Identification of a *cis* element co-localizing with a region composed entirely of A/T residues**

Deletion of the promoter from -183 to -171 (see Figure 3) resulted in a further decrease of 50- and 12-fold in the expression level as assayed by the stable and transient expression systems, respectively. Expression levels remained unchanged with further 5' promoter deletions to -145.

To confirm the location of a regulatory element between -183 and -171, small internal deletions were made by fusing the 3' deletion to -194 to the 5' deletions pAct15 Δ-183 and Δ-171. The internal deletion Δ-194/-171 had a 6-fold decrease in expression, while Δ-194/-183 had an expression level similar to the parental promoter (see Figure 5). The internal deletions Δ-194/-168 and Δ-194/-145 had approximately the same expression level as Δ-194/-171 in the stable and transient expression assays, indicating that a *cis* element overlaps with the



**Figure 2.** *Actin* promoter-luciferase expression vector. The *actin* 5' region-luciferase-2H3 3' terminator gene fusion are represented by the stippled region, the thick black line and the checkered region, respectively. The backbone, pAT153L, is denoted by a thin black line and the *Thy1* selectable marker is represented by a diagonally striped line. Restriction enzyme sites used to construct the 5' and internal deletions of the *actin* promoters are shown as well as the *Sall* insertion site of the *Thy1* selectable marker (see Materials and Methods). Arrows are used to illustrate the direction of transcription.



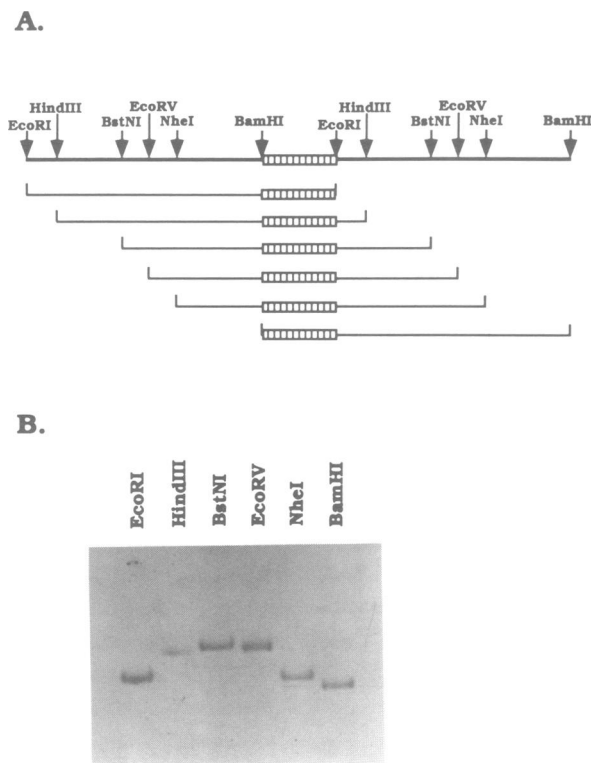
**Figure 3.** Properties of *Act15* 5' promoter deletions. The relative sizes of the 5' deletions are graphically represented and their end points shown. The translation initiation site is designated as +1. The transcription initiation sites are specified by an arrow. The *cis*-acting elements identified in this study are represented by symbols found above the parental promoter in the first line. The growth phase expression levels of the truncated promoters in both transient and stable expression assays are indicated ± the standard error of the mean normalized to the parental promoter which is assayed in parallel. The average specific luciferase activity (in light units/mg protein) is denoted for the construct containing the parental promoter. The transient expression values are the averages of at least three independent experiments conducted in duplicate. The stable expression values are the averages of three separate electroporations in which each population was assayed at least twice. N.D. indicates not determined.

region between -183 and -171. This region is composed entirely of dA and dT residues and contains a 9 bp palindromic sequence, TAATTATTA, and a 3 copy tandem repeat of the motif ATT. The difference in expression level between the 5' deletion and the internal deletion suggests part of the function of this element may be redundant. Because of the sequence composition, this region was named the A/T element.

**Identification of a cis element common to actin promoters**

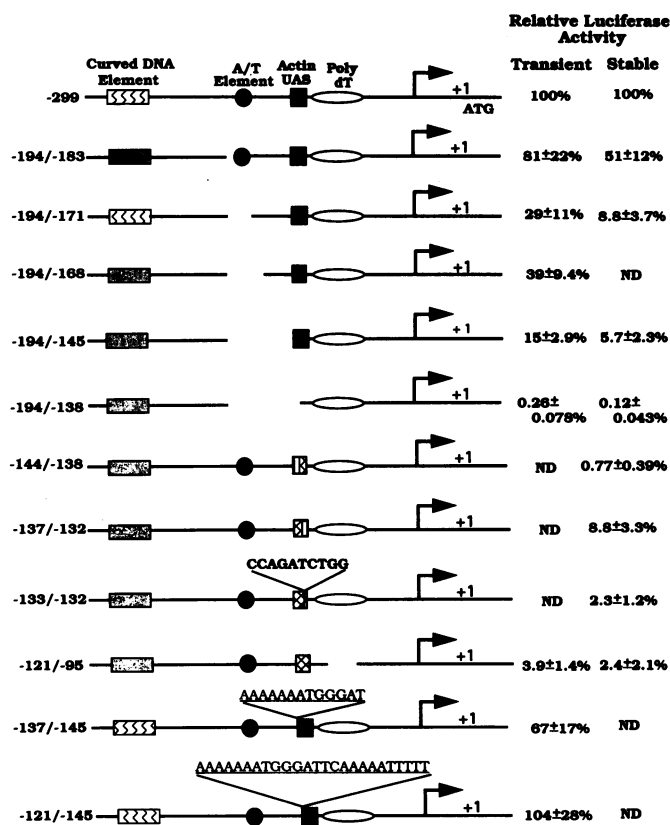
A 5' deletion from -145 to -138 resulted in further ~40- and ~8-fold decreases in stable and transient expression assays, respectively (see Figure 3). To confirm the potential importance of the region around -138, an internal deletion of this region was generated using the 3' deletion to -194. As shown in Figure 5, a 50-fold difference in expression level was observed when the internal deletions Δ-194/-145 and Δ-194/-138 were compared.

The sequence between -145 and -138 is AAAAAAA but is adjacent to the downstream sequence TGGGATT (see Figure 1A) that had been previously proposed to be a positive cis element in the *Act15* promoter (11). To directly examine the importance of this region, constructs that replace short sequences of DNA



**Figure 4.** Circular permutation assay of the *Act15* upstream sequence from -299 to -260 to test for curved DNA. (A) Structure of the region of the plasmid pRH190 that contains the *Act15* upstream sequence. The *Act15* promoter fragment from -299 to -260 is represented as a stippled box, inserted between the tandemly repeated region in the plasmid pCY4. Digestion of pRH190 individually by each restriction enzyme indicated releases a circularly permuted ~820 bp fragment. (B) Electrophoretic mobility of the circularly permuted DNA fragment. Short-wave UV photograph of an ethidium bromide stained 8% polyacrylamide gel containing the circularly permuted fragments from pRH190. Restriction enzyme digestion and electrophoresis were performed as indicated in Materials and Methods.

in and around this putative cis element with the *BgIII* linker, 5' GAAGATCTTC, were examined. The internal deletion Δ-144/-138, which replaces 5 dA residues with the *BgIII* linker, showed a 130-fold drop in expression compared to the parental construct (see Figure 5). The internal deletion Δ-137/-132, replacing the sequence GGATT with the *BgIII* linker, produced an 11-fold decrease in the expression level. The construct Δ-133/-132, which replaces a dT residue with the *BgIII* linker, exhibited a 42-fold drop in the level of expression. These sequence replacements extend the 3' border of this cis element to at least -132. The variability in the expression levels between these various promoter constructs may reflect the relative requirement for the deleted sequence or a change in spacing between other promoter elements. The region between -145 and -132 has been designated the actin UAS because it includes a sequence also found in the *Dictyostelium actin 6* promoter that



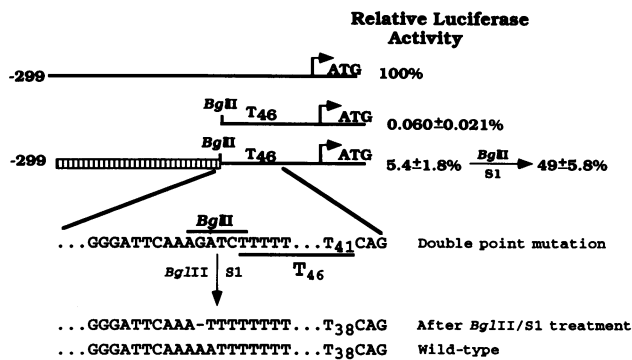
**Figure 5.** Properties of internally deleted *Act15* promoters. Internal deletions are graphically represented and the 3' and 5' deletion end points are shown. The translational initiation site is designated as +1. The transcription initiation sites are specified by an arrow. The cis-acting elements identified in this study are represented by symbols shown above the parental promoter in the first line. All the internal deletion constructs contain the *BgIII* linker, CCAGATCTGG, inserted at the junction of the 3' and 5' deletions and the *BgIII* linker sequence is written in bold above the position of the insertion in pAct15-133/-132. The sequences that are duplicated in pAct15-137/-145 and pAct15-121/-145 are underlined and written above the position of the duplication. The growth phase expression levels of the deleted promoters in transient and stable expression assays are indicated ± the standard error of the mean normalized to the parental promoter which is assayed in parallel. The transient expression values are the averages of at least three independent experiments conducted in duplicate. The stable expression values are the averages of three separate electroporations in which each population was assayed at least twice. N.D. indicates not determined.

was identified as a positive *cis*-acting element for that gene (see below).

**Mutations that alter a homopolymeric dT region affect promoter strength**

The *Act15* promoter has a 45 nucleotide homopolymeric dT [poly(dT)] region just upstream of the putative TATA box (see Figure 1A). The importance of this region was examined by creating an internal deletion, Δ-121/-95, that removed 25 of the 45 consecutive dT residues from the middle of the homopolymer dT region and inserted the *Bg*III linker, 5' GAA-GATCTTC. This deletion resulted in a 40- and 25-fold decrease in level of expression as assayed by stable and transient expression assays, respectively (see Figure 5). As compared to the 5' deletion pAct15 Δ-138, a further deletion to -114 causes a 7-fold decrease in luciferase expression (see Figure 3). Deleting the sequence between -138 and -114 removes the 3' portion of the actin UAS and 11 of the 45 consecutive dT residues. We have named the region containing 45 consecutive dT residues the poly(dT) element.

To examine the region between the actin UAS and the poly(dT) element, an *Act15* promoter containing site-directed mutations at -127 and -124 was constructed (see Materials and Methods). The double point mutation creates a *Bg*III site at -128 that lies at the border between the actin UAS and the homopolymer dT region (see Figure 1A and Figure 6 for a diagram of the constructs). As summarized in Figure 6, the double point mutation in the full length promoter caused an ~20-fold reduction in expression compared to the parental promoter. To further examine the effect of the nucleotide changes introduced by the site-directed mutagenesis, we removed the GATC sequence created by the site-directed mutation (see Figure 6B and Materials and Methods). When the 5' overhang from the *Bg*III site was



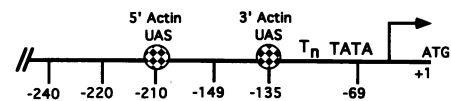
**Figure 6.** Analysis of the *Act15* promoter region between the actin UAS and the poly(dT) element. The *Act15* promoter 5' deletion to -123 and the reconstructed *Act15* promoter are shown. The translation initiation site is designated as 'ATG'. The transcription initiation sites are specified by an arrow. The stippled rectangle represents the fragment containing the *Act15* sequence from -299 to -128. The construct from which the site-directed mutations were removed by *Bg*III and S1 digestion are indicated by an arrow with '*Bg*III' and 'S1' written above and below it, respectively. The change in sequence that occurs when the promoters are digested with *Bg*III and S1 nuclease is shown at the bottom. The wild-type promoter sequence is shown for comparison. The growth-phase expression levels of the promoters in transient expression assays are indicated ± the standard error of the mean normalized to the parental promoter which is assayed in parallel. The values are the averages of three independent experiments conducted in duplicate.

removed from these constructs to recreate a region of dA residues immediately adjacent to the poly(dT) element, the expression level was increased 10-fold, restoring approximately wild-type levels of expression (see Figure 6A).

The results obtained from these mutations could be due to either the disruption of a positive element or the introduction of a new sequence element that inhibited promoter activity. To address the latter hypothesis, we examined two constructs containing all the sequence of the parental *Act15* promoter and a *Bg*III linker, Δ-137/-145 and Δ-121/-145 (see Figures 1A and 3). Analysis of these constructs showed that they had expression levels that were 67% and 104% of parental levels, respectively, suggesting that the sequence of the *Bg*III site *per se* did not inhibit promoter strength.

**The actin 6 promoter requires two actin UASs and a proximal region for full activity**

Since the *Act15* and *Act6* genes both have high levels of growth phase expression, we decided to characterize the *Act6* promoter, allowing us to compare the two promoters and identify common *cis*-acting elements. The *cis*-acting regulatory regions controlling growth phase expression in the *Act6* promoter were examined using a series of 5' and internal deletion constructs linked to the luciferase reporter gene (see Figures 1B and 2). These constructs were compared to an *Act6* promoter containing the sequences back to -722, which has been previously defined as the parental promoter based on its ability to confer a pattern of expression similar to that of the endogenous gene (6). Stable expression assays were used to identify *cis*-acting regulatory regions and



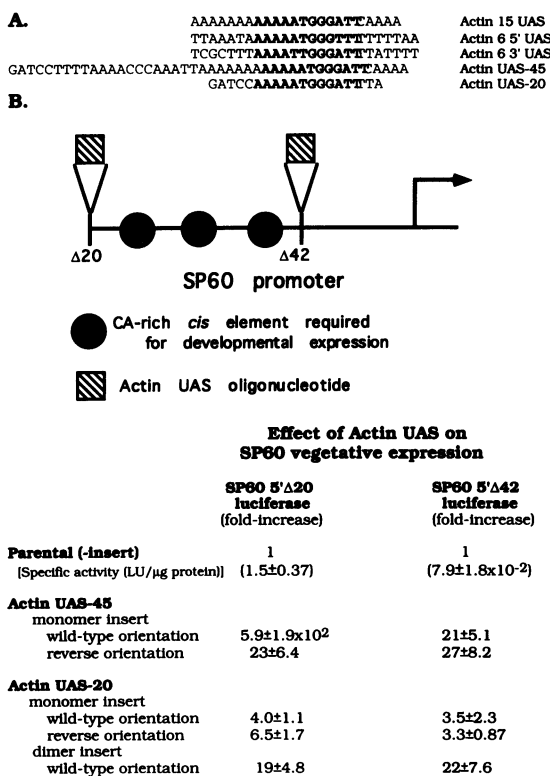
Construct	Relative Luciferase Activity
pAct6 -722 (parental)	100%
[Specific activity (LU/μg protein)]	(4.2±0.91x10 <sup>4</sup> )
<b>5' deletions</b>	
pAct6 -339	128±35%
pAct6 -240	66±5.3%
pAct6 -220	179±120%
pAct6 -210	19±6.4%
pAct6 -149	15±8.3%
pAct6 -135	0.12±0.073%
pAct6 -69	<0.003%
<b>Internal deletions</b>	
pAct6 -230/-210	43±17%
pAct6 -155/-135	0.68±0.60%
pAct6 -230/-135	0.070±0.012%

**Figure 7.** *Act6* promoter analysis. A diagram of the *Act6* promoter is given showing the relative positions of the two UASs. The various *Act6* promoter constructs assayed are summarized in this figure. The growth phase expression levels of these promoters in transient and stable expression assays are indicated ± the standard error of the mean. These values are normalized to the parental promoter which is assayed in parallel for all the experiments. The average specific luciferase activity (in light units/μg protein) is denoted for the construct containing the parental promoter. The stable expression values are the averages of two separate electroporations each assayed at least twice.



to define a minimal region sufficient to confer parental expression levels. In the course of sequencing promoter deletions, several errors in a previously published study were found. The corrected version of the sequence and the deletion end points are shown in Figure 1B. The most noteworthy change is the presence of only 3 dG residues and not 4 dG residues at -211, the position of a putative *cis*-acting element (6). Figure 7 summarizes the results of the deletion studies. (Note: in Figure 7, 5' deletions are designated pAct6 5' - #, where # denotes the nucleotide at the end point of the 5' deletion).

5' deletions of the promoter to -220 showed parental expression levels in both transient and stable expression assays. Thus, we find no evidence for the putative 'positive upstream element' previously identified between -597 and -570 (6), which was identified using RNA blots to assay expression levels. Deleting the sequence from -220 to -210 resulted in an ~5-fold decrease from parental levels of expression (see Figure 7). This removed part of a sequence similar to the actin UAS identified in the *Act15* promoter (see above), which we named the 5' actin



**Figure 8.** Analysis of actin UAS function. (A) The sequences of the three identified actin UASs from the *Act15* and *Act6* promoters are shown on the first three lines. The actin UAS-45 oligonucleotide that contains the *Act15* promoter sequence between -166 and -128 is on the fourth line. The actin UAS-20 oligonucleotide shown on the bottom line was designed based on the homology between those three actin UASs. A common core region similar in all five sites is written in bold. (B) The effect of actin UAS oligonucleotides on *SP60* vegetative expression. A schematic of the *SP60* promoter showing the end points of the promoters used for actin UAS insertion relative to the locations of the CAEs (represented by circles) required for developmental and cAMP-induced expression is presented. The fold-induction observed ± the standard error of the mean when the actin UAS oligonucleotides are inserted into *SP60* promoters is shown in the figure. The values obtained are the averages of at least two separate electroporations each assayed at least twice.

UAS based on this sequence similarity. A further 5' deletion to -149 did not alter the expression level, suggesting there were no additional promoter elements within this region.

Deleting the sequence between -149 and -135 resulted in an additional 125-fold decrease in expression (see Figure 7). This deletion removed part of a sequence similar to the actin UAS identified in the *Act15* promoter; we named this element the 3' actin UAS. A final deletion from -135 to -69 caused expression to decrease below the level of detection (see Figure 7). This deletion removes a nearly homopolymeric (dT:dA) stretch, located in a similar region to the *Act15* poly(dT) element (see above), and part of the putative TATA box (see Figure 1B). The deletion to -69 in the *Act6* promoter does not distinguish whether it is the removal of the poly(dT) element, the TATA box or both that was responsible for this decrease in expression. We note that deleting the poly(dT) regions that lie further upstream in the *Act6* promoter has no effect on expression level. The proximity of the poly(dT) region to the TATA box or transcription start site may be important to the function of the poly(dT) region. In the course of examining the *Act6* promoter, the parental *Act15* promoter was assayed in parallel to compare the differences in the strengths of the two actin promoters. The absolute expression level of the parental *Act15* promoter was ~4-fold higher than that of the parental *Act6* promoter (compare Figures 3 and 7).

A set of small internal deletions was constructed to more precisely define the actin UASs and to examine the relationship between the two actin UASs in the *Act6* promoter. (The internal deletions are designated pAct6-\*/-#, where \* and # denote the nucleotide at the end-point of the 3' and 5' deletions, respectively and contain a *Bgl*III linker, GAAGATCTTC, at the junction point.) The removal of only the 5' actin UAS (pAct6-230/-210) or the 3' actin UAS (pAct6-155/-135) resulted in ~2- and 140-fold decreases in expression relative to the parental promoter, respectively (see Figure 7), while deleting the 95 bp containing both actin UASs (pAct6-230/-135) resulted in a ~1400-fold decrease in expression from the parental *Act6* promoter. These results, including the small decrease in expression resulting from the deletion of the 5' UAS, were consistent with those observed with the 5' deletion constructs.

### The actin UAS is sufficient to promote growth phase-specific expression

Since the *actin 6* and *actin 15* genes are both expressed during the growth phase and both contain actin UASs, we tested the ability of oligonucleotides containing an actin UAS to confer growth stage-specific expression on a heterologous promoter, *SP60/cotC*, a prespore-specific gene that is normally not expressed during growth.

Two oligonucleotides containing actin UAS sites were synthesized (see Figure 8). One oligonucleotide, actin UAS-45, was 45 bp in length and contained the *Act15* promoter sequence from -166 to -128 (see Figure 1A.). The other oligonucleotide, actin UAS-20, was 20 bp and was derived from a consensus sequence of the three identified actin UASs (see Figure 8A). These were attached to the 5' end of the parental *SP60* prespore-specific promoter and a deletion, *SP60 5'Δ42*, that lacks the three CA-rich elements essential for cAMP-induced and developmental expression (21). The parental *SP60/cotC* promoter, *SP60 5'Δ20*, is induced from a very low level of growth phase expression by a factor of >10<sup>5</sup> during the developmental phase, while *SP60 5'Δ42* is not expressed at any stage of development (21).





the number of oligos inserted and sequence at the degenerate positions. Since the oligonucleotides can be inserted in the wild-type orientation (dT oligo in the coding strand and dA oligo in the non-coding strand) or the reverse orientation, we also examined preferences in the orientation of the inserts in the selected plasmids.

The *Act15-NPTI* expression vector also carries the origin of replication (*cis* element) from the endogenous *Dictyostelium discoideum* plasmid *Ddp2* (see Figure 9A) and were transformed into a cell line expressing the *Ddp2 trans* factor, which is required for autonomous replication of this plasmid (19). This system will allow the plasmid introduced into *Dictyostelium* to replicate extrachromosomally. This scheme maintains a constant copy number of the plasmid in *Dictyostelium* cells and thus prevents the selection of plasmids due to copy number differences. This extrachromosomally replicating plasmid also functions as a shuttle vector between *E. coli* and *Dictyostelium* to facilitate recovery of the plasmid for analysis of the oligonucleotide inserts.

#### Promoter strength and length of the poly(dT) element correlate with the G418 concentration to which a promoter can confer resistance

We first tested the G418 concentration to which various *Act15* promoter-NPTI fusions can provide resistance to examine the correlation between promoter strength and the ability to confer resistance to higher concentrations of G418. *Act15* upstream regions containing the sequences back to -299 [the *Act15* parental promoter (11)], -183 [which expresses at 21% of the parental level (see Figure 3)], -162 (which we expect to express at ~0.5% of the parental level based on other 5' deletions to -168 and -157), and -95 (which expresses at ≤0.003% of the parental level) were fused to the NPTI gene and transformed into *Dictyostelium* cells expressing the *Ddp2 trans* gene (see Figures 9A and 9C). The four plasmids conferred G418 resistance to maximum G418 concentration of 8000, 1000, 40 and 5 μg/ml, respectively. Cells from a 'mock' electroporation (no DNA) were able to grow in axenic medium containing 2 μg/ml G418, maintained a low steady-state number of cells at a G418 concentration of 5 μg/ml and died in medium containing 10 μg/ml G418. These results establish a correlation between promoter strength and the maximum G418 to which a promoter can confer resistance.

We used this selection scheme to examine the relationship between the number of dT residues in the poly(dT) element and promoter strength using *Act15* promoters with the upstream sequences to -299 and -162 and containing 45 (wild-type), 19 and 11 dT residues (see Figure 9; see Materials and Methods for details). Constructs with longer poly(dT) elements were able to provide resistance to higher concentrations of G418. pAct15 Δ-299 (the parental *Act15* promoter) provided resistance to 8000 μg/ml G418, while promoters with only 19 and 11 dT residues were able to provide G418 resistance to a maximum concentration of 1000 and 200 μg/ml, respectively. pAct15 Δ-162 was able to provide resistance to 40 μg/ml G418, but reducing the poly(dT) element to 19 and 11 dT residues in pAct15 Δ-162 resulted in promoters that were able to provide G418 resistance to only 10 μg/ml. These data establish a correlation between the length of the poly(dT) element and the level of G418 resistance.

#### Higher G418 concentrations select for promoter constructs having a larger number of polymeric (dT:dA) inserts

A library was made by inserting phosphorylated poly(dT) and poly(dA) oligos into pAct15 Δ-162 containing only 19 dT residues (see above, Figure 9 and Materials and Methods). This library was introduced into *Dictyostelium* cells and selected in medium containing 40 μg/ml G418. Plasmids were isolated and amplified in *E. coli* from 10 randomly selected clonal *Dictyostelium* cell lines growing in 40 μg/ml G418. Of the 10 plasmids, six had monomer inserts, three had dimer inserts and one had a trimer insert (see Table 1). The ratio of monomer, dimer and trimer inserts is roughly that observed in the input DNA from the library (see Materials and Methods). The plasmids from 22 clonal *Dictyostelium* cell lines selected at 400 μg/ml G418 were then isolated and examined. None had monomer inserts, one had a dimer insert, six had trimer inserts, one had a tetramer insert, two had a 5mer insert and 12 had rearrangements within the *Bam*HI fragment containing the promoter. The higher G418 concentration thus selected for promoters containing multiple oligonucleotide inserts and thus differs substantially from the distribution of inserts in the library, which contains predominantly monomer and dimer oligonucleotide inserts. The high proportion of rearranged plasmids may be related to observations that plasmids containing larger fragments of some *Dictyostelium* sequences rearrange during amplification in *E. coli* (see

**Table 1.** Sequence of oligonucleotide inserts recovered from the plasmids selected at 40 μg/ml G418

Insert	No. of inserts	Sequence in the coding strand of the recovered oligonucleotide(s)
A	1	T <sub>8</sub> CTCTCTTTGT <sub>9</sub>
B	1	T <sub>8</sub> ATATTTTGT <sub>8</sub>
C	1	T <sub>7</sub> CTATTTATAT <sub>2</sub>
D	1	T <sub>8</sub> CTGTATTICT <sub>9</sub>
E	1	A <sub>9</sub> TACAAATAGA <sub>8</sub>
F	1	A <sub>9</sub> CAAAGAGAAA <sub>6</sub>
G	2	T <sub>6</sub> TATTTTTTTT <sub>9</sub> T <sub>8</sub> GATTTTTCCT <sub>9</sub>
H	2	T <sub>7</sub> ATATCTTTTT <sub>9</sub> T <sub>8</sub> TGATTTTACT <sub>8</sub>
I	2	T <sub>8</sub> ATATGTTTCT <sub>9</sub> T <sub>8</sub> ACTT <sub>4</sub> TAT <sub>9</sub>
J	3	T <sub>8</sub> ATGTATAATT <sub>9</sub> T <sub>8</sub> GCGT <sub>4</sub> AAT <sub>9</sub> T <sub>8</sub> TAAT <sub>4</sub> GCT <sub>9</sub>

This table summarizes the oligonucleotides obtained in the plasmids selected at 40 μg/ml G418. The number of oligonucleotide inserts in each plasmid recovered is in the left hand column. The sequence of the oligonucleotide(s) from each recovered plasmid is listed in the right hand column. The nucleotides at the degenerate positions are written in bold. Some of the recovered nucleotides have undergone small deletions at their ends. B and H have lost one dT residue from an end. F has lost two dA residues from an end. G has lost three dT residues from an end. C has lost one dT residue on one end and seven dT residues on the other end.

Discussion). The isolated plasmids without rearrangements were reintroduced into *Dictyostelium* cells. In all cases they conferred G418 resistance at the concentration used to originally select them. The parental plasmid pAct15  $\Delta$ -162 with only 19 dT residues did not generate any G418 resistant colonies at 40  $\mu$ g/ml G418 in any trials. It should be noted that no example of the parental plasmid (plasmid lacking an insert) were isolated in these screens.

The inserts from the plasmids isolated above indicated that multiple oligonucleotide inserts into the deleted poly(dT) region were required to obtain cells capable of growth at 400  $\mu$ g/ml G418. To test this hypothesis, a library containing only monomer oligo inserts (see Materials and Methods) was introduced into *Dictyostelium* cells and selected at a G418 concentration of 400  $\mu$ g/ml. In two trials, no G418-resistant cells were obtained using the monomer library, while the construct containing the parental *Act15* promoter-NPTI fusion generated G418-resistant colonies in both cases, as expected from the results described above.

To determine whether the G418 selection had a bias for oligonucleotides inserted in one orientation over the other, the plasmids were isolated from the G418 resistant cell lines selected above and the inserted oligonucleotides were sequenced. In the plasmids selected at 40  $\mu$ g/ml G418, 13 out of the 15 oligonucleotide inserts had the poly(dT) oligonucleotide in the coding strand (see Table 1), exhibiting a preference for the maintenance of dT residues in the coding strand. However, of the six promoters that contained monomer inserts, two contained the poly(dA) oligonucleotide in the coding strand, indicating it is possible for a poly(dT) element with a substantial number of dA residues in the coding strand to function sufficiently well in our selection scheme. Twenty two *Dictyostelium* clones that grew at a G418 concentration of 400  $\mu$ g/ml were selected for further analysis. Ten of the 22 plasmids contain an *Act15* promoter region that did not show major rearrangements (see above). The oligonucleotide inserts in these 10 plasmids were sequenced and a similar preference for maintaining the dT residues in the coding strand was observed (data not shown). The poly(dT) oligonucleotide in 22 of the 34 oligonucleotides in these plasmids had been inserted such that the poly(dT) oligonucleotide was in the coding strand, while in nine cases the poly(dA) oligonucleotides were in the coding strand. In three cases, the inserts had small rearrangements that prevented the orientation of the insertion from being determined unambiguously.

#### The homopolymer dT element is not affected by small changes in sequence content

The sequence data from above was also used to determine if the selection exhibited a bias toward a particular nucleotide at the degenerate positions of the oligonucleotide inserts from the selected plasmids. Of the constructs recovered from cells selected at 40  $\mu$ g/ml G418, the ratio dG:dA:dT:dC in the coding strand at the degenerate positions was 13:26:20:16 (see Table 1), indicating a slight preference for dA and dT residues at the degenerate positions with 61% of those positions having dA or dT residues. We note that approximately half of the plasmids recovered had small deletions at the ends of the inserted oligonucleotides. When the plasmids isolated from the cells selected at 400  $\mu$ g/ml G418 were analyzed, there was a loss of the slight preference for dA and dT residues at the degenerate positions observed in the plasmids selected at 40  $\mu$ g/ml. The ratio dG:dA:dT:dC in the coding strand at the degenerate positions was 25:20:34:35, which may indicate that as the length of the

polymeric dT region increases the requirement for dT and dA residues may not be as stringent.

## DISCUSSION

### *Act15* promoter structure

One interesting feature of the regulatory regions identified in this work is their predominantly A/T base composition. Because the *Dictyostelium* genome is extremely A/T-rich, with the non-coding regions being about 90% A + T (26), previous analyses of *Dictyostelium* promoters had focused predominantly on relatively G + C-rich promoter regions, which might be expected to have higher specificities in this background. In this study, two of the four identified *cis*-acting elements, the A/T UAS and the poly(dT) element, consist entirely of dA and dT residues. The other identified *cis* elements, the curved DNA element and the actin UAS, are 90% and 79% A/T, respectively. Prior to this study, the only A/T-rich *Dictyostelium* promoter element that had been shown to be required is the A-rich element of the uridine diphosphoglucose pyrophosphorylase (UDPGP1) promoter (27) and the prespore-specific element (28). The oligo(dT) stretch that lies between the TATA box and transcriptional initiation site of nearly all *Dictyostelium* promoters has also been proposed to be an A/T-rich regulatory region in *Dictyostelium* (26). From our analysis and what is known about other *cis*-acting elements in other genes in *Dictyostelium* and other organisms, we expect that, except for the oligo(dT) element, the regulatory regions we have described are relatively short. It is, however, possible that one or more extend over larger distances. In the case of both *Act15* and *Act6* it should be emphasized that we have only examined the elements required for expression during vegetative growth and do not know whether these or other elements may regulate the increase in expression during early development.

The actin UAS sequence was initially identified as a sequence motif common to the upstream regions of several *actin* genes and called box 4 (10). Previous studies on the *Act15* promoter and the *Act6* promoter proposed that this common element has a key role in directing the expression of these genes (6,11). However, the previous studies identify this region on the basis of large 5' deletions. In this study, we definitively show, using sequence replacements within this element, that it is required for high level expression. It is interesting to note that the sequence replacements replacing only dA and dT residues have a greater effect than the one removing two of the three dG residues.

The curved DNA element overlaps with a sequence shown to contain an intrinsic bend. Although it is not known whether this structural feature of the DNA is important to this region's function, there are reports of curved or bent DNA having an important role in transcriptional regulation (29,30). It has been proposed that curved DNA functions by looping the DNA upstream of the curve back toward the transcriptional initiation site, thus allowing an upstream *cis* element to interact with proteins bound near the transcriptional initiation site. We do not favor this model, because the curved DNA element lies at the extreme 5' end of the parental promoter region, and it is unlikely that this DNA curve functions to bring a *cis* element back toward the transcriptional initiation site. It is possible that the structure of the DNA curve may be required for binding by a *trans* factor. The mammalian testis-determining factor SRY, which contains a HMG box, is an example of a DNA binding protein that recognizes the structural feature of a DNA bend (31).

Point mutations at -127 and -124 in the *Act15* promoter decrease the expression level 10- to 20-fold. Full activity is restored when the sequence GATC generated by the point mutations is deleted and the sequence in this region is restored to several dA residues followed by a long stretch of dT residues. The explanation we favor is that the site-directed mutations disrupt a positive *cis*-acting element. We postulate that this dA/dT junction region defines another *cis* element required for vegetative expression, although it is also possible that the actin UAS includes this dA/dT junction.

### **Act6 promoter structure**

The *actin 6* and *actin 15* genes have similar patterns of expression, but the *Act15* promoter has a higher absolute level of expression. The promoters contain very similar core regions within the ~110 bp upstream of the transcriptional initiation sites that may be responsible for generating the similar expression patterns. However, the *Act15* promoter contains several additional positive-acting elements, the curved DNA element, the A/T element and a longer stretch of homopolymeric dT residues that are not observed within the *Act6* promoter and these additional elements may be important in promoting the higher level of absolute expression from the *Act15* promoter.

Our results also suggest that the two actin UAS sequences of the *Act6* promoter are not equivalent. In both the 5' and internal deletions, the removal of the 5' actin UAS resulted in a small decrease in expression of ~2- to 5-fold, but removal of the 3' actin UAS resulted in an ~125- to 140-fold decrease in expression. We expect that the 3' actin UAS, AAAATTGGG-ATTTT, is a stronger promoter element than the 5' actin UAS, AAAAATGGGTTTTT. While it is also possible that the 3' actin UAS functions as a stronger element because it is positioned closer to the transcription initiation site, TATA box or poly(dT) element, we do not think that this is the case, since actin UAS-20, which has the same core UAS, functions to a similar level when up close and at a distance from the *SP60/cotC* core promoter (see Figure 8B).

### **The actin UAS**

This study of *Dictyostelium actin* promoters has identified promoter elements that are required for its growth phase expression. Moreover, one promoter element, the actin UAS, was shown to be sufficient to direct growth phase expression from a heterologous promoter. To date, the actin UAS was found to be common to the *Act15* and *Act6* promoters and not identified in other promoters. Sequence analysis of other *Dictyostelium actin* promoters shows that a similar sequence is located at a similar distance from the start site in other members of this family and suggests that these sequences may function as UASs in these genes as well (10). There are required *cis*-acting regions in the UPDGP (27) and ALF (3) promoters that contain a GGG sequence motif, but we did not consider those to be functionally related to the actin UAS because the flanking sequences differ and these two elements are required for cAMP induction, while *actin* promoters are not cAMP responsive (4; Hori and Firtel, unpublished observations).

The results indicate that the actin UAS can function as a growth stage enhancer element that can function at a distance from the transcription start site that is significantly further (> 600 bp) than it is present in the parental *actin* genes. Moreover, we obtained a level of expression that was ~15% of that of the *Act6* promoter containing only its 3' UAS (pAct6-149; see Figure 8B),

indicating that this sequence is sufficient to confer a significant absolute level of expression. The fact that it can function at different distances from the promoter and in different orientations suggests that the actin UAS has some properties similar to that of metazoan enhancers (32). The actin UAS is the first example of a short promoter element being defined as sufficient to confer growth phase expression to a non-growth stage gene in *Dictyostelium*.

*Actin* promoters are often chosen to over-express exogenous genes during vegetative growth in ectopic expression experiments (33). These experiments substitute the actin promoters' high vegetative expression for the expression pattern of the endogenous gene. An oligonucleotide with the ability to direct growth phase expression would make it possible to produce hybrid promoters that retain their endogenous expression pattern while also having the ability to be expressed during vegetative growth.

### **Analysis of the poly(dT) element**

The poly(dT) element was studied in detail using a genetic selection scheme. A high proportion of the plasmids isolated from this selection scheme were rearranged when a G418 concentration of 400 µg/ml was used (see Results). Because the rearrangement was observed in the *Bam*HI fragment containing the *Act15* promoter, it is unlikely that the plasmid could have provided G418 selection if the rearrangement occurred prior to or during the selection in *Dictyostelium*. It is most probable that the rearrangements occurred after selection in *Dictyostelium* and during the recovery and amplification of the plasmids in *E. coli*. The A/T-rich composition of *Dictyostelium* tends to increase the amount of recombination and rearrangement in *E. coli* (the Firtel laboratory, unpublished observation). Plasmids containing a large number of A/T-rich oligo inserts may have an increased possibility of rearrangement in *E. coli* and result in underestimating the selection for multiple oligo inserts.

Polymeric (dT:dA) promoter elements have been most intensively studied in *Saccharomyces cerevisiae*, including yeast poly(dA:dT) enhancers (34,35). Poly(dT:dA) regions often function as positively acting, constitutive promoter elements in *S. cerevisiae*. There is evidence supporting two mechanisms for the action of transcriptional activation by these poly(dT:dA) upstream regions. One model proposes that the poly(dT:dA) promoter elements act as protein binding sites for a protein that stimulates the rate of transcription (34). Another model hypothesizes the activity of the poly(dA:dT) enhancers may be related to the inability of nucleosomes to form on DNA containing regions of polymeric (dT:dA) DNA (35). It has been proposed that an open region of chromatin could be more easily transcribed (36).

Increasing the length of the poly(dT:dA) region in a yeast promoter has been shown to increase promoter strength. In *S. cerevisiae*, *adr2* promoter mutants with increased constitutive expression have been isolated and characterized. One class of promoter-up mutants was found to contain an increase in the length of a naturally occurring homopolymeric dT stretch from 20 to 54 or 55 nucleotides (23). In yeast, the longer poly(dA:dT) enhancer of the *ded1* gene is a stronger promoter element than the shorter *his3/pet56* poly(dA:dT) enhancer (36). The increase in expression observed with longer poly(dT:dA) stretches is consistent with the nucleosomal exclusion model because the ability of poly(dT:dA) DNA to exclude nucleosomes is length-dependent (37,38). Similarly, the strength of the *Act15* poly(dT) element is strongly dependent on its length.

The plasmids selected from the library using G418 resistance contain oligonucleotide inserts with dT, dC and dG in addition to dT residues at the degenerate positions. These results indicate that the region does not have to be exclusively homopolymeric, similar to the poly(dA:dT) enhancers in the yeast *his3/pet56* and *ded1* genes, which are predominately dT residues in the coding strand, but not completely homopolymeric (36). In addition, our experiments indicate no nucleotide is strongly favored over the others in *Dictyostelium*.

The *Dictyostelium Act15* poly(dT) element begins only ~10 bp upstream of the putative TATA box (see Figure 1A), while the poly(dA:dT) enhancers of the yeast *his3/pet56* and *ded1* genes lie ~200–300 bp upstream of their respective TATA boxes (36). Although these yeast elements are within a relatively short distance of their TATA boxes, it may be significant that the *Dictyostelium* poly(dT) element lies nearly adjacent to the TATA box. Poly(dT:dA) regions within ~350 bp of the putative TATA box in the *Dictyostelium Act6* promoter have been deleted with no effect. We propose that the poly(dT) element lying adjacent to the TATA box may be important for directing the binding of the TFIIID complex to the appropriate position in the 90% A/T composition (26) of *Dictyostelium* non-coding DNA.

#### Comparison of transient expression with stable expression assays

These studies comprise the first use of the transient expression system to examine a *Dictyostelium* promoter. The comparison of the transient expression system to stable expression assay is important to establish the more quickly accomplished transient expression assay as a tool in defining important promoter elements in *Dictyostelium*. Our results indicate transient assays are a reasonable approach for studying promoter structure in this system.

Both 5' and internal deletion constructs were assayed by both stable and transient expression assays (see Figures 3 and 5) and upon comparison, the constructs had consistent results between the two assays if the level of expression in the transient assay was  $\geq 3\%$  of the parental level. Below this level, it may be that the absolute level of expression in the transient system is too low to give reliable data. This may be because the absolute level of luciferase activity (~100-fold less than in assays using stable transformants) approaches the limit of detection for the system (see Figure 3). Another possible reason for the difference in the two assays could be the presence of DNA not fully packaged into chromatin in transient assays (39). This could produce some non-specific transcription not affected by promoter deletions.

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