

Developmental regulation of transcription of a novel prespore-specific gene (Dp87) in *Dictyostelium discoideum*

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SUMMARY

The Dp87 is a novel prespore specific gene of *Dictyostelium discoideum* which has a long open reading frame of 555 amino acids. The entire amino acid sequence had low but significant homology to the spore coat proteins, SP96 and SP70, of this organism. When a chimeric gene, containing a 1380 bp of the 5' upstream region of this gene fused with CAT gene, as reporter, was introduced into cells of this organism, it was expressed only in prespore cells of the slug. Transformation experiments, using chimeric genes, containing a series of 5' deletions of the upstream region, showed that -447 bp to -357 bp is an important *cis*-acting regulatory region for transcription. A nuclear factor(s) that specifically bind to this *cis*-acting region were detected from slug cell nuclei. Transformation experiments using a chimeric gene consisting of the 5' region between -666

bp and +149 bp of this gene, a β -galactosidase reporter and an actin 8 terminator, showed that the reporter gene was expressed as early as in aggregation streams, indicating that Dp87 become transcribed a few hours earlier than the other prespore-specific genes so far reported. This was confirmed by northern hybridization detected using an image plate analyzer. The fact that cells expressing Dp87 appeared at random in aggregation streams gives solid support to the idea that position-independent differentiation of prespore and prestalk cells, followed by their sorting, brings about pattern formation in this organism.

Key words: *Dictyostelium discoideum*, prespore differentiation, Dp87 gene

INTRODUCTION

During development of the cellular slime mold *Dictyostelium discoideum*, cells aggregate to form cell masses (slugs) and differentiate into two types of cells i.e. prestalk and prespore cells. The prespore cells occupy the posterior three-quarters of the slug and differentiate into spores of the mature fruiting body. Many developmental characteristics specific to prespore cells have been studied at the sub-cellular and molecular levels. Earlier works showed that the prespore-specific characters appeared as the tip is forming on a cell aggregate (Loomis, 1982; Tasaka et al., 1983). Northern hybridization analysis of cDNA clones complementary to prespore-enriched mRNAs showed that these accumulated almost simultaneously at the tipped aggregate stage (Barklis and Lodish, 1983; Mehdy et al., 1983; Dowds and Loomis, 1984; Ozaki et al., 1988).

The promoter regions of two prespore-specific genes, D19 and SP60, and of the prestalk specific genes, *ecmA* and *ecmB*, were used to drive the β -galactosidase gene and introduced into *D. discoideum* cells. The expression of these chimeric genes, detected histochemically (Early and Williams, 1989; Williams et al., 1989; Haberstroh and

Firtel, 1990), showed that both prespore and prestalk cells could be initially detected at the loose cell aggregate stage and that both cell types were dispersed randomly in the cell mass. Prestalk cells then sorted out to the tip and basal regions of the cell mass, leaving prespore cells occupying the middle.

The regulatory mechanisms of transcription of three prespore specific genes, D19, SP60 and SP96 have been studied (Early and Williams, 1989; Haberstroh and Firtel, 1990, 1991; Tasaka et al., 1992). These genes can be subdivided into two groups according to the intracellular distribution of their products. One group, including SP96 and SP60, is the spore coat protein genes whose products accumulate in prespore-specific vacuoles and are exocytosed during formation of mature spores, to construct the spore coat. The other, D19, is the gene for the cell surface protein, PsA, of prespore cells (Early et al., 1988). This *cis*-regulatory regions of SP96 and SP60 showed similarities, but differed from D19. The presence of nuclear factor(s) which specifically bind the *cis*-region of SP96 has been reported (Tasaka et al., 1992).

We have previously isolated a member of a new class of prespore enriched cDNA clone called Dp87 by differential

screening (Ozaki et al., 1988). Northern hybridization indicated that its mRNA begins to accumulate at the tipped aggregate stage and is enriched in prespore cells, but is then lost during fruiting body formation, in contrast to other prespore specific mRNAs. In vitro run-on assays, using nuclei isolated from cells at different developmental stages or separated prespore and prestalk cells, indicated that the Dp87 gene is transcribed only in prespore cells (Morio et al., 1991).

In this paper, we focus on the elucidation of the regulatory mechanisms of transcription of the Dp87 gene.

MATERIALS AND METHODS

Culture

Amoebae of *D. discoideum*, strain KAX-3 were grown in HL-5 medium and those of strain NC-4 were grown with *Klebsiella aerogenes* on SM agar.

Stalk cell induction by DIF

The mutant strain HM44, which is defective in production of DIF (Differentiation Inducing Factor, a low relative molecular mass stalk-specific morphogen) was used for stalk cell induction under submerged conditions (Town et al., 1976; Kopachik et al., 1983). Vegetative cells were incubated for 12 hours in the presence of 5 mM cAMP in stalk salt solution, harvested and washed in KK₂ buffer and incubated with or without 3000 U/ml of chemically synthesized DIF in stalk solution containing 5 mM cAMP.

Cloning of cDNAs and genomic fragments, sequencing and primer extension

The cDNA clone Dp87 (cDp87-2) has been isolated from a cDNA library constructed from poly(A)⁺RNA of slug cells by differential screening using poly(A)⁺RNA isolated from prespore and prestalk cells, as previously described (Ozaki et al., 1988). To isolate longer cDNA, the same library was rescreened using a cDp87-2 probe and cDp87-1 was isolated; this contained an almost full length cDNA. Southern hybridization analyses of nuclear DNA using these cDNA clones indicate that this gene is unique (data not shown). The restriction map near this gene is shown in Fig. 1. Based on this map, the genomic clones of gDp87-1 and gDp87-2 were isolated from mini-genomic libraries including an appropriate size of *Cla*I and *Pst*I fragments of genomic DNA which were constructed in the same way as described previously (Tasaka et al., 1990). DNA sequencing and the primer extension to determine the initiation site of transcription were conducted as described previously (Sanger et al., 1977; Tasaka et al., 1990).

Construction of chimeric genes and transformation

An *Eco*RV-*Alu*I genomic fragment of 1.4 kb was isolated from gDp87-1. The fragment includes 1380 bp of 5' upstream region and 130 bp of 5' untranslated region and 19 bp of translated region of the Dp87 gene. This genomic fragment was inserted in *Sma*I site of plasmid pPAVCAT which was kindly supplied by Dr C. K. Singleton (Singleton, 1987). In the resulting chimeric gene (pDp87CAT), the coding region starting from the initiation codon of the Dp87 gene was joined to the CAT coding region in frame, an extra 22 amino acids being added on the N terminus of the CAT protein. To construct mutated pDp87CAT genes, deleted in the 5' upstream regions, pDp87CAT was deleted unidirectionally by *Exo*III nuclease and mung bean nuclease from the 5' end, as described previously (Henikoff, 1984; Tasaka et al., 1992). The

joined part of the Dp87 fragment and pPAVCAT and the deleted end points (shown in Fig. 2A) were ascertained by DNA sequencing. Plasmids containing different chimeric Dp87-CAT genes were co-transformed by the calcium phosphate method into KAX-3 cells with plasmid B10SX, which contains the Neo^R selectable marker (Nellen et al., 1985, 1987).

The *Alu*I-*Alu*I fragment of pDp87-1 was inserted into the multicloning site of plasmid pDD GAL(H)⁺, kindly provided by Dr J. Williams (Jermyn and Williams, 1991). This plasmid (pDp87-gal) contained 666 bp of 5' upstream region and intact 5' untranslated region and 19 bp of coding region of the Dp87 gene, and the translation initiation codon of Dp87 was joined in frame with the -gal gene. It contained an additional 10 amino acids at the N terminus and had an actin 8 terminator. KAX-3 cells were transformed using pDp87-gal by electroporation (Howard et al., 1988). Transformants were screened and each clone was cultured in the same way as described previously (Tasaka et al., 1992).

CAT assay

The method for CAT assay was basically as described by Gorman et al. (1982). Cells, sampled from different developmental stages, were suspended in 250 mM Tris-HCl buffer (pH 7.9) with 5 mM EDTA, frozen quickly and stored at -80°C. Before assay, the cell lysate was thawed, additionally frozen and thawed three times and centrifuged at 15000 rev/minute for 10 minutes to remove cell debris. The supernatant was used for CAT assay and protein analysis. The preliminary experiments showed that there was no strong inhibitor or protease activity in the cell lysate to block the CAT assay.

Histochemical detection of β-gal gene

After growth cells were collected and spread on Nylon filters at 5×10⁶ cells/2.5 cm² put on a pad saturated with KK₂ phosphate buffer. The methods of fixation and staining cell masses were the same as described (Dingerman et al., 1989).

Isolation of nuclear extract and gel retardation experiment

5×10⁹ cells were harvested, washed with KK₂-phosphate buffer (20 mM, pH 6.4) containing 10 mM EDTA, washed finally with 0.2% NaCl, and suspended in 20 ml of nuclei-isolation buffer (NI buffer; 50 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 10% sucrose, 2% NP-40, and 0.5 mM PMSF), vortexed for 1 minute, and centrifuged at 1500 revs/minute for 5 minutes to remove unbroken cells. Pelleted cells were resuspended in the NI buffer and centrifuged again. The first and second supernatant were combined and centrifuged at 4500 revs/minute for 5 minutes to collect the nuclei. The nuclei were suspended in 300 μl of NI buffer, and an equal volume of solution II (10 mM Hepes pH 7.9, 600 mM NaCl, 10% sucrose, 5 mM MgCl₂, 0.1 mM EDTA, 0.5% NP-40, 1 mM DTT, 5 mM spermidine and 0.5 mM PMSF) was added, vortexed for 1 minute, and incubated at 4°C for 1-1.5 hours with occasional mixing. The solution was centrifuged at 15000 rev/minute at 4°C for 1 hour. The supernatant was dialyzed against solution III (10 mM Hepes, pH 7.9, 1 mM MgCl₂, 20% glycerol, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT and 0.5 mM PMSF), at 4°C for more than 2 hours and centrifuged at 10000 rev/minute at 4°C for 10 minutes and the supernatant was stored at -80°C.

The gel shift assay contained 0.1-1 ng of DNA (specific activity 0.1-5×10⁸ counts/minute per μg DNA), 0.5-1 μg of poly dA/dT, 1 μg of BSA and nuclear extract (approx. 10 μg of protein) and an appropriate concentration of competitor DNA and was incubated at room temperature or 4°C for 30 minutes. The mixture was applied to 5% polyacrylamide gel (0.5 mm thick) and

electrophoresed at 4°C for 2-4 hours at 150 V with 1× TBE (90 mM Tris-borate pH 8.0, 2 mM EDTA).

Isolation of total RNA and northern blotting analysis

Total RNA was extracted by the sodium dodecyl sulfate/phenol method or acid guanidinium thiocyanate-phenol-chloroform method and electrophoresed through 1% neutral agarose gel, transferred to nylon membrane, hybridized with ³²P-labeled DNA probes and analyzed using X-ray film or a Fuji image plate analyzer (Chomczynski and Sacchi, 1987; Ozaki et al., 1988; Morio et al., 1991).

RESULTS

DNA sequences of genomic and cDNA clones

The cDNA clone of cDp87-2 (1.2 kb) was isolated by differential screening, using poly(A)⁺RNA from prespore and prestalk cells, from a cDNA library constructed using poly(A)⁺RNA of tipped aggregate cells (Ozaki et al., 1988). Northern hybridization indicates that only one mRNA species of about 2.0 kb long is present in prespore cells. A cDNA clone, cDp87-1, which contains almost the full-length cDNA was isolated from the same library, using cDp87-2 as the probe. Southern hybridization indicated that the Dp87 gene is unique (data not shown), and that two genomic clones, gDp87-1 and gDp87-2, included the 5' side and the 3' side of the gene, respectively, as shown in Fig. 1.

The DNA of part of genomic clones gDp87-1 and gDp87-2, and the cDNA clone cDp87-1 were sequenced and compared. The genomic DNA sequence is shown in Fig. 2A. Only one long open reading frame was found in the cDNA clone. The comparison between the genomic clones and the cDNA clone indicates that there is one short intron near the 5' end of the coding region. The intron is heavily AT-rich and has general acceptor and donor sites (Grant et al., 1990). The initiation site of transcription was determined by the primer extension method, and is indicated in Fig. 2A (Tasaka et al., 1990, data not shown). The end of the cDNA clone is also shown in Fig. 2A. The end point of the transcription is not clear, but it should be near the end of the cDNA because the length of cDp87-1 is almost the same as the size of the mRNA and there is a putative poly(A) additional signal (AATAAA) at the 3' end of the cDNA. The 5' and 3' untranslated regions and the upstream and downstream regions of this gene are enriched with AT, which is common in *D. discoideum*.

The deduced amino acid sequence of the Dp87 gene is shown in Fig. 2B. It contains 555 amino acids and the molecular mass is calculated as 58,672 Da. At the N terminus, there is a typical leader sequence of 17 amino acids. The peptide has a long serine-alanine repeat at the C terminus and a threonine-rich region just before this repeat. Many cysteines (10.8%) are dispersed from the N terminus to the middle of the peptide. The peptide may be glycosylated, since it contains four possible N-glycosylation sites in the middle and some threonine residues. The peptide shows 27.9% overall similarity to SP70 and 25.5% to SP96, but

no homology to SP60, all of which are spore coat proteins. It shows no significant similarity to any other protein in the NBRF-PDB and SWISS-PROT protein data bases (Fosnaugh et al., 1989; Haberstroh and Firtel, 1990; Tasaka et al., 1990). Some highly homologous regions between it and SP70 or SP96 are dispersed throughout the peptide. In particular, a part of the C-terminal half of the Dp87 peptide, near the serine-alanine repeat shows high similarity to a part of the C-terminal half of SP70 (18/22 amino acids). The serine-alanine repeat of Dp87 is also highly homologous to that of SP96 (Fig. 2C). There is no conserved region among the three peptides.

Identification of cis-acting region for transcription

The Dp87 gene is transcribed only in prespore cells and its transcription is regulated by extracellular cAMP (Takemoto et al., 1990; Morio et al., 1991). To investigate the mechanisms regulating transcription, 1380 bp of 5' upstream DNA of Dp87 gene, or deleted fragments, were inserted in frame into the *Sma*I site of the 5' upstream region of the CAT gene of the transformation vector pPAVCAT. The chimeric genes were co-transformed into *D. discoideum* Ax3 cells with B10SX which includes the gene Neo^R allowing transformants to be selected (Nellen et al., 1987). The CAT activity of cloned transformants was assayed at the vegetative and slug stages. Each transformant was cultured and analyzed twice. All the extracts isolated from vegetative cells had no, or very weak, CAT activity and the average activity of slug cells of each clone is shown in Fig. 3A. Nine out of 11 independent transformants including the intact DNA fragment were strongly active at the slug stage. This suggests that the 5' upstream DNA fragments used include enough *cis* information for transcription at the slug stage. Although the activity of each clone varied, Southern hybridization showed that each transformant included several tens of CAT genes in tandem and the numbers integrated were almost the same among transformants (data not shown).

The CAT activity of the transformants including different lengths of the 5' upstream region of the Dp87 gene are

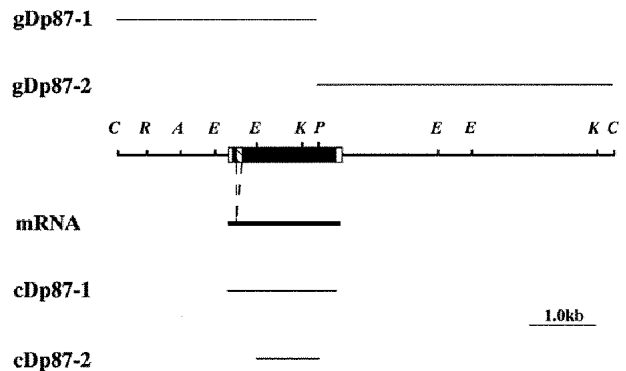


Fig. 1. Restriction map of the Dp87 gene, two cDNA clones and two genomic clones. The coding and non-coding regions are indicated by filled and open boxes, respectively. The intron is indicated by vertical white dashes. A, *AluI*; C, *Clal*; E, *EcoRI*; K, *KpnI*; R, *EcoRV*; P, *PstI*.

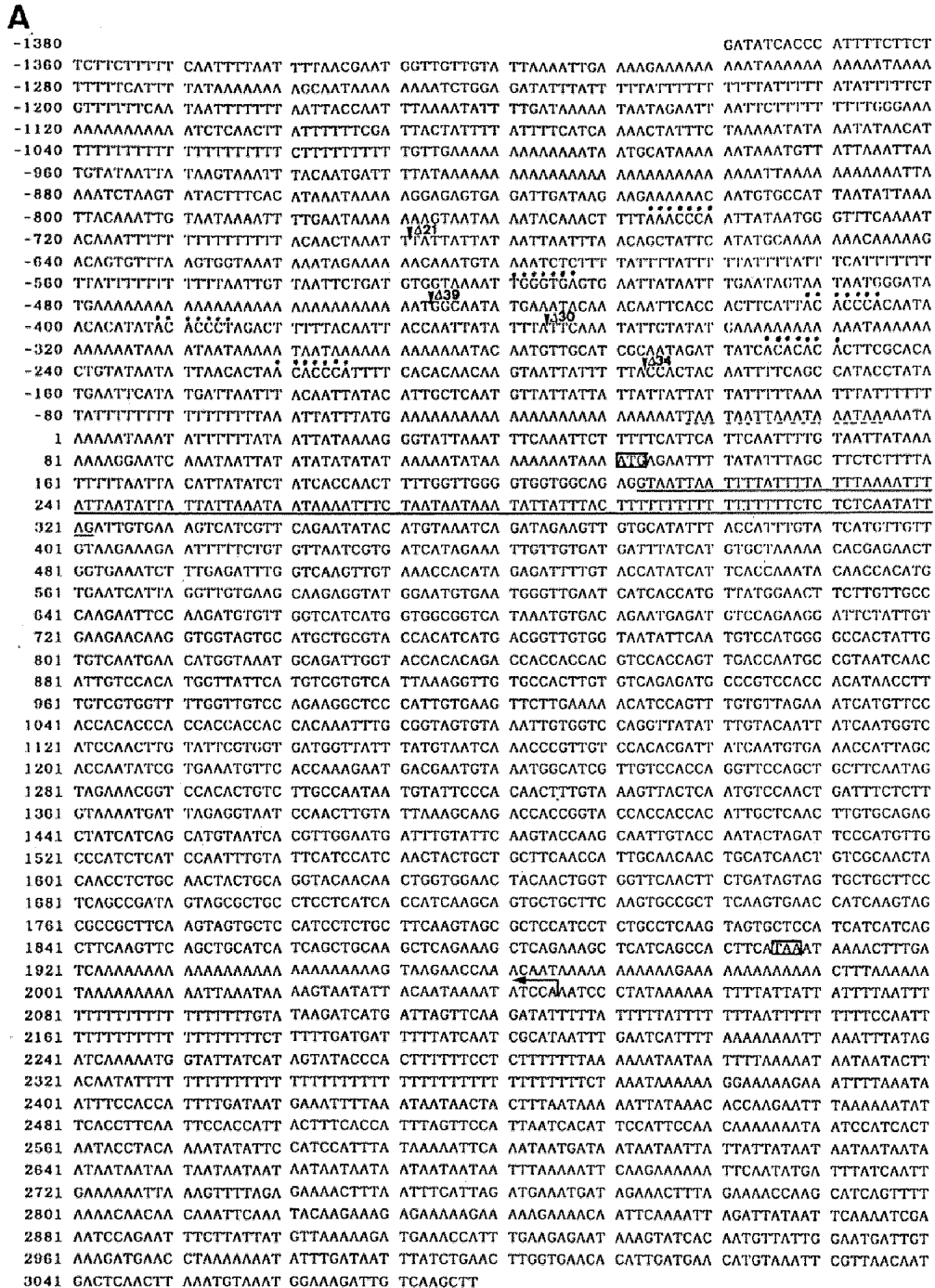


Fig. 2

summarized in Fig. 3A. All the transformants showed no CAT activity at the vegetative stage. Six out of seven A21-CAT transformants containing -689 bp to +149 bp expressed CAT activity at the slug stage. Four out of five A39-CAT transformants containing -447 bp to +149 bp also expressed CAT activity at the slug stage, while all (3 clones for each) of A30-CAT (-356 bp to +149 bp) and A34-CAT (-187 bp to +149 bp) did not express CAT activity at the slug stage. These indicate that there is a *cis*-reg-

ulating region from -447 bp to -356 bp related to transcription of Dp87 gene. Though the number of transformants examined was limited, the average activity of A21 and A39 showed a significant difference, suggesting that there is a regulatory element from -736 bp to -448 bp to increase the rate of transcription.

To investigate whether the 5 upstream regions used in the above experiments include enough information for pre-spore-specific transcription, slugs of some transformants

Nuclear factor binding to the *cis*-acting region

To identify any nuclear factor binding to the *cis*-acting region of Dp87 gene, a nuclear extract was isolated from slug cells and mixed with a 32 P-labeled DNA fragment -432 bp to -350 bp, which was almost the full length of the *cis* region (-447 bp to -356 bp), and the mixture applied to 5% polyacrylamide gel electrophoresis. As shown in Fig. 4B, a few retardation bands were observed. However, when the same unlabeled DNA fragment was added at increasing concentrations, only one band (indicated by the arrowhead) was competed out. This band was not competed out by DNA from outside the *cis* region or by heterologous DNA. These results indicate that there is a factor in the slug nuclear extracts that specifically binds

to the *cis* region. This nuclear factor should be a protein, because the band did not appear when the nuclear extract was pretreated with trypsin or heated at 90°C for 5 minutes (data not shown).

To further localize the specific binding sequence, shorter DNA fragments were synthesized and used for gel retardation experiments as probes or competitors (Fig. 4C,D). When the full-length DNA fragment was used as a probe and two shorter DNA fragments in this region were used as competitors, both shorter fragments competed for specific binding when present in a 10 times excess over the probe (Fig. 4C). In contrast, DNA fragments from outside the *cis* region could not compete. When the shortest DNA fragment (frag c) consisting of 13 base pairs was used as

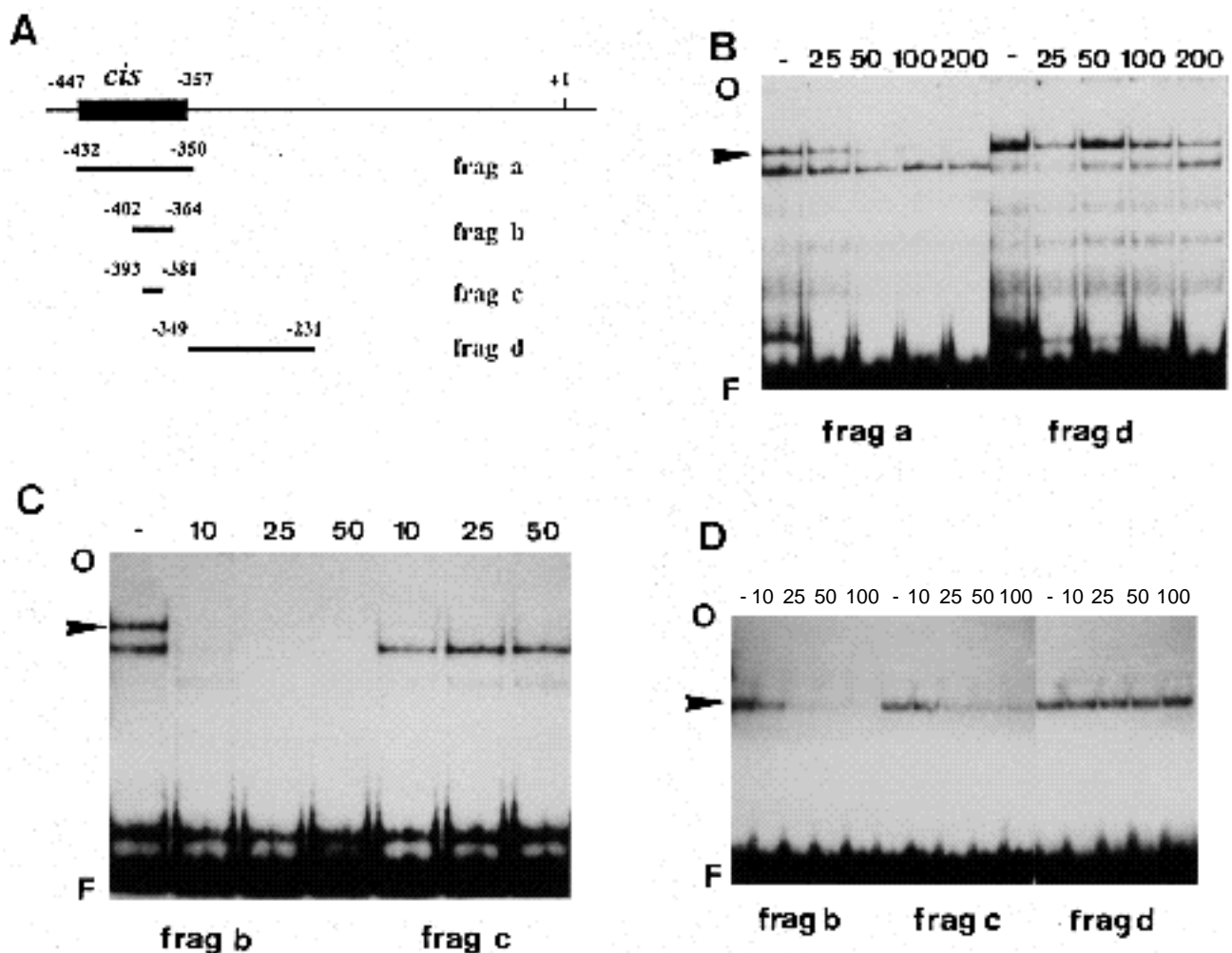


Fig. 4. Gel retardation assays. Cells were lysed with NP-40 to isolate nuclei. Nuclear proteins were extracted with 0.3 M NaCl. 32 P-labeled DNA fragments were mixed with the nuclear extract, incubated at 24°C for 30 minutes and applied to 5% polyacrylamide gel. The competitor DNA fragment used in each experiment is indicated below each figure. Above each lane, the concentration of the competitor DNA is given (molar excess over the probe). The lanes labeled - contained no competitor. Arrowheads mark the specific retardation band; F, the position of free probe and O, the original position. (A) Frag-a is from -432 to -350, frag-b from -402 to -364, frag-c from -393 to -381 (TACACCCTAGACT), and frag-d from -349 to -231. The *cis* region (from -447 to -357) as indicated by transformation experiments (Fig. 3) is shown by a filled box. (B) Frag-a was used as a probe. Different concentrations of homologous (frag-a, left) or heterologous (frag-d, right) DNA fragments were added as competitors to the incubation mixture. (C) Frag-a was a probe and frag-b (left) and frag-c (right) were competitors. In the case of frag-b, not only the specific retardation band (arrowhead), but also another retardation band just below the specific one disappeared. When frag-c, a part of frag-b, was used, only the specific band disappeared. (D) Frag-a was used as a probe. In this case, only one retardation band (arrowhead) was observed. Frag-b (left), frag-c (middle) and frag-d (right) were added as competitors.

a probe, only one retardation band was observed and it was competed out by the homologous or a slightly longer DNA fragment (Fig. 4D), but not by other fragments outside of this region (data not shown). This suggests that the retardation obtained with the shortest DNA fragment is sequence-specific.

Detection of Dp87 gene expression

To detect Dp87 gene expression histochemically, the DNA fragment from -666 bp to +149 bp was joined in frame to the -galactosidase gene of pDD GAL(H)⁺ and introduced into *D. discoideum* cells. Fig. 5A shows histochemical staining of transformant slugs. All the prespore cells occupying the posterior three-quarters of slugs were stained homogeneously. This indicates that the DNA fragment used for transformation contains enough information for specific transcription in prespore cells.

Surprisingly, histochemically stained cells were first detected in aggregation streams which radiate from an aggregation center (Fig. 5B). The number of stained cells was initially very low, but the intensity of the staining was about the same as that of slug cells. The number of stained

cells increased dramatically during aggregation and they were evenly distributed in loose aggregates as well as in aggregation streams (Fig. 5C). At a later stage, the tip and the basal part reach to the agar surface became unstained and this pattern of stained cells was basically maintained at the standing and migrating slug stages (Fig. 5A). When the same 5' upstream DNA fragment was inserted into a plasmid containing the -gal gene joined to the SP60 terminator instead of the actin 8 terminator used in the previous vector, the stained cells were first detected at a later aggregating stage before loose mound formation and the intensity of the staining was weaker. (The plasmid containing -gal gene and SP60 terminator was kindly provided by Dr Firtel of the University of California at San Diego; Haberstroh and Firtel, 1990.)

The stage when Dp87 transcription was first detected by histochemical staining was earlier than suggested by our previous northern hybridization experiments. This inconsistency is probably due to the difference in sensitivity of the methods used. To confirm this point, cells transformed by pDp87 -gal were allowed to develop synchronously and total RNA was isolated from vegetative, incipient aggrega-

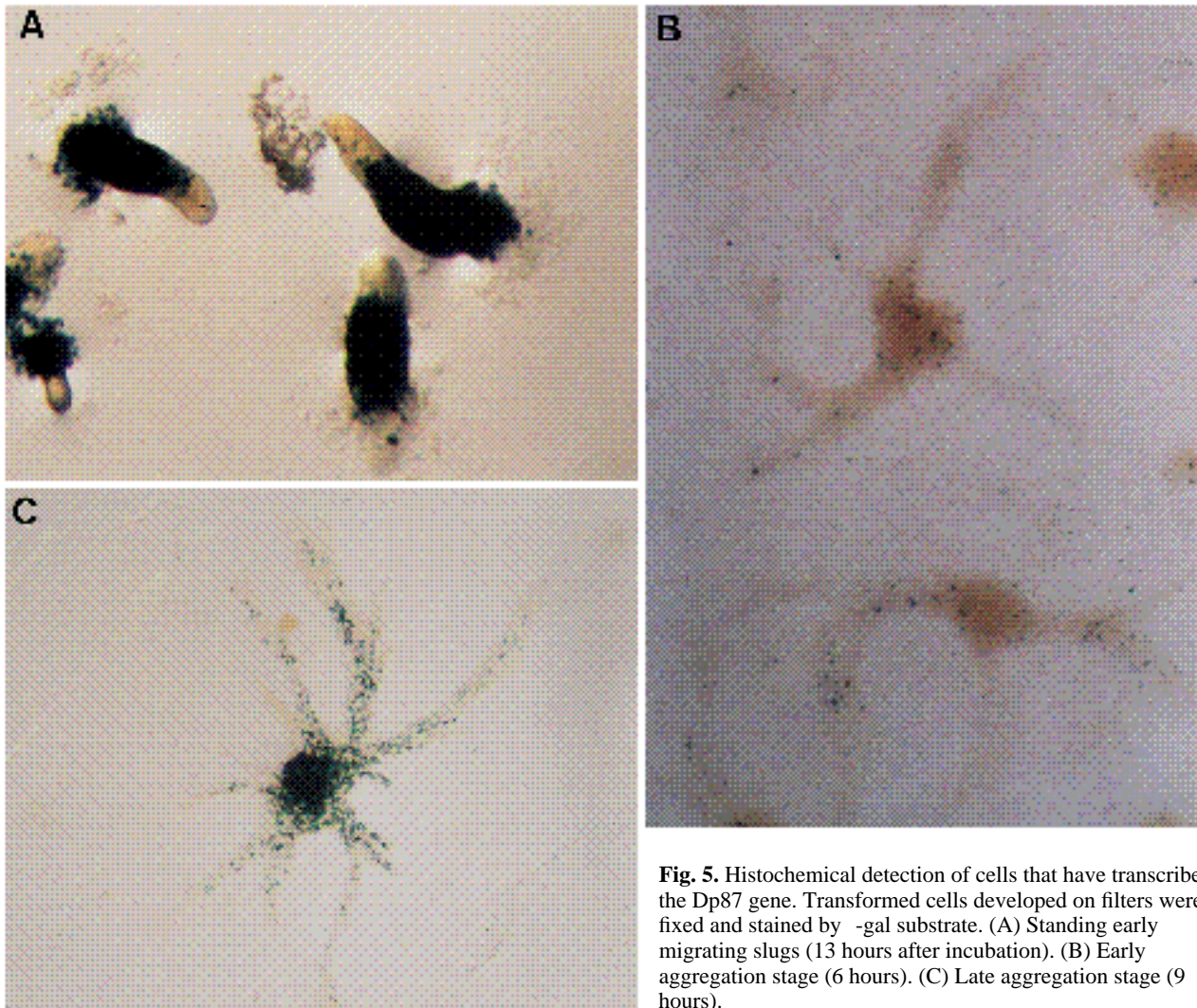


Fig. 5. Histochemical detection of cells that have transcribed the Dp87 gene. Transformed cells developed on filters were fixed and stained by -gal substrate. (A) Standing early migrating slugs (13 hours after incubation). (B) Early aggregation stage (6 hours). (C) Late aggregation stage (9 hours).

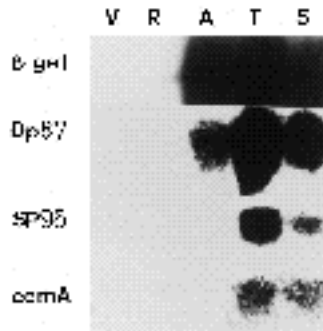


Fig. 6. Northern analyses of Dp87 gene expression using an image analyzer. Cells that transformed by pDd87 -gal were harvested at given developmental stages and total RNA was extracted by the single-step RNA isolation method (Chomczynski and Sacchi, 1987). Total RNA was electrophoresed on 1% agarose gel, blotted on nylon membranes and hybridized with 32 P-labeled cDNAs, -gal Dp87 SP96 and ecmA. The filters were analyzed by an image analyzer FUJIX BAS2000 (Fuji Photo Film Co., Ltd). V, vegetative growth phase; R, incipient aggregative (rippling) stage; A, stream stage; T, tipped stage; S, slug stage.

tive (rippling), stream, tipped aggregate and slug cells. After gel electrophoreses of these RNAs, the filters were hybridized with 32 P-labeled Dp87, -gal, Sp96 and ecmA cDNAs and the hybridization patterns were analyzed by a Fuji image plate analyzer. The results are shown in Fig. 6. Both Dp87 mRNA and -gal mRNA are detected at the stream, tipped aggregate and slug stages but not at the vegetative and rippling stages. In contrast, SP96 and ecmA mRNAs were first detected at the tipped aggregate stage.

Effect of DIF on Dp87 gene expression

Transcription of the prespore specific D19 gene is repressed by DIF, a prestalk and stalk cell inducer (Early and Williams, 1988). The effect of DIF on the expression of the Dp87 gene was examined using a DIF-defective mutant strain, HM44. After cells were preincubated with cAMP alone, chemically synthesized DIF was added to the incubation medium and isolated total RNA analyzed by northern hybridization. Under the experimental conditions, more

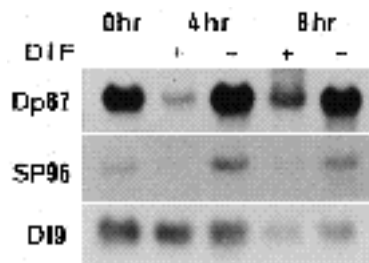


Fig. 7. The effects of DIF on the expression of prespore-specific genes. Vegetative HM44 cells were preincubated in the presence of 5 mM cAMP for 12 hours and then incubated with or without 3000 U/ml of DIF. Cells were harvested at 4 hours and 8 hours after DIF addition and total RNA was extracted for northern analyses. After electrophoresis, filters were hybridized with 32 P-labeled cDNAs Dp87 SP96 and D19 and exposed to X-ray film.

than 90% of cells differentiated into vacuolate stalk cells after 48 hours of incubation with DIF, but no stalk cells formed without DIF. Syntheses of both ecmA and ecMB mRNAs were clearly induced in the presence of DIF, in agreement with the findings of Kopachick et al. (1985) and Early and Williams (1988) (data not shown). It was shown that like D19 mRNA, Dp87 and SP96 mRNAs accumulated during the preincubation periods with cAMP (Fig. 7). After DIF addition, the levels of Dp87 and SP96 mRNAs declined within 4 hours, but that of D19 mRNA did not decrease appreciably during the first 4 hours, but then declined gradually.

DISCUSSION

In this paper, we characterized a novel prespore-specific gene, Dp87, whose transcription occurs only in prespore cells (Morio et al., 1991) and is controlled by exogenous cAMP (Takemoto et al., 1990). The amino acid sequence of the protein encoded by this gene shows low, but significant, similarity to the spore coat proteins, SP96 and SP70. Though this suggests that Dp87 is a member of the spore coat protein family, it has three distinct characteristics from the other proteins. First, Dp87 mRNA completely disappears at the culmination stage, as analyzed by northern hybridization (Ozaki et al., 1988). The disappearance of mRNA in culmination must be brought about by its degradation, for the transcription continues during culmination (Morio et al., 1991). Second, we have demonstrated that its transcription starts at an earlier aggregation stage than the others. Third, although the Dp87 gene product is stored in prespore vacuoles, it is discharged into the interspace of spores during spore formation, as detected by a specific antibody produced against the Dp87 peptide (Nakao et al., unpublished data). These facts strongly suggest that Dp87 is a novel type of prespore-specific gene.

The initiation of transcription of the Dp87 gene is the earliest event of prespore cell differentiation so far detected. Accumulation of -galactosidase from a Dp87-reporter construct starts earlier than suggested by previous northern analyses (Ozaki et al., 1988). However, in the present work using a more sensitive image plate analyzer (Fig. 6) shows that Dp87 mRNA is first detected at the aggregation stream stage, concurrently with appearance of -galactosidase activity in the transformant. The fact that both Dp87 mRNA and -galactosidase activity appeared at the same stage suggests that the -gal expression pattern correctly reflects the transcription pattern of Dp87 gene.

It was shown that both Dp87 and SP96 mRNAs rapidly declined within 4 hours after addition of DIF to HM44 cells preincubated with cAMP. In contrast, D19 mRNA did not decrease as rapidly as Dp87 and SP96, but eventually declined as well. In essence, transcription of all these prespore-specific genes was shown to be negatively regulated by DIF, in agreement with the previous study by Early and Williams (1988). As shown before, the transcription of both Dp87 and SP96 genes is positively regulated by exogenous cAMP in disaggregated slug cells (Takemoto et al., 1990). This suggests that the regulatory mechanism of transcription of these prespore genes may be similar. However, the data in Figs 5, and 6 and the evidence that cells transformed

by SP60-*LacZ* first showed β -galactosidase activity only after cell mounds were formed (unpublished data) indicate that transcription of the Dp87 gene begins before that of SP96 and SP60. This suggests that there may be at least two different stages of prespore differentiation, which are under different transcriptional control.

It has been generally believed that prespore and prestalk cells differentiate simultaneously after formation of a cell mound in the aggregation center. The present result, that prespore cells expressing β -galactosidase activity appeared at an earlier stage of aggregation, suggests that prespore differentiation may precede prestalk differentiation.

The present study clearly shows that prespore differentiation occurs independently of the position of cells in a cell mass. That β -galactosidase, produced by the chimeric Dp87 gene in transformants, is very stable was shown by the fact that disaggregated prespore cells maintained the enzyme activity long after Dp87 mRNA and β -gal mRNA were completely lost (data not shown). This probably explains why a few stained cells are occasionally observed in the anterior prestalk region of a migrating slug (Harwood et al., 1991). It is, therefore, most likely that the changes of distribution of β -galactosidase cells during development is caused by movement of cells that have already expressed Dp87. The developmental changes in the pattern of β -galactosidase-positive cells were the same as those detected previously using prespore specific antibodies or other transformants with SP60 or D19 (prespore) and *ecmA* (prestalk) promoters (Early and Williams, 1988; Williams et al., 1989; Haberstroh and Firtel, 1990). These results strongly support the idea that the construction of the prespore and prestalk pattern in slugs is brought about by sorting out of differentiated cells (Tasaka and Takeuchi, 1981).

The *cis*-acting region involved in transcription of the Dp87 gene was investigated by transformation experiments using various chimeric genes. A chimeric gene including the DNA fragment from -447 bp to +149 bp was transcribed only in prespore cells, while that including -355 bp to +149 bp was transcribed in neither vegetative nor slug cells. This indicates that the region between -447 bp and -356 bp is a *cis*-acting region for transcription. The sequence of this region was compared to that of the proposed *cis*-acting regions of the other prespore-specific genes such as SP96, SP60 and D19 (Early et al., 1988; Haberstroh and Firtel, 1990, 1991; Tasaka et al., 1992). The CA-box, 'ACACCCA', is observed in Dp87, SP96 and SP60, but not in D19, and the same sequence is also observed in the 5' upstream region of another spore coat protein SP70 (Fosnaugh and Loomis, 1991). In particular, a CA-box is included in the CAE-1 element, which is one of the regulatory regions of the SP60 gene (Haberstroh and Firtel, 1991). This suggests that this element may be important for transcription of these prespore genes, as discussed in our previous papers (Tasaka, 1991; Tasaka et al., 1992). As a gene including less than -355 bp was inactive in either vegetative or slug cells, whether the proposed *cis*-acting region is involved in cell-type- and stage-specific transcription or merely in general activation of transcription is not known.

A nuclear factor(s) that sequence-specifically binds to the *cis* region of Dp87 was identified, and gel shift assays using synthesized shorter DNA fragments showed that the factor

specifically binds to frag-c, which includes one of the CA-boxes. It was previously suggested that the same nuclear factor(s) may bind to the *cis* regions of both Dp87 and SP96 (Tasaka, 1991). It is also highly plausible that this factor(s) is similar to one of the CAE binding factors identified by Haberstroh and Firtel (1991). As this factor(s) binds to the *cis* region in a sequence-specific manner, it must constitute part of the regulatory machinery involved in the transcription of the Dp87 gene.

We are grateful to Dr C. Singleton of Vanderbilt University for the pPAVCAT vector, to Dr R. Firtel of University of California for the SP60-*LacZ* and Dr J. Williams of ICRF for the pDD GAL(H)⁺. We also thank Dr R. Kay of MRC for DIF and HM44. This work was supported by Grants-in-Aid (01044080, 02236101, 02258222, 02454018, 02640531, 03220101, 03254102) from the Ministry of Education, Science and Culture of Japan and by a grant from the Naito Foundation.

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(Accepted 2 January 1993)

Note added in proof

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with following accession number D13973.