Hypersensitive sites in the 5' and 3' flanking regions of the cysteine proteinase I gene of *Dictyostelium discoideum*

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ABSTRACT

The cysteine proteinase I gene of *Dictyostelium discoideum* is a developmentally regulated single copy gene. Specific sites in the 5' and the 3' flanking regions of the gene were cleaved by an endogenous nuclease when the gene was being transcribed. The majority of these sites were not cut when the gene was inactive. A dramatic change in the pattern of micrococcal nuclease and DNase I hypersensitive sites occurred in the 5' flanking region when transcription commenced at the 8 h stage of development. The major sites, doublets at -220/-300 bp and -670/-770 bp upstream of the transcription start site, corresponded to those cut by the endogenous nuclease. When transcription subsequently ceased the hypersensitive sites did not significantly change, indicating the gene remained in an activated state. The micrococcal nuclease hypersensitive sites in the 3' flanking region did not change significantly during development.

INTRODUCTION

Discrete DNA segments in the 5' flanking region of several genes show a very high sensitivity to attack by a variety of nucleolytic probes (1). These hypersensitive sites have been observed adjacent to genes encoding heat-shock proteins (2, 3), β -globin (4-6), histones (7), a *Drosophila* glue protein (8) and larval cuticle gene cluster (9), and on SV40 minichromosomes (10). Furthermore, these sites have been associated with many different sequences which are involved in gene expression and frequently their appearance has been correlated with developmental and tissue-specific gene activation (4-6, 8, 11-16). DNA sequences related to nuclease hypersensitive sites include promoter regions within approximately 200 bp upstream of the transcription start site (2, 3, 11, 12). The whole promoter region of the thymidine kinase gene, for example, becomes hypersensitive to DNase I and several restriction enzymes when

the gene is expressed (13, 17). Sequences further upstream from the transcription start site are also found to be nuclease hypersensitive. The developmentally regulated expression of the Sgs4 gene of Drosophila is controlled by a sequence located between -330 and -550 bp upstream of the transcription start site, which becomes accessible to DNase I when the gene is activated (8). These nuclease hypersensitive sites may represent nucleosome-free gaps up to 200 bp in length which facilitate interaction with the transcription machinery (6, 18, 19, 20). Hypersensitive sites associated with transcription are often found considerable distances upstream of the cap site (15, 16, 21, 22). Enhancer elements also exhibit DNase I hypersensitivity at or near them. The 72 bp repeat enhancer of the SV40 minichromosome is hypersensitive to DNase I (10, 14). Even translocation of the enhancer 3.5 kb upstream preserved its function and DNase I hypersensitivity (23). The enhancer elements of the genes coding for immunoglobulin κ light chain and μ heavy chain are located downstream of the transcription start site (24-26) and are related to a region of DNase I hypersensitivity (21, 27-31).

In the studies reported here we have analyzed the chromatin structure of a single copy gene in D.discoideum. The gene is developmentally regulated, transcription commencing at the 6th hour of development and ceasing after 12 h of differentiation (Pavlovic et al. submitted). Messenger RNA accumulation reaches a maximum between 8 and 12 h when it constitutes about 1% of the mRNA population (32,33). The messenger RNA can be accumulated prematurely when exogenous cAMP is present (34). It is approximately 1200 nucleotides in length and encodes a 36,000 dalton polypeptide (cysteine proteinase I) with a high degree of homology to plant and animal cysteine proteinases (33). Using the indirect end-labeling technique (2, 35) we have mapped the micrococcal nuclease, DNase I and endogenous hypersensitive sites at the 5' and 3' flanking regions of the gene. We find two doublets of hypersensitive sites in the 5' region, located -220/-300 bp and -670/770 bp upstream of the transcription start site, which are cut by all three nucleases when the gene is being transcribed. The two hypersensitive sites at -220 and -300

coincide with the gene proximal region of two unusually GC-rich sequences (J.G. Williams, personal communication) which implies these sequences may be involved in gene activation.

MATERIALS AND METHODS

Cell culturing, nuclei preparation, DNA extraction and digestion, gel electrophoresis, and nick translation were as previously (36) described.

Micrococcal Nuclease Digestion

Micrococcal nuclease digestion was performed by resuspending washed nuclei (1.5×10^9 /ml) from vegetative or differentiating cells in 3 ml of 20 mM Tris-HCl, pH 7.8, 0.5 mM CaCl_2 and incubating at 25°C with 12.5 units/ml enzyme (37). Incubation times varied from 15 sec to 5 min. The reaction was stopped by adding EDTA to 20 mM and sodium dodecyl sulfate to 0.5%. The samples were treated with 250 ug/ml proteinase K for 2 h at 37°C.

Genomic DNA was cut with micrococcal nuclease at 0°C. 20 ug of DNA in 200 ul of Tris-HCl, pH 7.8, 0.5 mM CaCl_2 was digested with 2 units of enzyme for 15 sec and 60 sec.

DNase I Digestion

For the DNase I digestion, washed nuclei were resuspended (1.5×10^9 /ml) in 15 mM Tris-HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 3 mM MgCl_2 and 0.25 M sucrose. The nuclei were digested with the following concentrations of DNase I for 1 to 5 min at 25°C: 75 units/ml (vegetative), 20 units/ml (8 h) and 120 units/ml (15 h). The reaction was stopped by adding EDTA to 20 mM and sodium dodecyl sulfate to 0.5%. The samples were treated with 250 ug/ml proteinase K for 2 h at 37°C.

Endogenous Nuclease Digestion

Endogenous nuclease digestion was carried out by resuspending nuclei (1.5×10^9 /ml) in 10 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl_2 , 1 mM CaCl_2 and incubating for 20 min at 25°C or 0°C. The reaction was stopped with 20 mM EDTA and 0.5% sodium dodecyl sulfate followed by treatment with proteinase K.

Isolation of Plasmid Inserts

50 - 100 ug plasmid DNA were digested with the appropriate restriction enzymes followed by fragment separation on 1.5%-2.0% agarose gels and staining with 0.5% ethidium bromide. A strip of

DEAE cellulose membrane (Schleicher and Schuell NA 45) was placed in an incision just ahead of the band to be recovered.

Electrophoresis was continued until binding was complete. To remove residual agarose the membrane was washed in low salt buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 150 mM NaCl). The bound DNA was eluted by incubating the strip in high salt buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1.0 M NaCl) for 30 min at 65°C. After washing the membrane with a small volume of the same buffer the eluted DNA was precipitated with 2.5 volumes of ethanol. Reprecipitation was from 0.25 M sodium acetate to remove residual NaCl.

Transfer of DNA onto Nitrocellulose Filters

DNA was hydrolyzed by incubating the gels for 10 min in 0.25 M HCl. Denaturing of the DNA was performed in 0.5 M NaOH, 1.5 M NaCl for 60 min followed by neutralizing the gel for 1 h in 1 M Tris-HCl pH 7.5, 1.5 M NaCl. The DNA was blotted onto a nitrocellulose filter using 20 x SSC as transfer buffer. The steps were carried out at room temperature. After rinsing in 10 x SSC the filters were dried and baked for 2 h at 80°C in a vacuum oven.

Hybridization

The nitrocellulose filters were prehybridized in 20 ml 6 x SSC, 5 x Denhardt's, 15 mM Na/PO₄-buffer pH 7.0, 1% N-lauroylsarcosine, 0.1% sodium dodecyl sulfate, 10% dextran sulfate and 250 ug/ml denatured calf thymus DNA at 68°C overnight. Hybridization was carried out for 16 h at 68°C in 10 ml of the same solution containing 1 ng/ml ³²P labeled excised plasmid insert (10⁹ cpm/ug DNA). Following hybridization, the nitrocellulose filters were washed two times in 0.5 x SSC, 0.1% sodium dodecyl sulfate for 5 min at room temperature and three times for 30 min at 68°C. The filters were exposed with 1 or 2 tungsten intensifier screens (Ilford) and X-ray film (Fuji).

RESULTS

Fig. 1a shows a restriction map of pDd11.7.10, the cDNA clone of the cysteine proteinase I gene (33, 34). The probes used to study the chromatin structure of the gene are indicated. Fig. 1b depicts the genomic map of the gene (38). Southern blot

a**pDd 11710** **b****Cysteine proteinase I**

Fig. 1a. pD11.7.10, cDNA clone of the cysteine proteinase I gene.

Restriction map and cDNA fragments isolated and used as hybridization probes. The symbols of the restriction enzymes used are: A, *Ava*II; B, *Bgl*II; E, *Eco*RI; H, *Hind*III; He, *Hae*III; S, *Sca*I.

(b) Genomic map of the cysteine proteinase I gene.

The symbols of the restriction sites are: A, *Ava*II; Al, *Alu*I; B, *Bgl*II; D, *Dde*I; E, *Eco*RI; H, *Hind*III; He, *Hae*III; Hf, *Hinf*I; S, *Sca*I.

analysis confirmed it is a single copy gene (32, 38), and determination of the entire nucleotide sequence of the cysteine proteinase I gene revealed that it contains five introns (39).

After mild digestion of nuclei with micrococcal nuclease DNase I or autodigestion by endogenous nuclease, the purified DNA

was cleaved with an appropriate restriction enzyme, size fractionated on agarose gels and transferred to nitrocellulose filters. Hybridization was performed with a short ^{32}P -labeled DNA fragment specific for one end of the chosen restriction cut within the genome. The fragments which extended from the chosen restriction site carrying the sequence complementary to the hybridization probe were visualized, the largest being the uncleaved restriction fragment. The size of the smaller fragments gave the position of the nuclease cleavage sites relative to the restriction site.

For the indirect end-labeling experiments, we have used the 517 bp ScaI - BglIII cDNA fragment of pDd11.7.10 (Fig. 1a). Southern blot analysis showed no cross-hybridization with any other DNA sequence under the hybridization conditions used. All the indirect end-labeling experiments mapping in the 5' direction were repeated with a 209 bp HindIII - ScaI cDNA fragment of pDd11.7.10 (Fig. 1a), which was subcloned in pUC12 (38). Identical results were obtained but the hybridization signal was considerably weaker (Fig. 6b).

To map the position of the hypersensitive sites in the 5' flanking region, the DNA fragments were cleaved with AvaII. The AvaII site in the coding region lies 5 bp downstream from the ScaI site (Fig. 1b). To map in the 3' direction the DNA was cleaved with BglIII (Fig. 1b). AvaII and BglIII, which cut once in the coding region of the cysteine proteinase I gene (Fig. 1b), both generate restriction fragments of at least 12 kb in the 5' and 3' flanking regions of the gene.

Endogenous Nuclease Cleavage

We had previously observed endogenous nuclease activity in isolated nuclei. We wanted to examine whether the accessibility of the flanking regions of the cysteine proteinase I gene to the endogenous nuclease changed during development, i.e. was related to gene regulation. Further, we wished to map the positions of the cleavage sites in the 5' and 3' flanking regions of the cysteine proteinase I gene using the indirect end-labeling technique described above.

Fig. 2 shows the endogenous nuclease sites in the 5' flanking region of the cysteine proteinase I gene at three

Fig. 2. Endogenous nuclease sites in the 5' flanking region of the cysteine proteinase I gene. Isolated nuclei from vegetative, 8 h and 15 h differentiated cells were incubated for 20 min at 25°C (lanes 3, 5 and 7) and at 0°C (lanes 2, 4 and 6). Lane 1: Genomic *D. discoideum* DNA digested with *Ava*II. The purified DNA fragments were cut with *Ava*II, separated on a 1.5% agarose gel, transferred to a nitrocellulose filter and hybridized with the 517 bp *Bgl*III - *Sca*I cDNA fragment (see Fig. 1a).

developmental stages. Nuclei from vegetative, 8 h, and 15 h stage cells were incubated on ice (Figs. 2 and 3, lanes 2, 4 and 6) or at 25°C for 20 min (Figs. 2 and 3, lanes 3, 5 and 7). The DNA was extracted and cut with *Ava*II or *Bgl*III for the 5' and 3' mapping respectively. The fragments were separated on 1.5% agarose gels, transferred to nitrocellulose and hybridized with the *Bgl*III - *Sca*I cDNA fragment of pDd11.7.10.

In vegetative (Fig. 2, lanes 2 and 3) and 15 h stage cells (lanes 6 and 7), when the gene was inactive, only weak endogenous nuclease cleavage was observed in the 5' region. At 8 h, however, when transcription was occurring, strong cleavage sites were observed at 1350, 1430, 1800, 1900, 4300, 4700 and 6400 bp upstream from the *Ava*II site (Fig. 2, lanes 4 and 5). The cap site coincides, within the resolution of mapping, with the site

Fig. 3. Endogenous nuclease sites in the 3' flanking region of the cysteine proteinase I gene. Isolated nuclei from vegetative, 8 h and 15 h differentiated cells were incubated for 20 min at 25°C (lanes 3, 5 and 7) and at 0°C (lanes 2, 4 and 6). Lane 1: Genomic *D. discoideum* DNA digested with BglII. The purified DNA fragments were cut with BglII, separated on a 1.5% agarose gel, transferred to a nitrocellulose filter and hybridized with the 517 bp BglII - ScaI fragment.

specifically cut by micrococcal nuclease in chromatin and pure DNA which is 1130 bp upstream from the AvaII site (J.G. Williams, personal communication).

Endogenous nuclease cutting in the 3' downstream region was also barely detectable in vegetative (Fig. 3, lanes 2 and 3) and 15 h cells (lanes 6 and 7), with the exception of cleavages at 6400 and 8000 bp downstream from the BglII site (Fig. 3, lanes 4 and 5). Cutting at the 8000 bp site was greatly increased. The end of the transcribed region is at least 1250 bp downstream from the BglII site but has not been mapped.

The maps of the endogenous nuclease cleavage sites in the 5' and 3' flanking region of the cysteine proteinase I gene are shown in Fig. 4 and Fig. 5 respectively.

Fig. 4. Map of the endogenous nuclease cleavage sites in the 5' flanking region of the cysteine proteinase I gene at the three stages of development indicated (See Fig. 2).

Micrococcal Nuclease Digestion

The micrococcal nuclease hypersensitive sites in the 5' upstream region of the gene are shown in Fig. 6. Micrococcal nuclease displays a strong DNA sequence specificity (40, 41). The micrococcal nuclease cleavage sites in naked DNA, 540, 700, 1130, 1400, 1850, and 2200 bp upstream of the *Ava*II site, were also observed in the nuclear digests of vegetative cells (Fig. 6b). However only the three sites 540, 950, and 1130 bp upstream of the *Ava*II site were present in all three developmental stages and in naked DNA (Fig. 6b). A dramatic change in the pattern of hypersensitive sites was observed between vegetative (Fig. 6a, lanes 1-4) and 8 h cells (Fig. 6a, lanes 5-8). Many of the sites in vegetative cells were absent from 8 h cells and the majority of the new sites corresponded to

Fig. 5. Map of the endogenous nuclease cleavage sites in the 3' flanking region of the cysteine proteinase I gene at the three stages of development indicated (See Fig. 3).

Fig. 6. Micrococcal nuclease hypersensitive sites in the 5' flanking region of the cysteine proteinase I gene.

(a) Isolated nuclei from vegetative cells, (lanes 1-4) 8 h (lanes 5-8) and 15 h differentiated cells (lanes 9-12) were digested with 12.5 units/ml micrococcal nuclease at 25°C. Incubation times were 30 sec (lanes 2, 6 and 10), 60 sec (lanes 3, 7 and 11) and 120 sec (lanes 4, 8 and 12). Control samples were incubated without enzyme for 300 sec at 25°C (lanes 1, 5 and 9).

(b) Genomic DNA was digested with 10 units/ml micrococcal nuclease at 0°C for 15 sec and 60 sec (lanes 2 and 3). DNA prepared from micrococcal nuclease digested nuclei (120 sec) isolated from vegetative and 15 h differentiated cells (lanes 1 and 4) were run alongside the naked DNA controls.

The purified DNA fragments were cut with *Ava*II, separated on a 1.5% agarose gel and transferred to a nitrocellulose filter. Hybridization was performed with the 517 bp *Bgl*III - *Sca*I cDNA fragment of pDd11.7.10 (see Fig. 1a.).

(c) Aliquots of the samples prepared from micrococcal nuclease treated nuclei, isolated from 8 h differentiated cells described in panel a lanes 5-8, were fractionated on a similar gel and hybridized with the 209 bp *Sca*I - *Hind*III fragment of pDd11.7.10 (lanes 1-4).

regions cut by endogenous nuclease. Most prominent were the pair of doublets 1350/1430 bp and 1800/1900 bp upstream from the *Ava*II site (Fig. 6a, lanes 5-8). These doublets did not arise from cutting within the sequences complementary to the 517 bp probe

Fig. 7. Micrococcal nuclease hypersensitive sites in the 3' flanking region of the cysteine proteinase I gene.

(a) Isolated nuclei from vegetative cells (lanes 1-4), 8 h (lanes 5-8) and 15 h differentiated cells (lanes 9-12) were digested with micrococcal nuclease (see Fig. 6).

(b) Genomic DNA was digested with 10 units/ml micrococcal nuclease at 0°C for 15 sec and 60 sec (lanes 2 and 3). DNA prepared from micrococcal nuclease digested nuclei (120 sec) isolated from vegetative and 15 h differentiated cells (lanes 1 and 4) were run alongside the naked DNA controls. The purified DNA fragments were cut with BglII, separated on a 1.5% agarose gel and transferred to a nitrocellulose filter. Hybridization was performed with the 517 bp BglII - ScaI cDNA fragment.

since they were also found when the 209 bp probe was used (Fig. 6c). Moreover, sequencing data revealed that the hypersensitive sites 1350 bp and 1430 bp upstream of the *AvaII* site coincide with two identical unusually GC-rich sequence elements (J.G. Williams, personal communication). A strong band appeared at 4700 bp. The 4300 bp site cleaved by endogenous nuclease in 8 h stage cells (Fig. 2, lane 5) was recognized by micrococcal nuclease at all stages of development (Fig. 6a, lanes 1-12). Hence, it is presumably always accessible. A change also occurred in the coding region, an 820 bp band appearing in 8 h

cells (Fig. 6a, lanes 6-8). The appearance of this band may reflect the loss of or a structural change in nucleosomes from the coding region (Pavlovic et al., submitted). The nuclei from 15 h cells (Fig. 6a, lanes 10-12) gave a pattern almost identical to 8 h cells (Fig. 6a, lanes 6-8) except that the 1800/1900 bp double band had been replaced by an 1850 bp band. Cutting within the coding region resembled vegetative cells, with the 700 bp and 760 bp bands reappearing (Fig. 6a, lanes 2-4 and 10-12). The strength of the 1350/1430 bp doublet was reduced in 15 h cells.

The changes in hypersensitive sites in the 3' flanking region of the gene resulting from transcription were only minimal (Fig. 7). Several endogenous nuclease cleavage sites, which appeared in 8 h cells (Fig. 7a, lane 5) but not in vegetative and 15 h cells, were accessible to micrococcal nuclease in the two latter stages (Fig. 7a, lanes 1-4 and 9-12). Thus, in vegetative cells (Fig. 7a, lanes 2-4) the sites at 1050 bp, 1700 bp and 2200 bp downstream from the BglII site were recognized, whereas in the 15 h developmental stage (Fig. 7a, lanes 10-12) these sites were also cleaved along with sites at 2000 bp, 2400 bp and 3100 bp. In 8 h cells (Fig. 7a, lanes 5-8) 4200 bp and 1600 bp bands typical of endogenous nuclease cleavage disappeared following longer micrococcal nuclease digestion times. They may be rapidly trimmed by the latter enzyme. The sites in naked DNA preferentially cut by micrococcal nuclease did not coincide with sites in chromatin, the exception being two sites 800 bp and 3600 bp from the BglII cleavage site (Fig. 7b).

DNase I Digestion

We have also examined the DNase I hypersensitive sites in the 5' flanking region of the cysteine proteinase I gene during development. The results are shown in Fig. 8. The hypersensitive sites at position 1350/1430 and 1800/1900 bp recognized by the endogenous and micrococcal nuclease also became sensitive to DNase I when the gene was transcribed at the 8 h stage (Fig. 8, lanes 7 and 8). An additional DNase I hypersensitive site was observed 1650 bp upstream of the AvaII site. When the gene was inactive (vegetative cells), no DNase I hypersensitive sites were detected within 4000 bp upstream from the AvaII site (Fig. 8, lanes 4 and 5). Moreover, after

Fig. 8. DNase I hypersensitive sites in the 5' flanking region of the cysteine proteinase I gene. Isolated nuclei were treated with DNase I: 75 units/ml (vegetative), 20 units/ml (8 h) and 120 units/ml (15 h) at 25°C. Incubation times were 60 sec (lanes 4 and 10), 120 sec (lanes 5 and 11), 240 sec (lane 7), 300 sec (lane 8) with and 300 sec without DNase I (lanes 3, 6 and 9). Genomic DNA was digested with 2 units/ml at 0°C for 120 sec (lane 2). Lane 1: Genomic *D. discoideum* DNA digested with *AvaII*. The purified DNA fragments were cut with *AvaII*, separated on a 1.5% agarose gel, transferred to a nitrocellulose filter and hybridized with the 517 bp *BglIII* - *ScaI* cDNA fragment (see Fig. 1a).

extensive digestion, the sequences at 1350/1430 and 1800/1900 bp appear to be less sensitive to DNase I than their boundaries (lane 5). At the 15 h developmental stage when transcription

Fig. 9. Map of the micrococcal nuclease and DNase I hypersensitive sites in the 5' flanking region of the cysteine proteinase I gene in vegetative cells, 8 h and 15 h differentiated cells (see Figs. 6 and 8). Arrows indicate micrococcal nuclease hypersensitive sites, triangles indicate DNase I hypersensitive sites.

ceases, the DNase I hypersensitive sites persisted with the exception of the 1650 bp band (Fig. 8, lanes 10 and 11). Two additional bands appeared at positions 1180 and 1300 bp respectively (Fig. 8, lanes 10 and 11).

Maps of the hypersensitive sites in the 5' and 3' flanking regions of the cysteine proteinase I gene are shown in Fig. 9 and Fig. 10 respectively.

DISCUSSION

The indirect end-labeling experiments showed that specific sites upstream and downstream from the cysteine proteinase I

Fig. 10. Map of the micrococcal nuclease hypersensitive sites in the 3' flanking region of the cysteine proteinase I gene in vegetative cells, 8 h and 15 h differentiated cells (see Fig. 7).

coding region were cleaved by an endogenous nuclease in 8 h cells, i.e. when the gene was being transcribed (Figs. 4, 5). This is unlikely to be a nonspecific effect involving "activation" of the endogenous nuclease at the 8 h stage, since an endogenous nuclease also cleaves the flanking regions of the ribosomal genes in vegetative cells (42). Some sites flanking the cysteine proteinase I gene and cut by endogenous nuclease in 8 h cells were still accessible to micrococcal nuclease and DNase I at developmental stages where transcription of the gene was not occurring. This suggests the sites were present but for some reason not cut by the endogenous nuclease. Perhaps the enzyme becomes associated with the gene and/or recognizes changes in the DNA structure occurring during transcription. However, we have no clear evidence that the nuclease is associated with the gene prior to nuclear isolation or that transcription is occurring during incubation of the nuclei.

In the 5' flanking region of the cysteine proteinase I gene a dramatic change occurred in the arrangement of micrococcal nuclease hypersensitive sites when the gene was turned on (Fig. 9). The major change was the appearance of two strongly cleaved doublets (approx. 220/300 and 670/770 bp upstream from the cap site). These sites were also cleaved by the endogenous nuclease. When the gene was repressed during later development the micrococcal nuclease hypersensitive sites found in vegetative cells did not reappear. The doublet closest to the cap site in 8 h cells was still present although weaker. The second doublet had been replaced by a single band. Almost identical results were obtained when nuclei were treated with DNase I (Fig. 9). The same sites in the 5' flanking region became hypersensitive to DNase I when the gene was transcribed. The only difference observed was an additional DNase I hypersensitive site 520 bp upstream from the cap site. No sequences were susceptible to DNase I within 3000 bp upstream from the transcription start in vegetative cells. At the 15 h developmental stage, when transcription of the gene ceased, the 520 bp band became considerably weaker, and two additional bands appeared 50 and 170 bp 5' of the cap site. The DNase I sensitivity of the two strongly cleaved doublets at -220/300 bp and -670/770 bp

persisted. Hence, although no longer transcribed (due, for example, to the absence of transcription factors), the gene may remain in a stable, activated state. This might explain the observation that when developing D. discoideum cultures are disaggregated the cells are capable of recapitulating the same sequence of previous morphological and biochemical changes in a fraction of the original time (43-45). A return to the repressed state may require DNA synthesis. We have incubated 8 h cells in nutrient medium and found that after one round of cell division the pattern of hypersensitive sites typical for vegetative cells reappeared.

The doublet of hypersensitive sites closest to the cap site appearing in 8 h cells coincide, within the resolution of mapping, with the gene proximal margin of two unusually GC-rich repeats (J. G. Williams, personal communication). Hence, factors activated or synthesized in the presence of elevated cAMP levels may recognize these sequences, changing the chromatin structure to the activated state. The second doublet of hypersensitive sites may be involved in the regulation of a gene transcribed in the opposite direction.

No significant change was seen in the pattern of micrococcal nuclease hypersensitive sites in the 3' flanking region of the gene during development (Fig. 10). The sites cleaved by endogenous nuclease at 8 h were cut by micrococcal nuclease at all three developmental stages.

The micrococcal nuclease hypersensitive sites were irregularly spaced in the 5' and 3' flanking regions immediately surrounding the coding region. Many of these sites were less than 160 bp apart, the minimum length required for a single nucleosome (46). We have obtained similar results with Dictyostelium ribosomal genes (in preparation). This suggests normal nucleosomes may not be associated with these regions, even when the gene is inactive. Should these regions become packed into normal nucleosomes, the gene may possess a supranucleosomal structure (47) and activation/transcription be impossible. If this is so, some machinery could exist to prevent normal nucleosomes associating with the flanking regions during DNA replication. In higher eukaryotes, protein factors binding to enhancer sequences might be responsible.

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