cis-Acting Site Controlling Bidirectional Transcription at the Growth-Differentiation Transition in Dictyostelium discoideum

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A pair of adjacent genes, impA and dia1, are divergently transcribed but expressed at different stages in the life cycle of Dictyostelium discoideum. The intervening 654-bp region carries cis-acting regions that are essential for transcription in both directions as well as repression of dia1 in growing cells. We have focused on a 112-bp region proximal to dia1 that is essential for bidirectional transcription. Analyses of a set of internal deletions showed that the sequence between positions 80 and 97 (TTGAATTTTTTGAATTT) is critical and that bases outside this region are dispensable. Site-directed mutations within this critical region confirmed the importance of this sequence for transcription both to the right and to the left. However, insertions of either 6 or 24 Ts into the run of 6 Ts separating the repeated GAA sequence had little effect on the functioning of the site in either direction, suggesting that factors recognize the half-sites TTGAATT separately. Inversion of the bases between positions 80 and 97 greatly reduced expression in both directions, indicating that orientation is critical for expression of both the nearby impA gene and the distal dia1 gene, which is more than 500 bp away. Comparison of 38 mutant constructs with multiple random variations in the region indicated that transcription factors may bind to a range of related sequences and still retain function. All functional constructs directed transcription both leftward and rightward, while all nonfunctional constructs were impaired for transcription in both directions. It appears that the same transcription complex controls transcription of both impA and dia1.

Differential regulation of gene transcription underlies most developmental transitions. DNA-binding proteins recognize sites in the DNA adjacent to genes and can either stimulate or inhibit the initiation of transcription. As a result, the pattern of transcription of each gene is determined, in part, by the specific arrangement of cis-acting sites. However, the motifs recognized by specific transcription factors and their arrangement relative to the transcriptional start site are poorly understood. The ability to efficiently introduce genetic constructs into strains of the social amoeba Dictyostelium discoideum together with the ability to precisely control synchronous development in large populations of cells make this system amenable to high-resolution studies on transcriptional regulation (5, 6).

Removing Dictyostelium cells from growth medium and suspending them in nonnutrient buffer results in a growth-to-differentiation transition (GDT) in which most genes expressed in the growth phase are turned off and the early developmental genes are turned on (4, 7). One of the genes that is rapidly turned off, impA, is found on chromosome 4 adjacent to dia1, a gene which is expressed within an hour of GDT (3). The function of these genes is unknown. They are divergently transcribed and share a 654-bp upstream region of DNA. There is a cis-acting site in the middle of this intergenic region which is recognized by a DNA-binding protein present in growing cells but not in cells which have developed for 4 h.

Deletion of this central portion of the intergenic region does not affect expression of impA but lifts the repression of dia1 such that it is expressed at high levels in both growing and developing cells (3). Stage specificity of expression of dia1 is largely determined by control exerted in this central 153- to 440-bp region. However, expression of dia1 is also dependent on a 112-bp portion of the intergenic region proximal to impA. Deletion of this region leaves 542 bp upstream of the ATG of dia1 but results in complete lack of expression of this gene. When deleting from the other end, a construct that carries this 112-bp region expresses impA normally, but further deletions into this region result in no expression of impA (3). It appears that this impA proximal region carries cis-acting sites necessary for transcription in either direction. Little is known about the control of bidirectional transcription, and so it was of interest to further dissect this region to determine whether there were separate cis-acting sites controlling transcription on each strand or a single motif of bases controlling transcription in both directions.

We have generated internal deletions of the 112-bp region of the impA-dia1 intergenic region using PCR with primers designed to cross the deleted portion. Based on the ability of these constructs to drive impA and dia1 in transformants, we were able to define the essential cis-acting site to the region between 80 and 97 bp upstream of the ATG of impA. Changes in specific bases within this site affected transcription in both directions equally, suggesting that expression of these genes depends on a common transcription factor acting bidirectionally.

**MATERIALS AND METHODS**

Cell culture and developmental conditions. Dictyostelium discoideum strain AX-4 and its derivatives were grown axenically in HL-5 with shaking at 22°C (8). Strains carrying reporter constructs were grown in HL-5 containing 50 μg/ml of G418. Cells were harvested during the exponential phase of growth and either immediately used to prepare RNA or washed twice in pad diluting fluid (8), suspended at 107 cells/ml in pad diluting fluid, and allowed to develop for 5 h at 22°C before being collected for preparation of RNA.

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Modification of the 112-bp region. The reporter vector Suika carrying the impA-dia1 intergenic region flanked by green fluorescent protein (GFP) and DsRed was constructed in an integrating plasmid that carries a G418 resistance cassette (1). The plasmid was digested with XbaI and SacI, and the 6-kb fragment was ligated to the 654-bp intergenic region fused to GFP in place of impA (3). DsRed with an act15 terminator that had been fused to the other end of the impA-dia1 intergenic region (3) was excised with XbaI and HindIII and inserted into the same position to generate Suika. We replaced an A at position 119 relative to the start of impA translation with a G by using PCR to generate an AvrII site to be able to replace the impA proximal 112-bp region with fragments ending in CiaI and AvrII sites.

Internal deletions within the impA proximal intergenic region were generated by PCR with primers that covered the targeted deletions and a primer that ended at the AvrII site that was generated in the vector at position 119. The primer for deletion Δ16-41 was paired with a primer that ends at the endogenous CiaI site of the reporter vector. Primers for the inversion of the critical region, I-GAA6T, as well as primers for the extensions of the intervening run of Ts, GAA12T and GAA30T, covered those regions and were paired with the primer that ends in the AvrII site for PCR. Primers for the site-directed variants covered those regions and were also paired with the primer that ended in the AvrII site for PCR.

The PCR products were cut with AvrII and CiaI and used to replaced the original bp 1 to 112 region of pAvrSuika. Two complementary oligonucleotides covering Δ46-114 and ending in CiaI and AvrII sites were linearized at 95°C for 3 min and annealed at 45°C for 30 min. The annealed product was inserted between the CiaI site and the AvrII site of pAvrSuika. All sequences in the final constructs were twice verified by sequencing.

Constructions of random sequence variants. The region between positions 41 and 100 was subjected to random base substitution by programming the synthesizer to use mixtures of nucleotides with 20% of bases other than that found in the endogenous sequence at each position. The resulting mixture of oligonucleotides was amplified by PCR with a primer pair ending in the endogenous ClaI and AvrII sequences (5'-ACCATCGATTGTGATT-3' and 5'-TGCTCAGTGGCCTAGGGCCAA-3'). The PCR products were digested with CiaI and AvrII and cloned into those sites in pAvrSuika. The mixture of ligated products was transformed into E. coli. AX4 cells were transformed with the cloned plasmids, each containing different sequences. All sequences in the final constructs were verified twice by sequencing and found indeed to have suffered a 20% mutation rate.

Flow cytometry. Cells were grown in HL-5 medium on the surface of petri dishes. To reduce background fluorescence, the medium was replaced with defined medium (FM) 2 days before the cells were collected for flow cytometry (8). Cells were pelleted by centrifugation and resuspended in 20 mM sodium potassium phosphate buffer (pH 6.4) before being analyzed in a FACScalibur (Becton Dickinson) as previously described (3). Fluorescence at 510 nm (GFP) was recorded.

Northern analysis. Total RNA was extracted with TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Samples containing 10 μg of RNA were separated by electrophoresis under denaturing conditions and blotted onto a MagnaGraph transfer membrane (Osmotics Inc.). DNA probes were labeled by random priming of the Klenow fragment of DNA polymerase on 200 ng probe. DNA in 30 μM dITP, dATP, and dGTP, 30 μM 32P-labeled dCTP, 10 mM Tris-HCl pH 7.5, 5 mM MgCl2, 7.5 mM dithiothreitol for 30 min at 37°C. Hybridization was performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt’s solution, 0.1% sodium dodecyl sulfate, 50% formamide, 100 μg/ml denatured salmon sperm DNA at 42°C after 1 h of prehybridization.

RESULTS

Expression of reporter construct. To facilitate measurement of transcription in both directions, we generated a construct in which the gene encoding short-half-life GFP replaced the coding sequence for impA and the gene encoding DsRed Express replaced the coding region of dia1. These reporter genes were separated by the normal 654-bp intergenic region, except that an A at position 119 was converted to a G to generate a unique AvrII site. This construct, pAvrSuika, was introduced into wild-type AX4 cells, and transformants were selected for growth in the presence of 50 μg/ml G418. When grown on bacterial lawns, plaques generated by these transformants resulted in no observable transcription in either direction, further refining the essential region. This construct retains the internally repetitive sequence (ATTTCIAGAATTCA), which is close to an inverted copy of the sequence (TTGAATTTTGAATT) that

rescence of DsRed in the center where the cells were developing (Fig. 1). This is the pattern expected from the expression of the endogenous impA and dia1 mRNAs (3).

Internal deletions. Using a series of PCR primers for the impA proximal region and a primer from outside the 112-bp region, we generated a series of internal deletions flanked by CiaI and AvrII sites. These were inserted into the full-length construct cut with CiaI and AvrII to position them in their natural context. The constructs with internal deletions were introduced into AX4 cells, and transformants were selected for resistance to 50 μg/ml G418. Expression levels of GFP and DsRed were estimated by analyses of Northern blots of RNA isolated from vegetative cells and cells which had developed for 5 h (Fig. 2).

Deletion of bases 16 to 41 had no effect on transcription in either direction. This deletion removes the bases directly adjacent to the transcribed region of impA as defined by the sequence of a cDNA (DDB0166497). Full expression of GFP showed that positioning the region carrying essential cis-acting sites 25 bases closer to the gene did not have significant consequences. The deletion removes a series of repeats of TAA which might function as a TATA-binding site for leftward transcription but appear to be dispensable. impA appears to be a TATA-less gene.

Deletion of bases 46 to 114 resulted in no observable transcription in either direction, confirming that this region carries a bidirectionally active cis-acting site. Deletion of bases 72 to 110 also resulted in no observable transcription in either direction, further refining the essential region. This construct retains the internally repetitive sequence (ATTTCIAGAATTCA), which is close to an inverted copy of the sequence (TTGAATTTTGAATT) that...
was deleted, but is clearly not sufficient for transcription in either direction.

A deletion of bases 65 to 73 or the larger region between positions 42 and 79 did not affect expression of GFP or DsRed, showing that the essential region lies between bases 80 and 110. Moreover, these deletions show that the sequence centered on position 70 (TTTGATTT) plays no essential role in transcription in either direction. The fact that deletion from positions 88 to 110 with insertion of three As drastically reduced expression in both directions while deletion from positions 98 to 111 had no appreciable effect indicates that bases between 88 and 97 play critical roles in bidirectional transcription. This was confirmed by the lack of expression in either direction from a construct in which bases 83 to 95 were deleted.

Together, these internal deletion studies delineate the critical region to bases between positions 80 and 97. This region carries the internally repeated sequence TTGAATTTTTGA ATT, on which we focused further attention. Although this region appears to be too small for two independent cis-acting sites controlling transcription in the leftward (impA) direction independently of transcription in the rightward (dia1) direction, separate sites could conceivably be interdigitated.

Modifications of the critical region. To determine whether the orientation of the critical region was important for transcription either leftward or rightward, we inverted the bases between positions 80 and 97 (Fig. 3A). Surprisingly, the construct carrying this inversion did not express either GFP or DsRed, although the endogenous sequence was present on the opposite strand (Fig. 3B). It appears that orientation matters for transcription in both directions.

We also inserted extra Ts between the pair of GAAs to determine whether spacing between the purine runs is critical (Fig. 3A). When the number of Ts in this region was increased from 6 to either 12 or 30, transcription in both directions was unaffected. These results suggest that there may be two independently recognized sites in this region that together determine bidirectional transcription.

Site-directed mutations. We synthesized variant sequences that differed only between positions 80 and 97 (Fig. 4A). In every case a purine was exchanged for a purine and a pyrimidine was exchanged for a pyrimidine. Full-length constructs with these variations were introduced into AX4 cells, and transformants were selected for resistance to 50 μg/ml G418. We found that transcription in both directions was normal for three out of the six constructs, showing that this sequence could tolerate up to three differences (V5) and retain function (Fig. 4). Two of the constructs (V3 and V4) expressed both GFP and DsRed but at significantly lower levels. The construct carrying variant V6 expressed very little GFP or DsRed (Fig. 4B). We looked for a pattern in the bases that...
were essential for transcriptional activity as well as those that could be replaced, and we were unable to define a motif from these data.

**Random variants.** We synthesized a set of random variants with altered sequences between positions 41 and 100. The oligo synthesizer was programmed to generate 20% substitution of bases in this region with any of the other three bases. The variants were cloned into the ClaI and AvrII sites of the reporter construct and introduced into AX4 cells, and transformants were selected for resistance to 50\(\mu\)g/ml G418. A set of 38 independent transformants was chosen, and the region was sequenced. Sequences of the region between positions 80 and 97 were aligned (Fig. 5A). While there were some base changes in the upstream region between positions 41 and 79, analyses of the internal deletions showed that they were irrelevant (Fig. 2).

Flow cytometry was used to measure the level of GFP expression in strains transformed with each of the 38 random variants (Fig. 5B). Before each analysis the signal was calibrated to give a median value of 1.5 to 3.0 for the fluorescence of the host cells lacking a GFP construct (strain AX4). The median for cells carrying a construct with the wild-type intergenic region driving GFP (Suika cells) was always \(10\). Histograms of the fluorescence showed that cells carrying six of the random variants expressed GFP at levels not significantly different from those of cells carrying the wild-type construct. Moreover, the spread between individual cells in the strains was similar to that of the host cells.

**FIG. 3.** Inversion and extension of the critical region. (A) The sequences of the construct in which the region between positions 80 and 97 was inverted (I-GAA6T) and those from the constructs with insertions of 6 and 24 Ts (GAA12RT and GAA30T) were aligned. (B) Northern analyses of RNA from cells of strains carrying the various constructs probed for GFP or DsRed. RNA was collected from growing cells for analyses of GFP and from developed cells for analyses of DsRed.

**FIG. 4.** Sequences of site-directed mutations and their activity. (A) Sequences of designed variants were aligned. Numbering is from the start of translation of ImpA (reverse complement ATG). (B) Northern analyses of RNA from cells of strains carrying the various site-directed mutations probed for GFP or DsRed. WT is the strain transformed with the plasmid carrying the unmodified intergenic region. RNA was collected from growing cells for analyses of GFP and from developed cells for analyses of DsRed.
carrying different constructs was not significantly different. The remaining 32 strains carrying random variants expressed GFP at considerably lower levels and had median fluorescence values of less than 4 (Fig. 5B). While a few of these expressed the genes at levels somewhat higher than the no-construct wild type (AX4), the variance between independent measurements of the median values for the weakly expressing strains indicates that this is not significant.

The fluorescence signal for DsRed was not sufficiently strong to be measured by flow cytometry. Therefore, we collected RNA from the 38 strains after 5 h of development and prepared Northern blots. Scanning the blots after hybridization with a probe for DsRed showed that the 6 strains which were positive for expression of GFP were also positive for expression of DsRed, while the remaining 32 strains expressed DsRed at close-to-background levels (Table 1). While there were some differences in the levels of expression in the opposite directions, they were small compared to the variance. The results indicate that transcription in both directions is equally affected by the sequences in the random variants.

While no simple motif could be recognized by comparing the sequences of the functional and nonfunctional constructs, some of the random variants were informative. Focusing on the essential region between positions 80 and 97, it can be seen that there is a single base change, a T to G at position 87, in the nonfunctional construct R43 (Fig. 5A). This position is invariant in the functional constructs generated by site-directed mutagenesis or random variation (Fig. 4 and 5). It is possible that this base is critical to the motif. There is only a single change in the nonfunctional construct R42, an A to T at position 84, but this same change is observed in the functional constructs R14 and R20. There are other changes in the region of 80 to 97 in the functional variants which may be compensatory. Likewise, there is a single base change in the nonfunctional construct R12, a T-to-C transition at position 86, but this same change occurs in the functional site-directed mutant V5. The site-directed construct also has a T-to-C transition at position 82 and an A-to-G transition at position 93, either of which might be compensatory.

**DISCUSSION**

To further investigate the growth-differentiation transition in *Dictyostelium*, we constructed the Suika vector in which the fluo-

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**FIG. 5.** Sequences of random synthetic variants and their activity. (A) The constructs were sorted on the basis of their activity. The six functional constructs are at the top, separated from the 32 nonfunctional constructs. Sequences of the region between positions 80 and 97 were aligned. While there were base changes in the upstream region, analyses of the internal deletions had shown that they were irrelevant. (B) Flow cytometry of cells from strains carrying the random synthetic variants. WT is the strain transformed with the plasmid carrying the unmodified intergenic region. AX4 cells are from the untransformed host. The histograms show the number of cells (y-axis) with the measured GFP fluorescent intensity (x-axis). The fluorescence-activated cell sorting was adjusted to set the median for the fluorescence of the host cells lacking a GFP construct (strain AX4) between 1.5 and 3. All analyses were repeated at least three times.
Repression of dial during growth is dependent on the central region, while transcription in both directions is dependent on a 112-bp region proximal to impA (3). By constructing and analyzing the activity of a series of internal deletions, we have been able to further delineate the essential region to bases between positions 80 and 97 and show that it is necessary for transcription in either direction. Inversion of this region greatly reduced transcription in both directions, suggesting that the orientation of binding factors is critical to transcriptional activation. This partly explains why the highly related inverted copy between positions 45 and 59 is not sufficient to direct transcription when deletions eliminate bases between 83 and 95 (Fig. 2). While proper orientation might be expected to affect a gene that is within 100 bp, as it is for impA, it is somewhat unexpected for a site to be orientation dependent for a gene 500 bp away, as it is for dial. In addition to a transcription factor recognizing the site between positions 80 and 97, transcription of dial might require orientation-dependent association with a factor that binds at an adjacent cis-acting site that is not required for impA transcription. This hypothetical site would have to lie between positions 98 and 128, since a construct extending from the start of impA to position 128 fused to the region 100 bp upstream of the translational start of dial is sufficient for rightward transcription (data not shown).

There are two copies of GAA that stand out from the runs of Ts surrounding the critical region. They are separated by 6 Ts, but we found that extending this homopolymer to either 12 Ts or 30 Ts had little effect on the ability of the region to direct bidirectional transcription. Since extending the intervening sequence by six bases repositions the second GAA on the DNA double helix, proteins that bind this site must be quite flexible or recognize the half-sites, TTGAATT, in a manner that permits long-range interactions. Since the deletion construct Δ88-110, in which the second half-site was replaced with three As, showed some residual activity, it appears that the first half-site can be partially functional. The second A in this site is conserved in all functional constructs and may be critical.

Many point mutations that affect one or the other half-sites retain bidirectional function (Fig. 4). The constructs with site-directed mutations that have lost some or all activity (V3 and V6) have changes in the Ts between the GAAAs. Conversion of these bases to C appears to significantly affect recessive sites. The T that precedes the first half-site by two bases was changed to C in construct V1 but did not seem to affect the transcription. This hypothetical site would have to lie within 100 bp, as it is for impA. It is somewhat unexpected for a site to be orientation dependent for a gene 500 bp away, as it is for dial. In addition to a transcription factor recognizing the site between positions 80 and 97, transcription of dial might require orientation-dependent association with a factor that binds at an adjacent cis-acting site that is not required for impA transcription. This hypothetical site would have to lie between positions 98 and 128, since a construct extending from the start of impA to position 128 fused to the region 100 bp upstream of the translational start of dial is sufficient for rightward transcription (data not shown).

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One of the most comprehensive studies of protein-DNA recognition requirements was carried out on the binding of the

### TABLE 1. Comparison of gene expression in cells carrying random synthesized variants

| Variant | GFP     | DsRed  |
|---------|---------|--------|
| R4      | 70.0 ± 8.4 | 69.8 ± 10.3 |
| R6      | 84.9 ± 23.6 | 55.7 ± 10.7 |
| R9      | 103.7 ± 20.3 | 68.6 ± 5.5 |
| R14     | 94.5 ± 20.3 | 61.4 ± 8.0 |
| R20     | 70.0 ± 5.7  | 98.6 ± 25 |
| R31     | 42.8 ± 9.9  | 69.8 ± 10.3 |
| R2      | 11.6 ± 3.4  | 18.0 ± 3.1 |
| R3      | 9.6 ± 1.0   | 11.4 ± 4.2 |
| R5      | 18.3 ± 5.5  | 16.4 ± 1.1 |
| R7      | 10.1 ± 1.5  | 7.8 ± 2.2 |
| R8      | 12.0 ± 2.1  | 9.7 ± 5.4 |
| R11     | 14.0 ± 3.8  | 9.1 ± 3.6 |
| R12     | 15.6 ± 3.6  | 9.8 ± 4.9 |
| R13     | 14.7 ± 1.4  | 14.5 ± 5.6 |
| R15     | 11.8 ± 0.2  | 10.3 ± 5.2 |
| R17     | 20.8 ± 3.7  | 26.5 ± 0.3 |
| R18     | 13.8 ± 2.7  | 12.0 ± 2.3 |
| R19     | 10.4 ± 1.7  | 8.4 ± 1.3 |
| R21     | 12.6 ± 0.5  | 13.7 ± 8.8 |
| R22     | 17.5 ± 5.3  | 35.2 ± 14.3 |
| R23     | 10.9 ± 1.4  | -2.6 ± 4.4 |
| R24     | 9.4 ± 1.2   | 11.8 ± 14.3 |
| R26     | 13.7 ± 3.6  | 7.2 ± 7.0 |
| R27     | 30.5 ± 9.7  | 19.6 ± 5.6 |
| R28     | 12.9 ± 2.8  | 6.4 ± 5.0 |
| R29     | 11.6 ± 0.7  | 18.1 ± 1.0 |
| R32     | 11.9 ± 2.8  | 27.5 ± 4.5 |
| R33     | 12.5 ± 4.0  | 16.8 ± 1.1 |
| R35     | 12.9 ± 3.7  | 6.5 ± 3.6 |
| R36     | 12.1 ± 0.4  | 10.1 ± 0.3 |
| R37     | 11.6 ± 0.5  | 4.6 ± 1.5 |
| R38     | 11.9 ± 2.8  | 13.2 ± 0.2 |
| R39     | 10.0 ± 0.2  | 13.7 ± 0.8 |
| R41     | 40.3 ± 6.1  | 14.6 ± 6.9 |
| R42     | 15.4 ± 5.9  | 10.4 ± 7.3 |
| R43     | 18.1 ± 3.8  | 5.2 ± 1.9 |
| R44     | 17.0 ± 6.6  | 3.7 ± 0.6 |
| R45     | 17.3 ± 4.3  | 9.7 ± 2.9 |

a The median GFP fluorescence for each strain was calculated relative to that of cells carrying the complete (wild-type) impA-dia1 intergenic region, which was set at 100. The median background fluorescence of no-construct wild-type cells (AX4) was 13.7 ± 0.2 relative to the wild-type level of fluorescence. The DsRed signal for each strain measured on Northern blots with NH1 images digitized relative to that of cells carrying the complete (wild-type) impA-dia1 intergenic region, which was set at 100. Data are the averages of at least three independent measurements; standard deviations are given. Strains that expressed the genes at more than half the wild-type rate were considered to carry functional constructs, while those that expressed the genes at less than 40% of the wild-type level were considered to carry nonfunctional constructs.
Salmonella phage P22 repressor Mnt to its own operator (2). Using variant synthetic oligonucleotides, the Stormo lab was able to establish relative binding affinities and construct position-specific matrices of binding energies for bases at each position in an 18-bp sequence. Their results supported the independent base assumption that the total free energy is the sum of the free energy contribution from each base. However, these experiments were carried out in vitro with purified components. Our in vivo results do not support the assumption that cis-acting function is the sum of the contribution of each base in the motif but suggest that transcription factors may bind to a range of related sequences and still retain function. Nevertheless, the coordinate response of expression of GFP and DsRed in each of the 44 variants strongly indicates that both rightward and leftward transcription depend on the same signals.

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