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The effects of transcription on the nucleosome structure of four *Dictyostelium* genes

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ABSTRACT

Micrococcal nuclease digestion of Dictyostelium discoideum nuclei from various developmental stages was used to investigate transcription-related changes in the chromatin structure of the coding region of four genes. Gene activity was determined by Northern blotting and nuclear run on experiments. During strong transcription of the developmentally regulated cysteine proteinase I gene, a smear superimposed on a nucleosomal ladder was observed, indicating perturbation of nucleosomal structure was occurring. However, two other developmentally regulated genes, discoidin I and pSC253, showed only slight nucleosome disruption during high levels of transcription. The chromatin structure of a fourth gene (pCZ22) was disrupted throughout development, even at those stages where transcription was greatly reduced. We suggest that although nucleosome structure can be transiently perturbed by the passage of the transcription complex in vivo, the degree of perturbation and the speed with which nucleosomes reassemble is also influenced by the DNA sequence.

INTRODUCTION

An understanding of the control of gene expression in eukaryotes will depend in part on knowledge of the chromatin structure of active genes and their coding regions. However, there is still controversy regarding the presence or absence of nucleosomes in transcribing chromatin and, although correlations between certain features of chromatin structure and gene expression have been found, their causal relationship is unclear (1-5).

Evidence is accumulating that the nucleosomes associated with the coding region of transcribing ribosomal genes take on an altered structure or may dissociate from the DNA (6-10). The active genes are particularly sensitive to micrococcal nuclease. We found a continuous distribution of rDNA fragments was produced following digestion of Dictyostelium discoideum nucleoli with micrococcal nuclease (6,7) or methidiumpropyl-EDTA-iron(II) (11). The loss of periodicity may result from changes in nucleosome structure but also from an undefined linker length between the

nucleosomes. However, electron microscopy data showed strongly transcribed rDNA contains rows of transcription complexes so tightly packed that nucleosomes could not form between them (7,12,13). Although nucleosomes preclude extensive crosslinking of DNA with trimethylpsoralen, crosslinking in the coding region was extensive in highly active ribosomal genes without disruption of the transcription complexes (13). Moreover, treatment with psoralen in the presence of 2 M NaCl increased crosslinking in the nucleosome-containing inactive chromatin but not in the coding region (13). Finally, the coding region of actively transcribing genes was accessible to restriction in contrast to the inactive spacer (7).

Biochemical studies on the chromatin structure of the coding region of genes transcribed by RNA polymerase II have produced conflicting results (14-18; reviewed in ref. 19). Indeed, recent *in vitro* studies have been contradictory. Neither SP6 RNA polymerase nor rat liver polymerase II could initiate transcription on promotors located within a nucleosome (20,21). However, Lorch et al. (21) found these polymerases could displace histones from the DNA during read-through transcription while Losa and Brown (22) reported SP6 polymerase did not displace the histone octomer from the *Xenopus* 5S gene *in vitro*. Lorch *et al.* (23) showed the contradiction was not due to differences in the method of assembling nucleosomes in the two laboratories. They confirmed that, during transcription, nucleosomes persist on the 258 bp fragment containing a *Xenopus* 5S RNA gene placed downstream of the SP6 promotor. However, transcription by SP6 polymerase of undefined rat liver DNA sequences carrying nucleosome monomers and joined to the SP6 promotor resulted in the displacement of histones from the DNA. The authors suggest that the *Xenopus* 5S gene is exceptional and that SP6 polymerase displaces histones from most DNA sequences *in vitro*. Nevertheless, the question of what happens to nucleosomes during transcription *in vivo* remains unresolved.

In this paper we have used micrococcal nuclease digestion to detect transcription-related changes in the chromatin structure of the coding region of four *D. discoideum* genes. These genes are all transcribed by RNA polymerase II. We have also sought changes in the accessibility of the coding region of one of these genes (cysteine proteinase I) to restriction enzymes, since in ribosomal genes such changes could be correlated with gene activity (7). We recently reported the appearance of

hypersensitive sites in the 5' flanking regions of the cysteine proteinase I gene when transcribed (24).

MATERIALS AND METHODS

Cell culturing, nuclei preparation, DNA extraction, gel electrophoresis and nick translation were carried out as previously described (7).

Micrococcal Nuclease Digestion

Washed nuclei (1.5 x 10^9 per ml) (25) were resuspended in 3 ml 20 mM Tris-HCl, pH 7.8, 0.5 mM $CaCl_2$ and incubated at 25°C with either 80 units/ml (nuclei from vegetative cells) or 50 units/ml (differentiating cells) micrococcal nuclease (26). Incubation times varied from 15 s to 5 min. The reaction was stopped by adding EDTA to 20 mM and sodium dodecyl sulphate to 0.5%. The samples were treated with 250 µg/ml proteinase K for 2 h at 37°C and DNA extracted (7,24).

Restriction Enzyme Digestion

Nuclei (10^8 per ml) were resuspended in 0.5 ml 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT and incubated for 30 min at 37°C with 200 units of either EcoRI, HindIII or HinfI. The reaction was stopped by adding EDTA to 20 mM and sodium dodecyl sulphate to 0.5%. The lysate was treated with proteinase K and DNA extracted.

Nuclear Run on Reactions

Preparation of nuclei and nuclear run on reactions were essentially performed as described by Crowley et al. (27). Tight cell aggregates were dissaggregated in a Dounce glass homogenizer with 10 strokes before NP40 was added. Plasmids were linearized with the appropriate restriction endonucleases, heat denatured and spotted in 6 x SSC onto nitrocellulose filters using a Schleicher and Schuell dot blot apparatus. 2 µg plasmid DNA were loaded per spot. The nitrocellulose strips were baked for 2 h in vacuo at 80°C. Prehybridization was carried out in 6 x SSC, 5 x Denhardt's solution, 0.1% SDS, 1 mM EDTA, 50% formamide, 100 µg/ml denatured salm sperm DNA and 100 µg/ml yeast core RNA for 16 h at 42°C. Hybridization was performed with fresh hybridization buffer containing 1 x 10^6 cpm/ml labeled RNA, for 48 h at 42°C. The nitrocellulose filters were washed in 0.5% SSC, 0.1% SDS twice for 5 min at rt and subsequently twice for 1 h at 65°C.

Isolation of Total Cellular RNA

Cells were harvested from growing and differentiating cultures as previously described and washed in K/PO_4 buffer. The cells were lysed in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 300 mM NaCl, 1% sodium dodecyl sulphate and 250 µg/ml proteinase K (about 5 x 10^7 cells/ml) and incubated for 60 min at 37°C. Nucleic acids were extracted as described above; precipitated with 2.5 volumes of ethanol, washed with 80% ethanol and taken up in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 300 mM NaCl and 100 µg/ml DNase I (Boehringer, Grade 1). Following incubation for 60 min at room temperature, sodium dodecyl sulphate to 0.5% and 250 µg/ml proteinase K were added. After a further incubation for 60 min at room temperature, phenol extraction was performed as above. RNA was dissolved in water (gel electrophoresis) or binding buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% sodium dodecyl sulphate) for poly(A^+) RNA isolation.

Gel Electrophoresis of DNA and RNA

DNA fragments were separated as in (7). RNA was analyzed on horizontal gels containing 1.5% agarose and 6% (v/v) formaldehyde. The electrophoresis buffer was 20 mM sodium phosphate buffer (pH 7.6), 3% formaldehyde. RNA was solubilized in 50% deionized formamide, 6% formaldehyde, 20 mM sodium phosphate buffer (pH 7.6) and incubated for 15 min at 55°C. After rapid cooling, a 1/10 volume of 50% glycerine, 0.025% bromophenol blue was added. Electrophoresis was at 30 V for 16 h at room temperature; the electrophoresis buffer was continuously recycled (28,29).

Clones

pDd11.7.10: cDNA clone of the cysteine proteinase I gene in *D. discoideum* (30,31). pDd812: cDNA clone of the discoidin I gene (32-34). pCZ22: cDNA clone of a constitutively expressed, multiple copy gene (35,36). PSC253: genomic clone of a developmentally-regulated, single copy gene (35,36). The 4.2 kb NdeI-EcoRI fragment was subcloned into pUC12. The 3.2 kb AluI fragment of the transcribed region was used as a hybridization probe.

Transfer of DNA and RNA onto DBM Paper

DBM paper was prepared and DNA transferred as previously described (7). Agarose gels containing RNA were incubated in water for 5 min at 60°C and then rapidly cooled to room temperature in water. The RNA was hydrolyzed by incubating the gels for 20 min in 50 mM NaOH at room

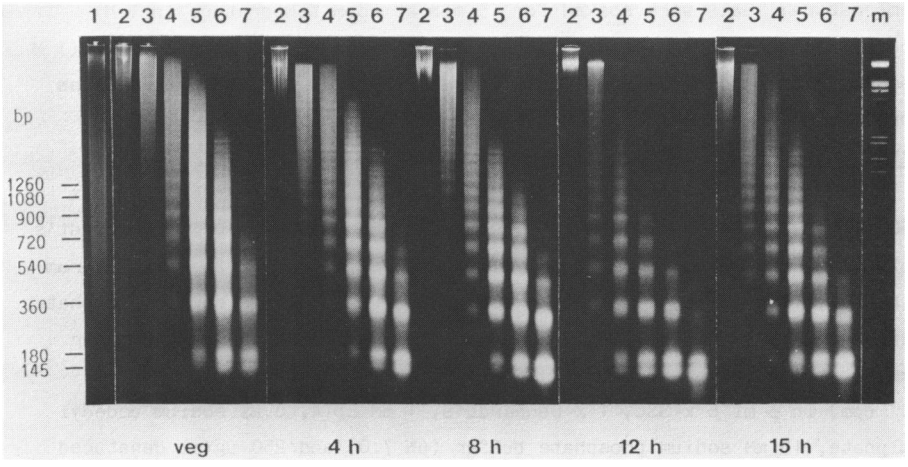

Fig. 1. Micrococcal nuclease digest of Dictyostelium nuclei. Isolated nuclei from the various developmental stages indicated were incubated at 25°C with 80 units/ml (vegetative cells) or 50 units/ml micrococcal nuclease (differentiating stages). Incubation times were 15, 30, 60, 120 and 300 s with (lanes 3 to 7) or 300 s without micrococcal nuclease (lane 2). Pure nuclear DNA was digested with 10 units/ml at 0°C (lane 1). The purified DNA fragments were separated on a 2.3% agarose gel. Marker (lane m): DNA double-digested with EcoRI and HindIII.

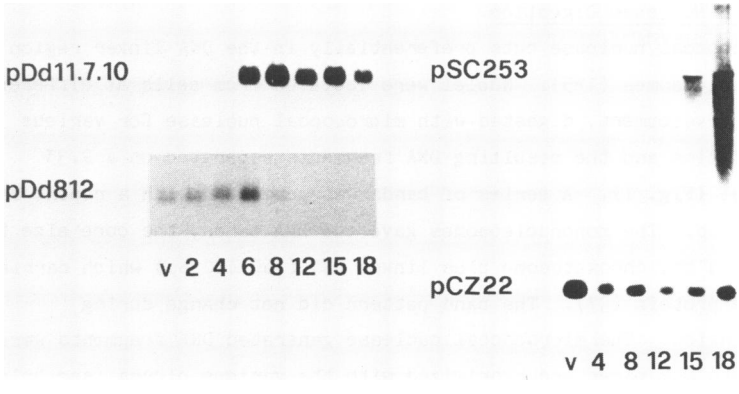

Fig. 2. Accumulation of the cysteine proteinase I (pDd11.7.10) discoidin I (pDd812), pSC253 and pCZ22 mRNA during development. DNA was extracted from vegetative cells (veg) and from cells at the indicated times after the onset of development (2 h, 4 h, 6 h, 8 h, 12 h, 15 h and 18 h). In each lane 5 µg total cell RNA were separated on 1.5% agarose gels containing 6% formaldehyde and blotted to DBM paper. Hybridizations were performed with the nick translated 800 bp BglII-HaeII cDNA fragment of pDd11.7.10, the 330 bp HindII-EcoRI cDNA fragment of pDd812, the 3.2 kb AluI fragment of pSC253 and pCZ22.

temperature. Gels were soaked for 15 min at room temperature in 1 M sodium acetate buffer (pH 5.0) and once in cold (4°C) blotting buffer (1 M sodium acetate, pH 5.0) for 15 min. Transfer of RNA to DBM paper was as for DNA (7).

Hybridization

DBM paper was prehybridized by incubating overnight at 68°C in 6 x SSC (1 x SSC contains 0.15 M NaCl, 0.015 M sodium citrate), 1 x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin), 1% glycine, 0.1% sodium dodecyl sulphate, 15 mM sodium phosphate buffer (pH 7.0) and 250 µg/ml denatured calf thymus DNA. The DBM paper was hybridized with about 50 ng of nick-translated DNA (approx. 4 x 10^7 cpm) in 5 ml 6 x SSC, 1 x Denhardt's, 1 mM EDTA, 0.2% sodium dodecyl sulphate, 15 mM sodium phosphate buffer (pH 7.0) and 250 µg/ml denatured calf thymus DNA for 20 h at 68°C. Following hybridization of DNA or RNA filters, they were washed six times with 0.5 x SSC, 0.1% sodium dodecyl sulphate, 1 mM EDTA for 30 min at 68°C. The filters were exposed with 1 or 2 tungsten intensifier screens (Ilford) and X-ray film (Fuji). To reuse the DBM paper it was stripped three times for 1 min in boiling water and prehybridized as described above.

RESULTS

Micrococcal Nuclease Digestion

Micrococcal nuclease cuts preferentially in the DNA linker region between nucleosomes (1-5). Nuclei were isolated from cells at different stages of development, digested with micrococcal nuclease for various lengths of time and the resulting DNA fragments separated on a 2.3% agarose gel (Fig. 1). A series of bands was generated with a repeat of around 180 bp. The mononucleosomes gave two DNA bands, the core size DNA (146 bp) and the chromatosome plus linker DNA (166-180 bp) which carries an H1-like protein (37). The band pattern did not change during differentiation. The micrococcal nuclease generated DNA fragments were blotted onto DBM paper and hybridized with the various clones (see below).

Clone pDd11.7.10. The plasmid contains a 1000 bp insert of cDNA complementary to mRNA of the cysteine proteinase I gene (30,31). The developmental kinetics of the expression of the cysteine proteinase I gene were determined using Northern blot hybridization (Fig. 2) and nuclear run on reactions (Fig. 3).

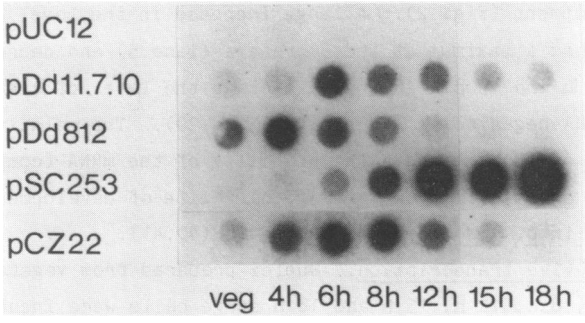

Fig. 3. Nuclear in vitro transcription during development. Plasmids were linearized with the appropriate restriction endonucleases and 2 µg per dot of the indicated clones were spotted onto nitrocellulose strips. The filter strip were hybridized with 1 x 10^6 cpm/ml of (^{32}P) UTP labeled run on transcripts of AX-2 nuclei isolated from the indicated stages.

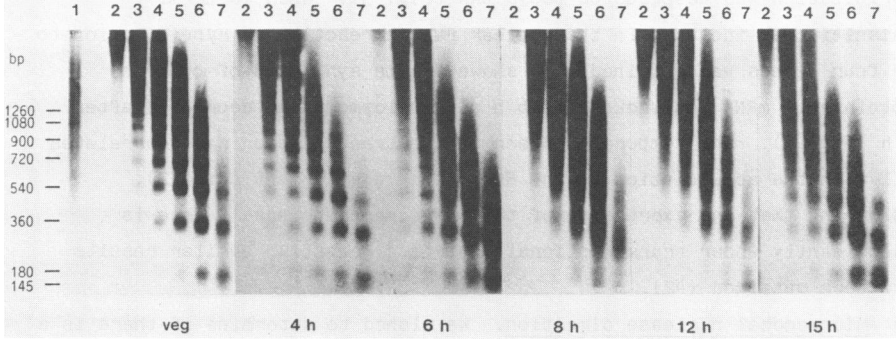

Fig. 4. Chromatin structure of the cysteine proteinase I gene during differentiation. Isolated nuclei from vegetative cells, 4 h, 6 h, 8 h, 12 h and 15 h stages of development and pure nuclear DNA were digested with micrococcal nuclease. Incubation times were as in Fig. 1; digested genomic DNA is in lane 1. Purified DNA was separated on 2.3% agarose gels, transferred to DBM paper and hybridized with the 800 bp BglII-HaeIII cDNA fragment of pDd11.7.10.

(a) Changes in cysteine proteinase I mRNA concentration during development. Equal amounts (5 µg) of total cellular RNA isolated from cells of the indicated developmental stages were size fractioned on 1.5% agarose gels, containing 6% formaldehyde. The RNA was transferred to DBM paper and hybridized with the 800 bp BglII - HaeIII cDNA fragment of pDd11.7.10 (24,31). The mRNA of the cysteine proteinase I gene was present at a very low concentration in vegetative cells, 2 h and 4 h

stages of development (Fig. 2). A large increase in the level occurred after 6 h, reached a maximum at the 8 h stage (lane 5) and decreased slowly after 12 h and 15 h. At the 15 h stage the level of cysteine proteinase I mRNA began to fall rapidly (31,38,39). The relatively high level remaining at 15 h reflects the stability of the mRNA (compare with Fig. 3). After aggregation, the average half-life of developmentally regulated mRNAs in D. discoideum is about 4 h (40,41).

(b) Nuclear in vivo transcription. Nuclei prepared from vegetative cells, 4 h, 6 h, 8 h, 12 h, 15 h and 18 h stage cells were incubated in the presence of ATP, CTP, GTP and (α-^{32}P) UTP for 30 min at 22°C using 10^7 nuclei per reaction, typically between 5 x 10^7 cpm (vegetative nuclei) and 1.5 x 10^7 cpm (18 h nuclei) were incorporated into RNA. The nuclear run on experiments have been repeated with different preparations of nuclei from AX-2 and AX-3 cells revealing very similar results (not shown). No hybridization was detected to pUC12 DNA (Fig. 3). When 10 µg/ml α-amanitin was included in the nuclear run on reaction no hybridization to the four clones was obtained (not shown). The synthesis of cysteine proteinase I mRNA was induced by 6 h of development and decreased after 12 h (Fig. 3). The temporal pattern of the transcription rate correlates well with the accumulation of the RNA during development (Fig. 2), indicating that the expression of the cysteine proteinase I gene is predominantly under transcriptional control. Recently, similar results have been obtained (42).

(c) Micrococcal nuclease digestion. We wished to determine if there is a change in the micrococcal nuclease pattern when the cysteine proteinase I gene becomes activated.

In Fig. 4, DNA fragments from similar gels to that shown in Fig. 1 were transferred to DBM paper and subsequently probed with the 800 bp BglII - HaeIII cDNA fragment of pDd11.7.10 (24). The typical nucleosome pattern was seen in vegetative and 4 h stage cells. The background was low and, even at the shortest digestion times, discrete bands were generated. However, at the 6 h stage a smearing of the pattern (i.e. a continuous distribution of DNA fragments) was observed and reached a maximum at the 8 h stage. Diffuse bands of the nucleosomal repeat were still apparent behind the smear. At the 12 h stage the smearing was reduced and by 15 h of development the vegetative cell pattern had reappeared. Pure nuclear DNA digested with micrococcal nuclease and probed with the cDNA of the cysteine proteinase I gene gave a smear (Fig.

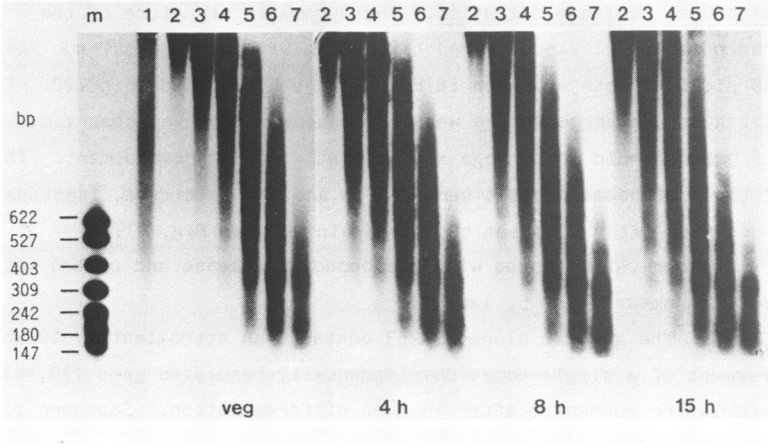

Fig. 5. Chromatin structure of the gene hybridizing to pCZ22. Isolated nuclei from vegetative cells, 4 h, 8 h and 15 h stages of development and pure nuclear DNA were digested with micrococcal nuclease. Incubation times as in Fig. 1; digested pure DNA is in lane 1. Marker (lane m): pBR322 DNA cut with HpaII.

4, lane 1). A comparison between the digestion kinetics of vegetative and 8 h stage cells showed that the coding region of the cysteine proteinase I gene was not digested more quickly when transcribed.

Clone pCZ22. pCZ22 is a cDNA complementary to a gene which is constitutively expressed during development (35,43). We have used Southern blot analysis to confirm that the gene exists in multiple copies per genome (41).

(a) Changes in pCZ22 mRNA concentration during development. Fig. 2 shows a Northern blot hybridization of pCZ22 to total cellular RNA from various stages of development. A high concentration of RNA complementary to pCZ22 was seen in vegetative cells. In 4 h cells the level had decreased but then remained constant for the rest of development.

(b) Nuclear in vitro transcription. The synthesis of pCZ22 mRNA during development up to 12-15 h (Fig. 3) correlates well with the mRNA accumulation in the corresponding stages (Fig. 2). The low level of hybridization of (^{32}P) UTP labeled pCZ22 mRNA in vegetative cells is not due to a very high concentration of pCZ22 RNA in the prepared nuclei. Hybridization of in vitro transcribed RNA from vegetative cells to 1,2,4 and 8 µg pCZ22 DNA immobilized on nitrocellulose membranes revealed no differences in the intensities of the hybridization signals (results not shown). Transcription decreased during the latter stages of development.

(c) Micrococcal nuclease digestion. The chromatin structure of the coding region of pCZ22 was examined by probing with micrococcal nuclease. In Fig. 5 similar blots as shown in Fig. 1 were hybridized to pCZ22. In all the stages a smear between a weak nucleosome repeat was observed. The extent of smearing did not change significantly during development. The sizes of the nucleosomal repeat were 180 bp and its multimers, identical to the ones seen after ethidium bromide staining (see Fig. 1).

Pure nuclear DNA digested with micrococcal nuclease and probed with pCZ22 showed a smear (Fig. 5, lane 1).

Clone pSC253. The genomic clone pSC253 contains an approximately 10 kb EcoRI fragment of a single copy, developmentally regulated gene (39,40). mRNA accumulation commences after 15 h of differentiation. Southern blot analysis confirmed it is a single copy gene (44).

(a) Changes in pSC253 mRNA concentration during development. No mRNA complementary to pSC253 was detected in vegetative, 4 h, and 8 h stage cells whereas at the 12 h stage a very faint band was seen (Fig. 2). After 15 h of development this band strongly increased and several minor bands appeared. After 18 h of development a smear was superimposed over these bands. We identified a number of mRNAs in addition to the two described by Lodish and coworkers (35,36).

(b) Nuclear in vitro transcription. Fig. 3 shows that induction of synthesis of pSC253 mRNA was already detectable at 6 h of development. The transcription rate increased during later development reaching a maximum at 18 h. However, in vivo, the mRNA was first detectable at 15 h (Fig. 2), suggesting that expression may be at least partly under post-transcriptional control.

(c) Micrococcal nuclease digestion. Gels similar to that shown in Fig. 1 were blotted and hybridized with the 3.3 kb AluI fragment of pSC253 (Fig. 6). The classical nucleosomal repeat was seen in all stages of development. The background between the bands was low although a slight increase was observed in 18 h nuclei. Due to the formation of spore and stalk cells, which produce cellulose-rich cell walls, nuclei could not be isolated from later stages when the gene becomes fully active. Like all other clones tested, the nucleosome repeat size in the coding region of pSC253 was about 180 bp and did not change during development.

Clone pDd812. pDd812 is a cDNA clone containing 570 bp of the discoidin I mRNA (32,33). The discoidin I genes of D. discoideum (strain Ax2) constitute a family of three closely related, co-ordinately regulated

genes (32-34). By 6 h the discoidin I mRNA level has increased to about 1% of the total poly(A^+) RNA (32,45-48). We used the 330 bp HindIII-EcoRI cDNA fragment of pDd812 as a hybridization probe (32). Pseudogene fragments have been found in several strains of D. discoideum (48) and Southern blot analysis with our probe detected some additional, although weak bands (44).

(a) Changes in discoidin I mRNA concentration during development. The Northern blot which had previously been probed with pDd11.7.10 (Fig. 2) was stripped and rehybridized with the pDd812 fragment (Fig. 2). Low levels of mRNA complementary to pDd812 were present in vegetative cells and increased to a maximum at 6 h. At the 8 h stage the concentration of mRNA had considerably dropped and in later stages no discoidin I mRNA was observed.

(b) Nuclear in vitro transcription. The transcription rate of mRNA complementary to pDd812 was maximal at 4 h of development and decreased rapidly after 6 h (Fig. 3). These results confirm previous work showing that the synthesis of pDd812 mRNA is predominantly under transcriptional control (32).

(c) Micrococcal nuclease digestion. In vegetative cells a nucleosome repeat and some smearing between the bands was seen (Fig. 7, veg). In the 4 h and 6 h stages the smearing was reduced and vanished totally after 15 h of development (Fig. 7, 4 h, 6 h, and 15 h). The size of the nucleosomal repeat did not change during development and was a multimer of 180 bp.

Restriction Enzyme Digestion

The chromatin of the coding region of the D. discoideum ribosomal genes is only accessible to restriction enzymes when being transcribed (7). The sites in the nucleosome-packaged non-transcribed spacer were not cleaved. We wished to ascertain whether the coding region of the cysteine proteinase I gene becomes accessible to restriction endonucleases when the gene is turned on.

Nuclei of vegetative, 6 h, 8 h and 15 h stage cells were digested with 200 units EcoRI or HindIII for 30 min at 37°C. The DNA fragments were purified and the HindIII digested samples cut to completion with BglII. The resulting DNA fragments were size fractioned on a 1.5% agarose gel, transferred to DBM paper and hybridized with the 800 bp BglII-HaeIII cDNA fragment of pDd11.7.10 (Fig. 8b).

Fig. 8a shows the accessibility of the coding region of the cysteine

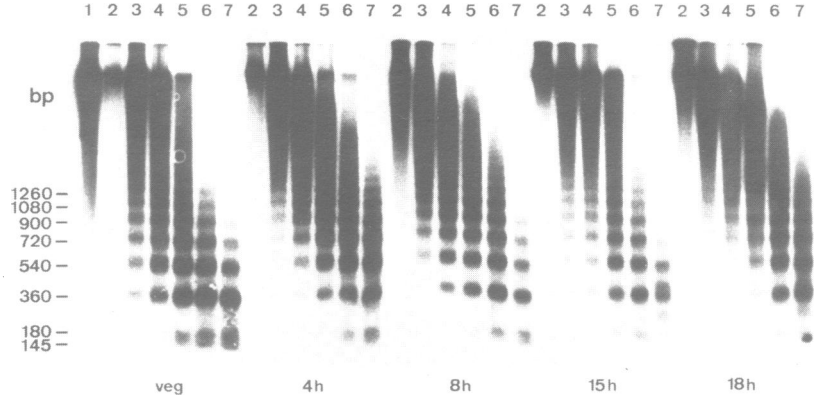

Fig. 6. Chromatin structure of the gene hybridizing to pSC253. Isolated nuclei from vegetative cells, 6 h, 8 h, 12 h, 15 h and 18 h stages of development and pure nuclear DNA were digested with micrococcal nuclease. Incubation times as in Fig. 1; digested pure DNA is in lane 1. Hybridization was with the 3.2 kb AluI fragment from the transcribed region.

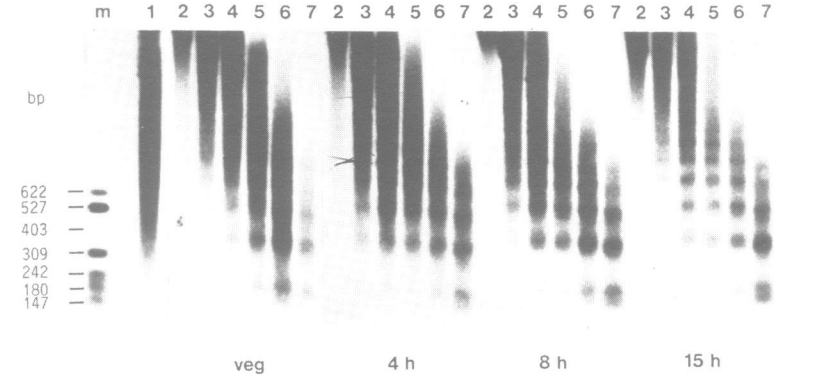

Fig. 7. Chromatin structure of the discoidin I gene during differentiation. Isolated nuclei from vegetative cells, 4 h, 6 h and 15 h stages of development and pure nuclear DNA were digested with micrococcal nuclease. Incubation times as in Fig. 1; digested pure DNA is in lane 1. Hybridization was with the 300 bp HindIII-EcoRI cDNA fragment of pDd812. Marker (lane m): pBR322 DNA cut with HpaII.

proteinase I gene to EcoRI and HindIII during development. Both enzymes recognize a 6 bp sequence. Complete digestion of pure nuclear DNA with EcoRI resulted in two double bands, 475 bp and 440 bp in length, originating from the coding region (Fig. 8a, lane 1). When nuclei from

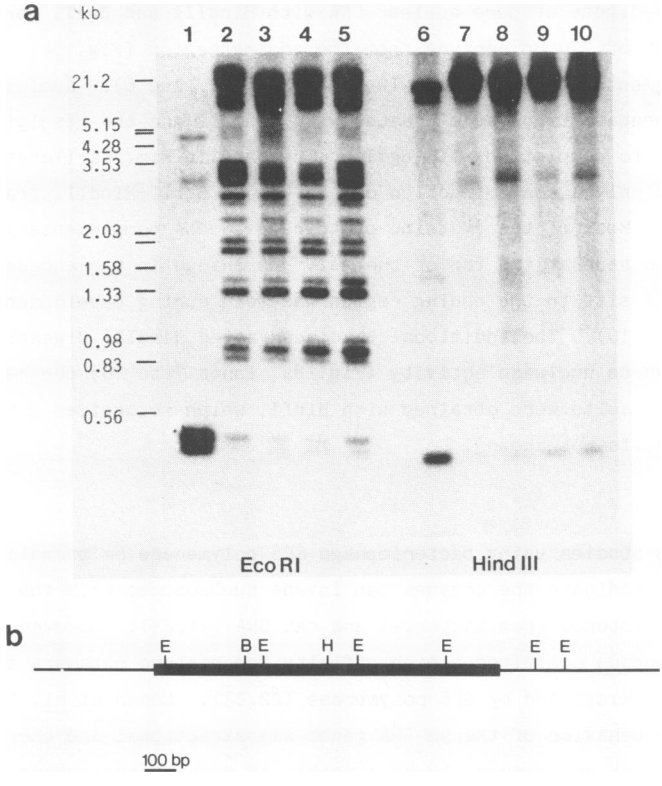

Fig. 8. Sensitivity of cysteine proteinase I chromatin to restriction endonuclease digestion. (a) Nuclei from vegetative, 6 h, 8 h and 15 h differentiated cells were incubated for 30 min with EcoRI (lanes 2 to 5) or with HindIII (lanes 7 to 10) at 37°C. The DNA fragments were purified and the HindIII digested samples cut with BglII. Lane 1 shows pure nuclear DNA cut with EcoRI, lane 6, pure nuclear DNA cut with HindIII and BglII. The DNA fragments were size fractioned on a 1.5% agarose gel, blotted, and hybridized with the 800 bp BglII-HaeIII cDNA fragment of pDd11.7.10. (b) Restriction enzyme cleavage map of the cysteine proteinase I gene. The symbols of the restriction enzymes used are: B, BglII; E, EcoRI; H, HindIII.

the various developmental stages were digested with EcoRI (Fig. 8a, lanes 2 to 5), the EcoRI fragments originating from the coding region were only excised to a minimal extent, giving very weak hybridization signals. No significant change in the accessibility of the EcoRI sites in the coding region between the four developmental stages was seen (Fig. 8a, lanes 2 to 5). A large number of additional fragments were obtained which represented incomplete digestion by EcoRI.

A double digest of pure nuclear DNA with HindIII and BglII generated a DNA fragment of 420 bp derived from the coding region (Fig. 8b). The adjacent fragments were more than 10 kb (Fig. 11a, lane 6). Nuclei of the four developmental stages were treated with HindIII and the resulting DNA fragments cut to completion with BglII. The data in Fig. 8a (lanes 7 to 10) show that only a small fraction of the 420 bp BglII-HindIII fragment was released. Most of the cysteine proteinase I cDNA complementary sequences were seen at the top of the gel. No change in the accessibility of the HindIII site in the coding region was seen during development (Fig. 8a, lanes 7 to 10). The additional band seen after HindIII digestion was due to endogenous nuclease activity (Fig. 8a, lanes 7 to 10; see ref. 24).

Similar results were obtained with HinfI, which recognizes a 5 bp sequence (results not shown).

DISCUSSION

In vitro studies using bacteriophage SP6 polymerase or mammalian RNA polymerase II indicate the enzymes can invade nucleosomes from the ends and displace histones from bacterial and rat DNA (21,23). However, the 5S gene of *Xenopus* exhibited a high affinity for histone octamers and these were not displaced by SP6 polymerase (22,23). Lorch *et al.* (23) suggested the behavior of the 5S RNA genes was exceptional and the passage of RNA polymerase would normally be expected to disrupt nucleosomes *in vivo*. In theory, the relative rates of transcription and nucleosome assembly would determine the extent to which a transcribed region is packaged into nucleosomes. Psoralen crosslinking experiments with ribosomal genes of *D. discoideum* suggest that nucleosomes and transcription complexes can occur together on the same coding sequence (13), implying that any alteration in nucleosome structure must be transient, nucleosomes rapidly reforming following transcription. However, Brown (cited in ref. 23) has suggested the stability of the histone octamer on 5S DNA *in vitro* resembles the *in vivo* behavior of most nucleosomes, the nucleosomes formed *in vitro* on small DNA fragments being less stable than nucleosomes *in vivo*. The aim of the present work was to determine whether disruption of nucleosome structure correlates with transcription *in vivo*.

The results with the developmentally regulated cysteine proteinase I gene (pDd11.7.10) indicate perturbation of nucleosome structure occurs during transcription. Analysis of the gene by micrococcal nuclease

digestion and blot hybridization showed that the coding region is in regularly repeating units (nucleosomes) when the gene is inactive. Following activation a continuous distribution of DNA fragments was observed. This lack of periodicity, which was seen as a smear in electrophoresis, may result from an undefined linker length between the nucleosomes, loss of the nucleosomes or a significant change in their structure (7).

However, the results with the other three genes studied did not support the general conclusion that transcription consistently leads to displacement of histones from the transcribed region.

pCZ22 mRNA was present during all stages of development and levels were particularly high in vegetative cells. Nevertheless, nuclear run on experiments consistently showed relatively low levels of transcription in vegetative and 18 h cells compared to the other developmental stages examined. However, the nucleosome array was equally disrupted at all stages.

Nuclear run on experiments indicated a single copy gene (genomic clone pSC253) was first transcribed at 6-8 h although engaged RNA was only readily detectable at 15-18 h of development. This suggests post-transcriptional regulation of expression occurs (49). Although the rate of transcription was similar at 12, 15 and 18 h, a slight smearing of the nucleosomal pattern first appeared at 18 h. Hence, there appears to be strong transcription at times when no chromatin perturbation is apparent.

Similarly, although nuclear run on experiments indicated transcription of the discoidin I gene was very high during early development, consistent with the mRNA rising to about 1% of the cellular poly(A^+) RNA by 6 h (34,46,47), only weak perturbation of the nucleosomal structure occurred. This was not due to differential expression of the three discoidin I genes since they are under co-ordinate control (34,47,48).

How can these results be reconciled with the model of Lorch et al. (21,23)?

The apparent lack of nucleosome perturbation during transcription of the pSC253 and discoidin I genes may reflect low densities of transcription complexes and rapid assembly of nucleosomes. However, the transcription rates of these two genes appear to be comparable to those of the cysteine proteinase I gene. The minimal nucleosome disruption of the discoidin I gene may result because transcriptional activity is restricted

to only a sub-set of cells. Immuno-histochemistry indicates the cells have variable amounts of antigen and some cells may never express discoidin I (50).

Such explanations are not relevant to the results obtained with the pCZ22 gene, whose nucleosome structure was disrupted even when the level of transcription was relatively low.

Considered together our results throw doubt on the proposal that the passage of the polymerase causes the release of nucleosomes from the vast majority of genes in vivo (23). The extent of nucleosome perturbation by the polymerase may not be determined exclusively by the transcription rate (density of transcription complexes) but also by the DNA sequence. Differences in the affinity of DNA sequences for the histone octamers may not only influence their release during transcription but also the rate at which they are reassembled. In such a model, the pSC253 and discoidin I genes would have a relatively high affinity for the histones. Hence, only high transcription rates would result in detectable perturbation of nucleosome structure. The degree of perturbation is, of course, difficult to assess since micrococcal nuclease digestion may only detect drastic changes (such as almost complete displacement of the histone octamer). The smaller the degree of disruption, the more rapidly could nucleosome reassembly occur.

The pCZ22 gene would have a relatively low affinity for the histone octamers, resulting in their ready release during polymerase passage and comparatively slow reassembly. Thus, even a relatively low transcription rate would result in a chromatin structure susceptible to micrococcal nuclease digestion. Alternatively, the pCZ22 locus might be in a region of the chromosome that is constantly in an open chromatin configuration. Thus, transcription would not be required to maintain this chromatin phenotype. Rose and Garrard (51) found that both the active κ-IgL gene and its non-transcribed allelic partner in a variant plasmacytoma cell line exhibit apparent unevenness in nucleosomal spacing and histone H1 deficiency.

Unlike ribosomal genes of D. discoideum (7) and the hyperactive secretory protein gene of Chironomous (52), the coding region of the actively transcribed cysteine proteinase I gene failed to become more accessible to restriction enzymes. Similarly, BspR1 restriction sites within the active mouse kappa immunoglobulin gene appeared well protected even against a high excess of enzyme and although micrococcal nuclease

digestion gave a smeared pattern of DNA fragments (53,54). Hence, the polymerase II transcription complex may protect DNA from endonuclease digestion more efficiently than the polymerase I transcription complex. However, the disagreement with the Chironomous results is puzzling.

DNase I hypersensitive sites appear upstream of the cysteine proteinase I gene when transcription commences at 6 h and remain when transcription has almost ceased during the latter stages of development (24). Unlike transcription, activation of the gene does not lead to perturbation of the structure of nucleosomes on the coding region.

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