A Dictyostelium prespore-specific gene is transcriptionally repressed by DIF in vitro

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Summary

One important role of DIF, the stalk cell-specific inducer of Dictyostelium, may be to divert cells from the spore cell pathway of differentiation. The D19 gene encodes an mRNA which is highly enriched in prespore over prestalk cells in the migratory slug. We show, using a mutant defective in DIF accumulation, that the concentration of D19, and several other prespore mRNA sequences, decreases in the presence of exogenous DIF. There is evidence that both transcriptional and post-transcriptional controls operate to regulate expression of these genes. We have performed in vitro nuclear transcription and mRNA half-life analyses, and find that DIF acts at the transcriptional level to repress the accumulation of the D19 mRNA.

Key words: Dictyostelium discoideum, DIF, prespore gene, transcription.

Introduction

Cells in the anterior one fifth of the Dictyostelium slug normally differentiate to form dead, vacuolated stalk cells while the majority of cells in the posterior region differentiates into spores. Cyclic AMP is the chemotactant responsible for cellular aggregation and cAMP signalling is thought to persist during slug migration (reviewed by Schaap, 1986). In disaggregated cells, shaken rapidly in suspension, the local extracellular cAMP concentration is reduced and several prespore-specific genes have been shown to have a reduced transcription rate and reduced mRNA stability (Landfear et al. 1982; Mangiarotti et al. 1983). These effects can be overcome by the addition of cAMP (Mangiarotti et al. 1983). Thus, prespore gene expression is regulated by cAMP at both a transcriptional and a post-transcriptional level.

Two substances, adenosine and DIF, have been shown to be capable of repressing prespore cell differentiation. In aggregation-competent cells, shaken in suspension, addition of adenosine inhibits cAMP-induced prespore gene expression (Weijer & Durston, 1985; Wang et al. 1986). In intact slugs, reduction of the adenosine concentration by enzymic depletion results in the appearance of prespore cells in the prestalk zone (Schaap & Wang, 1986). DIF is a low molecular weight chlorinated compound of known structure (Morris et al. 1987), which will induce isolated amoebae to differentiate into stalk cells (Town et al. 1976). If slugs are disaggregated and incubated in the presence of DIF, then prespore gene expression is repressed (Kay & Jermyn, 1983). The effect of DIF on the expression of individual genes can best be studied in vitro using the HM44 mutant which is blocked in the accumulation of DIF but remains DIF-responsive (Kopachik et al. 1983). If cells of the HM44 strain are incubated with cAMP for approximately 10 h, and DIF is then added, at least 90% of the amoebae form stalk cells. In the absence of DIF only 1% of the cells differentiates into stalk cells. After incubation with cAMP the cells contain prespore-specific proteins, distinguishable by 2-D gel electrophoresis and the addition of DIF suppresses their expression (Kopachik et al. 1985).

The D19 cDNA clone derives from an mRNA sequence that is highly enriched in the prespore region of the slug (Barklis & Lodish, 1983). We have established the sequence of the D19 gene and shown it to encode PsA, a cell surface glycoprotein of prespore cells (Grant et al. 1985; Early, Williams, Meyer, Por, Smith, Williams & Gooley, manuscript submitted). PsA is known to be specifically recognized by a monoclonal antibody, MUD-1 (Krefft et
al. 1983). Using this gene, we have investigated the mechanism whereby DIF acts to repress specific prespore gene expression.

Materials and methods

Dictyostelium discoideum growth and development

The mutant strain HM44 (Kopachik et al. 1983) was grown in association with Klebsiella aerogenes on SM agar plates (Sussman, 1966). Stalk cell induction was performed in submerged monolayer culture as described by Kopachik et al. (1985). Cells were incubated in the presence of 5 mM-cAMP for 10 h and the buffer was replaced with fresh buffer additionally containing 3000 units DIF-1 ml⁻¹ (Morris et al. 1987). Plates containing nogalamycin, which was added to 300 µg ml⁻¹ 5 min prior to the addition of DIF, were placed in a light-proof box to prevent inactivation of the drug. Nogalamycin was the generous gift of the Upjohn Co., Kalamazoo, MI, USA.

RNA isolation and Northern transfer

One plate of cells, containing approximately 4 x 10⁷ amoebae, was used to prepare RNA for each timepoint of the stalk cell induction. The cells were pelleted and frozen in dry ice. The frozen pellets were directly resuspended into 1 ml of buffer containing 50 mM-Hepes, 40 mM-MgOAc, 20 mM-KCl, 0.5% SDS, 200 mM-NaCl and 0.5% diethylpyrocarbonate. Total nucleic acid was prepared by repeated phenol–chloroform extraction. Aliquots (5 µg) of RNA were electrophoresed through a 1% denaturing formaldehyde gel and transferred to nitrocellulose filters (Maniatis et al. 1982). The filters were prehybridized for 4 h at 37°C in 50% formamide, 5% SSC, 10xDenhardt’s solution, 100 µg ml⁻¹ heat-denatured salmon sperm DNA, 1% SDS and hybridized in the same conditions overnight. The filters were probed with cDNA clones labelled with 32P by the method of Feinberg & Vogelstein (1983). The filters were washed four times for 15 min each in 2xSSC, 1% SDS at 65°C before autoradiography.

Isolation of nuclei and in vitro nuclear transcription

Synthetically active nuclei were prepared and stored as described by Jacobson (1976) and used for in vitro nuclear RNA synthesis as described previously (Williams et al. 1987). The labelled RNA was hybridized to Southern transfers bearing restriction digests of the D19 cDNA clone and of a constitutively transcribed sequence, 1G7. The filters were prehybridized at 37°C in a buffer containing 50% deionized formamide, 5% SSC, 200 µg ml⁻¹ BSA, 0.2% SDS, 300 µg ml⁻¹ Ficoll, 500 µg ml⁻¹ poly rA and 100 µg ml⁻¹ wheat germ tRNA. After a 4 h prehybridization the 32P-labelled transcripts were added to this buffer and hybridized for two days at 37°C. The filters were washed four times for 15 min each in 50% formamide, 1% SSC, 0.1% SDS at 37°C.

Results

(A) The addition of DIF to HM44 cells decreases the concentration of prespore-specific mRNA sequences

In a standard stalk cell induction, HM44 cells are incubated in tissue-culture plates with cAMP alone for 10 h and then with cAMP and DIF for an additional 12 h (Kopachik et al. 1985). After 10 h in the presence of cAMP, prespore-specific proteins are present and addition of DIF leads to their disappearance. In order to examine this process at an RNA level, the D19 cDNA clone was used to probe a Northern blot of RNA extracted from HM44 cells harvested during a stalk cell induction (Fig. 1). The amount of DIF used was sufficient to transform more than 85% of amoebae into morphologically recognizable stalk cells after a 12 h incubation period. Total RNA was extracted from cells harvested just before the medium change and at intervals afterwards. After the 10 h cAMP preincubation, the cells contain D19 mRNA and, in the absence of DIF, the level of D19 mRNA remains constant. When DIF is present, the level of D19 mRNA declines such that, approximately 4 h after DIF addition, it is at about 10% of its former level. This low level is maintained throughout the remaining incubation. The residual D19 mRNA may derive from those amoebae that never mature into stalk cells. It takes about 90 min for the amount of D19 mRNA to be halved. Two other prespore mRNA sequences, those corresponding to the cDNA clones PL3 and D7 (Barklis & Lodish, 1983; Oyama & Blumberg, 1986), were found to be repressed by DIF with identical kinetics (Fig. 2).

(B) DIF acts to repress D19 gene transcription

Nuclear run-on assays were used to determine if the repression of D19 by DIF is mediated at the level of gene transcription (Fig. 3). Transcriptionally active nuclei were isolated from HM44 cells during a stalk cell induction. Again the cells were incubated in

Fig. 1. The kinetics of repression of the D19 mRNA by DIF. The symbol 0 indicates the time at which DIF was added to HM44 cells, after 10 h preincubation with cAMP alone. The time of incubation in the presence or absence of DIF is shown in hours. Total nucleic acid was purified and analysed by Northern transfer using the D19 cDNA clone as a probe, as described in the methods section.
Transcriptional repression of gene by DIF

Fig. 2. Comparison of repression of the D19 with the repression of two further prespore genes. HM44 cells were incubated for the times shown in the presence or absence of DIF, after 10 h incubation in medium containing cAMP alone. Total nucleic acid was extracted and analysed by Northern transfer as before. A mixture of the D7 and PL3 cDNA clones was used to probe the lower filter. The Northern transfer probed with the D19 cDNA clone derives from a separate experiment, but in both cases greater than 90% of amoebae formed stalk cells after 12 h incubation in the presence of DIF.

Fig. 3. Determination of the rate of D19 gene transcription during stalk cell induction. Transcriptionally active nuclei were prepared from HM44 cells incubated in the presence or absence of DIF for the times shown, following 10 h preincubation with cAMP. 32P-labelled RNA from the nuclear run-on assays was used to probe Southern blots of the 1G7 and D19 cDNA clones. The restriction enzyme digestion separates the insert from the plasmid vector band, which then acts as a control for background hybridization. 1G7, a cDNA cloned into the Smal site of pUC8, was digested with EcoRI and HindIII, giving a 500 bp insert fragment. D19, a cDNA cloned into the BamHI site of pBR322, was digested with HindIII and SalI, giving a 1.3 kb insert fragment. In both cases, 2 μg of DNA was used per track.

Within 90 min of DIF addition, the signal obtained for D19 decreases approximately tenfold. After 4 h incubation with DIF there is a further decrease in D19 transcription such that only a faint signal is obtained after a very long exposure of the autoradiogram. In contrast, where DIF was not included in the medium, the signal intensity remains constant over the 4 h period. Thus DIF almost completely abolishes transcription of the D19 gene. Comparing the transcription rate with the rate of decline in cytoplasmic mRNA levels in the same experiment (Fig. 2), we conclude that a major part of the repression of D19 observed upon DIF addition can be explained by a reduction in nuclear transcription.

(C) The stability of D19 mRNA is unaffected by DIF addition

The average half-life of individual mRNAs in Dictyostelium developing aggregates of AX-3 cells has been estimated to be between 3 and 4 h (Mangiariotti et al. 1982). If the D19 mRNA turned over in HM44 cells with this half-life, a 50% reduction in mRNA levels 90 min after DIF addition would not be possible, even if transcription ceased immediately. Furthermore, the D19, PL3 and D7 genes are known to be regulated at both a transcriptional and a post-transcriptional level by cAMP (Landfear et al. 1982; Barklis & Lodish, 1983). Hence, experiments were performed to determine whether or not the repres-
sion by DIF also has a post-transcriptional component.

These experiments utilized the RNA synthesis inhibitor nogalamycin, which has been successfully used to determine mRNA half-lives in germinating Dictyostelium spores (Ennis, 1981; Kelly et al. 1985). A stalk cell induction was again performed using HM44 cells. After a 10 h incubation with cAMP alone the medium was changed and supplemented with nogalamycin at 300 μg ml⁻¹. 5 min later, DIF was added to half of the plates and cells were harvested at various times after nogalamycin addition, up to a total of 4 h. Cells were also harvested just prior to drug addition and a terminal timepoint from cells incubated with DIF, but without nogalamycin was included to check the induction. A Northern blot of total RNA extracted from the cells was probed with a mixture of the pdD63 and the D19 cDNA clones (Fig. 4). The low level of pDd63 mRNA synthesized in the presence of nogalamycin, compared to that induced in its absence, demonstrates the effectiveness of the drug. At 4 h, with DIF alone, D19 mRNA levels are much reduced compared to zero times. In the presence of the drug, the rate of decay of the D19 mRNA is not altered by the addition of DIF. The half-life of the mRNA is about 90 min in both the presence and absence of DIF. This value, taken in conjunction with the result of the nuclear run-on assays, is consistent with the effect of DIF being mediated purely at the transcriptional level.

In an experiment where DIF was added 30 min prior to nogalamycin, the subsequent rate of decay of the mRNA was not significantly different from that observed with the protocol described above (data not shown). Hence, it is unlikely that nogalamycin is interfering with DIF action by preventing the synthesis of a DIF-inducible component necessary for destabilization.

**Discussion**

Previous observations have shown that DIF acts to repress prespore cell differentiation (Kay & Jermyn, 1983; Kopachik et al. 1985). We have now shown that DIF represses the expression of the D19, PL3 and D7 prespore genes in HM44 cells. The mRNA species that hybridize to these cDNAs have all been assigned to the prespore II class on the basis of their time of appearance and sensitivity to extracellular cAMP (Chisolm et al. 1984). All three mRNA sequences disappear from HM44 cells on exposure to DIF with similar kinetics. It seems likely, therefore, that they are all repressed by DIF through a similar mechanism.

The rate of transcription of the D19 gene, determined from nuclear run-on assays, is reduced in the presence of DIF while the half-life of the mRNA is unaffected. Given the relatively short, 90 min, half-life of the mRNA, the reduction in transcription rate is sufficient for transcriptional regulation primarily to account for the observed rate of mRNA disappearance. The induction by DIF of two prestalk-enriched mRNA sequences, pDd63 and pDd56, has also been shown to be mediated at a transcriptional level (Williams et al. 1987; Ceccarelli et al. 1987). The response to DIF of the pDd63 gene is very rapid as new transcripts are detected within 15 min of the addition of DIF. We cannot accurately judge the speed with which DIF affects D19 transcription because it is intrinsically difficult to determine the precise onset of repression when an mRNA has any significant degree of stability. We have also used the MUD-1 antibody (Krefft et al. 1983) to examine the effect of DIF on the level of D19 protein in HM44 cells (data not shown). If DIF is added after 10 h incubation with cAMP alone, there is no further net accumulation of protein over the next 14 h, but the pre-existing low level of protein does not decline. This implies that DIF does not cause a rapid turnover of prespore protein, and hence its regulation of gene transcription is of primary importance in mediating prespore repression.

DIF has been proposed to act by interfering with the cAMP-mediated induction of prespore gene expression (Wang et al. 1986). In aggregation-competent NC4 cells, incubated in monolayer culture,
DIF and cAMP act antagonistically. DIF represses cAMP-receptor interactions at low cAMP concentrations and reduces the cAMP relay response (Wang et al. 1986). However, our experiments have been performed using a saturating, 5 mM, concentration of cAMP, where DIF does not prevent cAMP binding to the receptor. Possibly, therefore, DIF is counteracting cAMP induction of D19 expression at some intracellular stage of signal transduction. Such a mechanism would provide greater scope for specificity of repression and, consistent with this, not all genes induced by cAMP are repressed by DIF in HM44 cells (Jermyn et al. 1987). It would also explain the absence of mRNA destabilization, since this is apparently dependent upon the disruption of cAMP signalling (Mangiarotti et al. 1982, 1983). Adenosine is thought to block cAMP-induced prespore differentiation directly, by inhibition of cAMP binding (Wang et al. 1986). Unlike DIF, therefore, adenosine might be expected to exert both a transcriptional and post-transcriptional effect on prespore gene expression.

When sections of developing aggregates are stained with MUD-1, a region of nonstaining cells is observed at the top of the mound (Krefft et al. 1984). Possibly, therefore, factors such as DIF and/or adenosine may be repressing prespore differentiation in this region. Adenosine seems a likely candidate for such a role since the enzymes that hydrolyse cAMP to generate it are enriched in the prestalk region (Arman et al. 1980; Brown & Rutherford, 1980). Also, enzymic reduction of adenosine levels in the slug leads to prespore differentiation in the prestalk region (Schaap & Wang, 1986). However, exposure to high adenosine levels does not lead to an increase in the total proportion of prestalk tissue in the slug, while the analogou experiment using DIF results in a major reduction in the proportion of presumptive stomatocytes (D. Traynor and R. Kay, personal communication). This does not necessarily indicate a more important role for DIF but may simply reflect differences in the levels and spatial distribution of the exogenously added DIF and adenosine. As DIF and adenosine act at different stages in the cAMP signal transduction pathway, one attractive idea is that they might function synergistically to divert cells from the prespore pathway of differentiation. Further analysis of the precise mechanism of repression should help to elucidate their relative contribution to this process.

References


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