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Developmental Regulation of DNase I-Hypersensitive Sites in *Dictyostelium discoideum*

KATHLEEN AYRES,[1] WILMA NEUMAN,[1] WALTER G. ROWEKAMP,[2] AND STEPHEN CHUNG[1]*

Department of Biological Chemistry, University of Illinois at Chicago, Chicago, Illinois 60612,[1] and Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg 1, Federal Republic of Germany[2]

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We have studied two regions of *Dictyostelium discoideum* chromatin and identified several DNase I-hypersensitive sites in these regions. One of these sites is located about 300 to 500 bases upstream of the transcriptional start site of a gene that is expressed at all stages of development. This site is present in both vegetative cells and postaggregation cells. Another hypersensitive site is associated with a gene that is expressed only after the multicellular stage. This site is located about 400 bases upstream of the start site, and it is present only in postaggregation cells. Thus, much like higher eucaryotes, *D. discoideum* contains DNase I-hypersensitive sites that may be involved in the regulation of the genes with which they are associated.

Dictyostelium discoideum is an excellent system to study how cell-cell interaction regulates gene expression during development. During the feeding stage, *D. discoideum* exists as free-living amoeboid cells. Upon starvation, up to 10^5 of these apparently homogeneous cells aggregate to form a multicellular organism which eventually develops into a fruiting body consisting of spore cells and stalk cells (16). Previous experiments have shown that during the transition from the single-cell stage to the multicellular stage, new surface molecules which make cells adhere to each other tightly are synthesized (9, 22), and soon thereafter, approximately 2×10^3 to 3×10^3 genes are induced (3). Some of these postaggregation genes are prespore specific, while others are specific for prestalk cells (2, 20), suggesting that communication between cells within the aggregate directs cellular differentiation. Pulsatile addition of cyclic AMP to a starved cell suspension can mimic cell aggregation and induce some but not all of these cell type-specific genes (4), indicating that cyclic AMP is one of the messengers relaying signals between cells to direct differentiation. Some postaggregation specific genes can only be induced by tight cell-cell contact (4, 5), indicating that interactions between cell surface molecules or other as yet unidentified diffusible molecules may serve as signals to regulate transcription of these genes.

As a first step toward understanding how cell-cell interaction regulates expression at the molecular level, we analyzed the changes in chromatin conformation during gene induction. We studied a gene that is induced at the multicellular stage and a gene that is expressed at all stages of development. In this report, we showed that the chromatin of these genes is interrupted with regions that are hypersensitive to DNase I digestion. These hypersensitive sites are located several hundred bases upstream of the transcriptional start sites, and they appear only when the genes are transcriptionally active, suggesting that they may play a role in regulating the expression of these genes.

* Corresponding author.

MATERIALS AND METHODS

Cells. *D. discoideum* AX3 was used for these experiments (15). Conditions for the axenic growth and development of this strain have been described previously (1, 3, 5).

Northern blot hybridization. RNA blot hybridization was done by the method of Thomas (30). Formamide-formaldehyde-denatured RNA was run in 1.5% agarose gel containing formaldehyde. RNA blotted onto nitrocellulose paper was immobilized by baking in a vacuum oven at 80°C. The filters were then incubated with nick-translation labeled DNA probes at 40°C for 48 h. The hybridization buffer was 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.02 M sodium phosphate (pH 6.5)–2.5 mM EDTA–0.08% bovine serum albumin–0.08% polyvinylpyrrolidone–0.08% Ficoll–100 μg of sonicated, denatured calf thymus DNA per ml. After hybridization, the filters were washed with 0.1× SSC and 0.1% sodium dodecyl sulfate and exposed to Kodak XAR5 film with a Du Pont Cronex Lightening-Plus screen at −70°C.

DNA sequence determination. The *Eco*RI-*Xba*I fragment of SC29 (see Fig. 1B) was subcloned into pHSG359, sequentially digested by exonuclease BAL 31 from the *Eco*RI site, and religated with *Bam*HI linker. A number of appropriate deletions were recloned as *Bam*HI-*Pst*I fragments into pUC8 cleaved with *Bam*HI and *Pst*I. These deletions were sequenced by a modification of Sanger's dideoxynucleotide sequencing method (27), using the Biolab primer no. 1201. Approximately 80% of the sequence was additionally verified by Maxam and Gilbert sequencing (18).

S1 mapping using end-labeled DNA. S1 mapping was carried out essentially as described by Maniatis et al. (17) and Garriga et al. (8). The 265-base-pair (bp) *Alu*I-*Eco*RI fragment was labeled at the 5′ ends and hybridized to vegetative mRNA. Hybridizations were for 4 to 14 h at 40°C. Hybridizations were conducted in 10 μl of solution containing 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–400 mM NaCl–1 mM EDTA–80% formamide. S1 nuclease digestion was for 2 h at 22°C with 500 to 4,000 U of enzyme per reaction. After ethanol precipitation, the S1-digested samples were dissolved in a solution of 80% formamide–10 mM NaOH–1 mM EDTA–0.1% xylene

cyanole–0.1% bromphenol blue. The samples were then boiled for 5 min and loaded onto 5% polyacrylamide gels containing 7 M urea–0.09 M Tris–0.09 borate–2.5 mM EDTA. There was a 49-base S1-resistant fragment (data not shown), indicating that the 3′ splice junction of the intron is 49 bases from the right *Eco*RI site, in good agreement with the predicted position of 816 shown in Fig. 4.

Primer extension. The primer extension methodology employed was essentially that of Ghosh et al. (10) as described by Lee and Roeder (14). The 5′ end-labeled restriction fragments were hybridized with either 10 μg of poly(A) RNA or bakers' yeast RNA in 10 μl of solution containing 40 mM PIPES (pH 6.4)–400 mM NaCl–1 mM EDTA–80% formamide for 4 to 6 h at 42°C. The hybrids were precipitated with ethanol and dissolved in 10 μl of solution containing 80 mM NaCl–5 mM MgCl–50 mM Tris hydrochloride (pH 8.3)–2 mM dithiothreitol–1 mM of each deoxynucleotide triphosphate. Reactions were initiated by addition of 5 U of reverse transcriptase and continued at 37°C for 3 h. Reactions were terminated with the addition of EDTA to 10 mM and extracted with phenol and chloroform. The samples were precipitated with 50 μg of yeast RNA and analyzed in 5% acrylamide gel as described in the previous section.

Purification of "naked" DNA for DNase I digestion. Vegetative cells were lysed in 4% Cemusol Nonidet P40 (Fluka), and the nuclei, collected by centrifugation, were lysed by 4% Sarkosyl (Sigma). The DNA was banded in a CsCl-ethidium gradient and further purified by three extractions with phenol containing 1% sodium dodecyl sulfate.

Purification of nuclei from *D. discoideum* cells. Three to 6 liters of *D. discoideum* vegetative cells was grown to a density of 1×10^6 to 2×10^6 cells per ml for each nuclear DNA isolation. Cells were harvested by centrifugation (Sorvall RC3B and H6000A rotor at 2,000 rpm for 4 min) and washed with 0.4% saline. Developmental cells were washed with PDF (3), plated, and allowed to develop for 15 h, after which the slugs were disaggregated and the nuclei were isolated. The nuclei were isolated by a modification of the method of Hewish and Burgoyne (11; D. D. Blumberg, personal communication). The cells were suspended at 0°C in lysis buffer (34 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris hydrochloride, pH 7.4, 1 mM EDTA, 15 mM beta-mercaptoethanol, 0.2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], 0.15 mM spermine, 0.5 mM spermidine, and 1.5 mM phenylmethysulfonyl fluoride) containing 0.5% Cemusol Nonidet P-40 to a concentration of 1×10^8 to 2×10^8 cells per ml. The sample was divided into aliquots and vortexed for 30 s in 50-ml plastic screw-capped tubes. The cells were diluted with the addition of 0.5% Cemusol Nonidet P-40 lysis buffer to a concentration of 10^8 cells per ml and vortexed for an additional 30 s per sample. The samples were centrifuged at 3,000 rpm (Sorvall H6000A rotor) for 5 min at 0°C. If the nuclear pellet was white, it was washed in lysis buffer plus 0.1% Cemusol, vortexed for 5 s, and pelleted at 3,000 rpm for 5 min. Otherwise, the cells were lysed a second time with lysis buffer plus 0.5% Cemusol and washed with 0.1% Cemusol in lysis buffer. This nuclear pellet was washed twice with lysis buffer without phenylmethylsulfonyl fluoride or detergent, followed by one wash with digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris hydrochloride, pH 7.4, 0.5 mM dithiothreitol, 0.25 M sucrose, 1 mM CaCl, and 10 mM MgCl) (31).

Identification of DNase I-hypersensitive sites. The final washed nuclear pellet was suspended in digestion buffer at a concentration of 7.8×10^8 nuclei per ml and treated with DNase I (Worthington, "DPFF") at 37°C for 3 min. DNase activity was stopped by the addition of EDTA to 12.5 mM and sodium dodecyl sulfate to 2%. The samples were extracted three times with phenol and ethanol precipitated in the presence of 0.2 M sodium acetate. The precipitated nucleic acids were dissolved in NET (15 mM NaCl, 1.5 mM EDTA, 5 mM Tris hydrochloride, pH 8.0) and treated with RNase A at 25 μg/ml and RNase T_1 at 25 U/ml for 60 min at 37°C in the presence of 0.5% Sarkosyl. The RNases had been pretreated at 100°C for 10 min to inactivate DNase activity. The enzymes were removed by phenol extraction and precipitated with ethanol. The DNA was dissolved in TE buffer (10 mM Tris hydrochloride, pH 7.4, and 1 mM EDTA), and samples were electrophoresed on 1% agarose gels to determine the extent of DNase I digestion. Before restriction endonuclease digestion, the DNA samples were passed over Sephadex G-50 (fine) equilibrated with TE buffer. The DNA samples were then digested with appropriate restriction enzymes and analyzed by Southern blot hybridization (29).

RESULTS

Mapping of the genes. Since the primary objective of our research is to understand the role of DNase I-hypersensitive sites in the developmental regulation of gene expression, we did not study the region of the chromatin around the actin or discoidin gene even though these genes are well characterized. These genes are expressed primarily at the preaggregation stage, but there is a low level of expression of these genes in vegetative cells as well. Therefore, it is likely that the hypersensitive sites associated with these genes may be present at all stages of development. We chose to study a clone called SC253, which contains a gene that is induced only at the multicellular stage, and as a control, a clone called SC29, which contains three genes that are expressed in vegetative cells as well as at all stages of development (5).

The location of gene 253 in the genomic DNA clone SC253 has been determined by a combination of DNA sequence information, S1 mapping, and primer extension experiments. Detailed description of the results of these experiments will be presented elsewhere (W. Neuman et al., submitted for publication), and the location of 253 is shown in Fig. 1A. The transcription start site and the polyadenylation site are about 3,800 and 190 bases, respectively, from the right-hand *Eco*RI site. The nucleotide sequence around the transcription start site of 253 is shown in Fig. 2.

Clone SC29 hybridized to three mRNAs (Fig. 3). A previous report showed that this clone contains only one gene (5). This is probably due to the fact that it was analyzed in a glyoxal gel, where the RNA was denatured by glyoxal, and was run in an agarose-acrylamide composite gel. The two high-molecular-weight RNAs were not resolved in these gels, and the small RNA was probably hydrolyzed by the sodium hydroxide used to treat the gel to uncouple RNA from glyoxal before transfer. The approximate location of the three genes in SC29 (Fig. 1B) was determined by using fragments of the clone as probes in Northern blot hybridization (Fig. 3). The open bars below the restriction map (Fig. 1B) indicate the restriction fragments used to probe the Northern blots. The three genes are called 29A, 29B, and 29C as indicated.

Since we need only one gene as control, we chose to map 29C in greater detail because it is the smallest gene and, consequently, would be easier to sequence. An 840-base portion of SC29 from the right-hand *Eco*RI site, spanning the *Ava*I site, was sequenced (Fig. 4). The sequence information

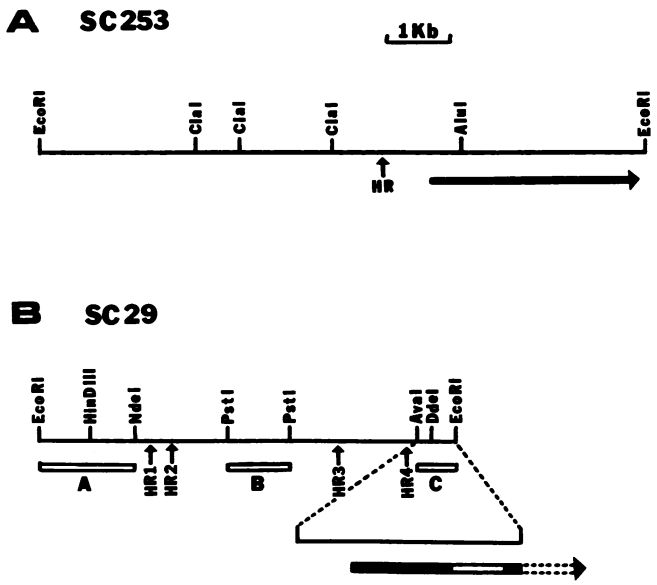

FIG. 1. Maps of genomic DNA clones SC253 and SC29. For the sake of clarity, not all the restriction sites of *Alu*I on SC253 are shown, and not all the *Pst*I and *Dde*I sites on SC29 are shown. The open bars indicate the restriction fragments that hybridized to RNA and represent the approximate locations of genes 29A, 29B, and 29C. The horizontal arrow in panel A represents the transcription start site and direction of transcription of gene 253. The horizontal arrow in panel B represents the transcript from 29C. The open area indicates the location of the intron, and the broken arrow indicates that the location of the 3′ end of the gene has not been determined. HR, Hypersensitive region.

indicates that SC29 contains only the 5′ end of 29C. The ATG at position 495 signals the beginning of an open reading frame that is interrupted by an intron at position 654 to 815. Located at position 397, approximately 200 bases upstream of the translation initiation codon, is the *D. discoideum* TATA box TATAAAAA (13), indicating that transcription of 29C initiates between positions 405 and 494.

The transcription start site was more accurately mapped by primer extension experiments. The 265-bp *Alu*I-*Eco*RI fragment was extended to 440 bp with reverse transcriptase, using vegetative mRNA as template (Fig. 5). This places the 5′ end of the transcript 175 bases upstream of the *Alu*I site, around position 423 as shown in Fig. 4. The existence of the intron was confirmed by S1 mapping (see Materials and Methods). These physical mapping data show that our interpretation of the DNA sequence information is correct. The location of 29C is represented in Fig. 1B.

DNase I-hypersensitive sites. We used a modified version of the nucleus isolation procedure developed by Hewish and Burgoyne (11) to isolate nuclei from vegetative cells. Cells were first lysed with the detergent Cemusol Nonidet P-40, and the nuclei were collected by centrifugation. After several washes to remove the detergent, a small amount of DNase I was added to digest the chromatin briefly. DNA was then purified from the nuclei by phenol-chloroform extraction, digested with restriction endonuclease, and analyzed in a Southern blot hybridization using labeled SC29 as a probe (Fig. 6A). The control lane (Fig. 6A, lane 6) is purified DNA digested with *Eco*RI. It shows one band (labeled a) corresponding to the SC29 fragment. In the lanes where the nuclei were digested with increasing amounts of DNase I (lanes 2 to 5), additional bands (labeled b, c, d, e, f,

```
                                                                      60
TCGATAAAAT AATTTAACCA TAAAAAAAAA AAAAAAAAAA AATATATGGA TAATTGTTTA
                                                                     120
AATAAGTTTT TATAAATTGG CAAACAAAAA ATATCATCAT AATTACACAT TGGGTACTTT
                                                                     180
TTTTTTTTAA AAAAATTAAA AATTTAGTAG CAAGTGGGTT AGTGTGGGTT TTAAATTGTG
                                                                     240
GTTTAACCTT TTAATGTTAG AAATAGGAGA TGAAATAAAA AATTTTTNNC CCGTATCATT
                                                                     300
ATAGTATGAT TTGTAAATTA TTATAATAAA TATAATTATT ATATAAATAA TATCAAAAAA
                                                                     360
AAAAAAAAAA AAAAAAACCA ATCCATTTTT ACAATTAGGA AAATAATCAA CACTAATATA
                                                                     420
AAAAATAAAA TAAATTAAAA TTTTATAACA AAAAGAATAG ATTTTTTTTT TTATTTGTTT
                                                                     480
TTTTTTACTG TGTTAATAGG GGGTTGGGGG GTTAATAATT TGTAATTTAA AATAATATTA
                                                                     540
TCATATGATT TGTTTTGAAC AACATATATA TTTATACATA TACACCATAA AAATACTATA
                                                                     600
TACATAATCA TTGGGAAATC TCATTTTACT TATCACATTG TTTTATTGTT ATTTTTTACA
                                                                     660
TAGTAAAATT TATTTTTGGA TATTTTCAAT TTGATTTCAA TTAAAAAAAA CAAAATGCTG
                                                                     720
TCAAATTATT TATTAAATAT TTTTAATTGG GCATAGTATG TATATGATGA ATGTCAAGTA
                                                                     780
GATAACTGTT TTTGTTGTTT CTATTAGCTG TTGCAATAGC TTCCAATAGT AGTAGTTTTT
                                                                     840
TTTTTTTAAT TTGTTTTTTA TTTTTTTTAT AATTTCATCT GTTATCATTA CCACATTTAT
                                                                     900
ATGGTAAATA ATTAATATTT GATCAATTAT AATATTTTAT TTTATTTTAA TTTTTAAAAT
                                                                     960
ATTTTTTTAA AATATATTTT TTTTTTGTAT AAATAGAAGT AATAATTTTT TTTTTTTATC
                                                                    1020
ACTTCATTTT TTATAAAATA AAACAAATAA AAAACAAAGA AAAAAAAAAA AAAAAACTAA
                                                                    1080
AAAAAAAAAA AAAACCAACC AAAAATTTAT TTATTAAAAT TGCAACCATG AATAAAATAT
                                                                    1120
ATTTAATATT AATTTTATTC ACTTTTGTTG GTATAATTTT
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FIG. 2. Nucleotide sequence of the promoter region of gene 253. The first base is 4,678 bases from the right-hand *Eco*RI site. The consensus TATA sequence is underlined, the initiation codon ATG is boxed, and arrowheads indicate the possible transcription start sites.

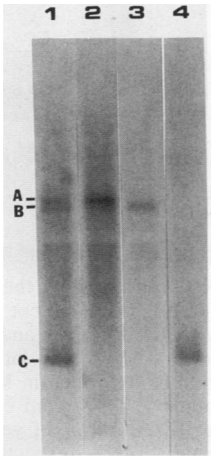

FIG. 3. Northern blot hybridization to determine the approximate locations of the genes on SC29. A 2-μg sample of poly(A)-containing RNA from vegetative cells was loaded on each lane and hybridized to: (lane 1) SC29 DNA, (lane 2) the *Eco*RI-*Nde*I fragment (see Fig. 1), (lane 3) the *Pst*I-*Pst*I fragment, or (lane 4) the *Ava*I-*Eco*RI fragment.

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                                                                    60
CCAAAAAATC AAAAAAAANC CCAATCACNT AAAAATTTAA TCAGCAGTAA TAAAAAAAAA
                                                                   120
AATTTTCAAA TAAAATTTTT TTTGACATTG TTGAGCAAAA ATTTTTATTT TTATATTTTT
                                                                   180
TTTTTTTTTG TTTTTGATTA AAAAAAAAAA AAAATTGTAA AATGTTTTAT TCAAACATAT
                                                AvaI               240
CCTAATTTTA TTTTTAAATT GGGATTTATT ATGGGTTGTN ACAACCCGAG ATGATGATTT
                                                                   300
TACCAAAAAG ATTTTTTTTT TTTATGGAGT TTTTAGGTAC CCTGGTAAGT TGAATAAAAT
                                                                   360
ATCTAAAAAC CAGTATTTTT CGAATCTTTT TTATTTTTTT TTAAATTTTT ATTTTTTATT
                                                                   420
TTATTTTTTA TTTTTTTTTT TGCAAATTTG GTTGCCTATA AAAACTTTTT TTATTTTTAT
                                                                   480
TTTTATCATT TTATATTTTT ATCAATCAAT TTATTTATTT CACTTTATTT TGACTTTTAC
                                                    DdeI           540
TTTTGTATTC CAAAATGAGA TTCATTTTAG TTTTAGTTTT AATTTTGGGC TTAGTCTCAT
                                                                   600
CATCATTTGG TATCAATGTT GACAAGAATG AAGTATCTCA AACCGAATTA CAATCAGCTC
                                                          AluI
                                                                   660
GTGACTTTAG TTCATTCGCT ATCGGCTTTG CAGAGGGTAT TGAAATTAGT TTGGTAAGTT
                                                                   720
TAAAATTTAT TATTTTATAG TTTTCCCAAA AACAACTTTT TTTTTTTTTT TTTTTTTTTT
                                                                   780
TTTTTTTTTT TTTCAAACAT TTTTTATTTG ATATTAAATT ATATTATATA CAATTTTATT
                                                                   840
TTACATTCTC TAATGATATT AAATTTTATA TATAGACTGG TAATCTTCAT AAATGTGTCG

-----approximately 20 bases to EcoRI site
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FIG. 4. Nucleotide sequence of a portion of gene 29C and its 5′ flanking region. The restriction sites mentioned in the text and the *D. discoideum* TATA box are underlined. Arrowheads indicate the possible transcription start sites. The translation initiation codon ATG is boxed. The intron sequence is bracketed and underlined.

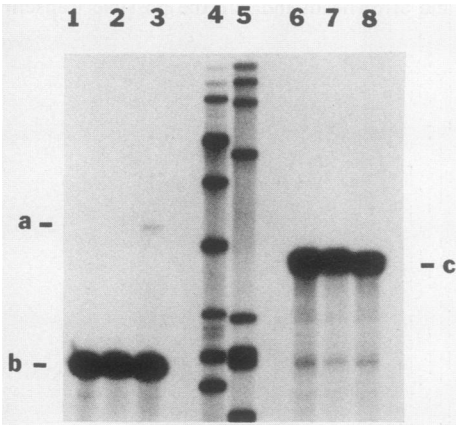

FIG. 5. Primer extension mapping of the transcription start site of 29C. The *Ava*I-*Eco*RI fragment was cut by *Alu*I (see Fig. 3) to generate a 370-bp *Ava*I-*Alu*I fragment and a 265-bp *Alu*I-*Eco*RI fragment. Both fragments were 5′ end labeled and subjected to primer extension reaction as described in Materials and Methods. Lanes 1, 2, and 3 contained 40,000 cpm each of the *Alu*I-*Eco*RI fragment plus either no RNA, 10 μg of yeast RNA, or 10 μg of poly(A)-containing vegetative RNA, respectively. Molecular weight markers: lane 4, pBR322 DNA cut by *Alu*I; lane 5, ϕX174 DNA cut by *Hae*III. Lanes 6, 7, and 8 contained 40,000 cpm each of the *Ava*I-*Alu*I fragment plus either no RNA, 10 μg of yeast RNA, or 10 μg of poly(A)-containing vegetative RNA, respectively. Band a is the 440-bp product of the primer extension reaction with the *Alu*I-*Eco*RI fragment.

and g) appear. These additional bands are generated by double-strand cuts by DNase I at the hypersensitive sites. In the first lane, where the nuclei were not digested with DNase I, some of these bands are also seen. These are probably generated by endogenous endonucleases digesting the same hypersensitive sites. These sites are not specific for DNase I, but rather represent regions on the chromatin where the DNA is more accessible to macromolecules. Other nonspecific endonucleases such as DNase II and micrococcal nuclease have also been shown to digest these sites preferentially (19, 32). DNase I digestion of purified DNA did not generate these bands (Fig. 7), indicating that preferential digestion at the hypersensitive sites is peculiar to chromatin.

Regulation of DNase I-hypersensitive sites. In the previous section we showed that *D. discoideum* chromatin is interrupted with DNase I-hypersensitive sites. To see whether

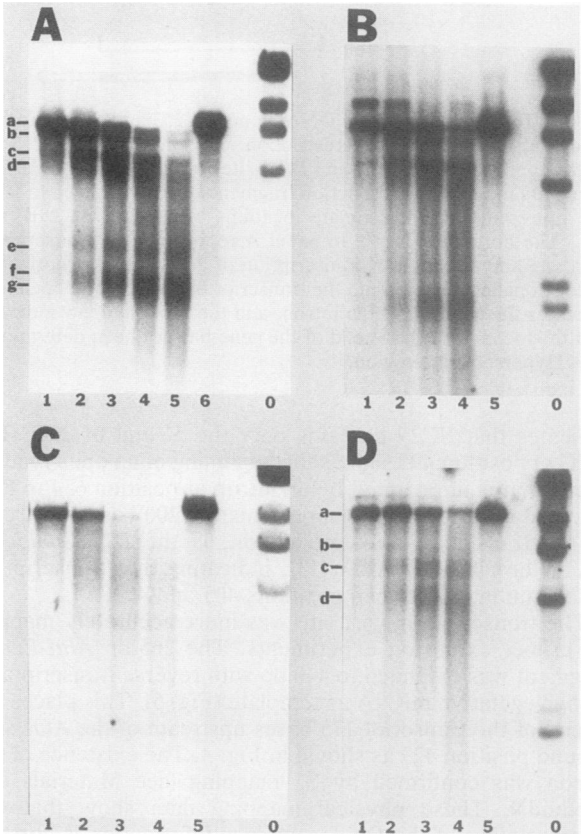

FIG. 6. Identification of DNase I-hypersensitive sites. Nuclei were prepared from the cells and treated with DNase I as described in Materials and Methods. DNA purified from the DNase I-treated nuclei was then digested to completion with *Eco*RI and blotted onto nitrocellulose paper for hybridization. (A) DNA from vegetative nuclei treated with 0, 19, 56, 112, and 150 U of DNase I per ml (lanes 1, 2, 3, 4, and 5, respectively). Lane 6, Purified genomic DNA digested with *Eco*RI. The filter was hybridized with ^{32}P-labeled SC29 DNA. (B) DNA from post-aggregation nuclei treated with 0, 19, 56, or 112 U of DNase I per ml (lanes 1, 2, 3, and 4, respectively) and hybridized to labeled SC29 DNA. Lane 5, Purified DNA digested with *Eco*RI. (C) DNA from vegetative nuclei treated with 56, 112, 150, and 205 U of DNase I per ml (lanes 1, 2, 3, and 4, respectively) and hybridized to SC253 DNA. Lane 5, Purified DNA digested with *Eco*RI. (D) DNA from postaggregation nuclei prepared and treated the same way as for panel B except that it was hybridized to labeled SC253 DNA. Lane 0 in each panel is the *Hin*dIII-cut lambda DNA size marker.

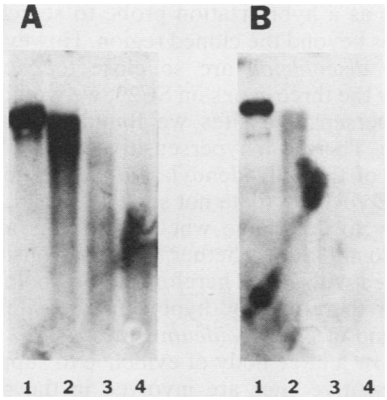

FIG. 7. DNase I digestion of naked DNA. DNA was purified as described in Materials and Methods. Each lane contained 10 μg of DNA before digestion. Lanes 1, 2, 3, and 4, in each panel were treated with 0, 0.19, 0.38, and 0.57 U of DNase I per ml, respectively. The digestion conditions were the same as those for nuclei as described in Materials and Methods. The filter in panel A was hybridized to labeled SC29 DNA, and that in panel B was hybridized to SC253 DNA.

the appearance of the hypersensitive sites is regulated during development, we chose to study the region represented by clone SC253. This clone contains one gene that is transcribed only when the cells reach the multicellular stage and form tight cell-cell contacts. Nuclei were extracted from postaggregation cells, digested with DNase I, and analyzed by Southern blot hybridization as described in the previous section (Fig. 6D). In addition to the main band (Fig. 6D, band a) there were three minor bands (b, c, and d) that hybridized to SC253 DNA. We believe that bands c and d denote a hypersensitive site in that region. Band b is probably an artifact because it is present even in the control lane (lane 5), where purified DNA was digested with restriction endonuclease alone. This band is more prominent in earlier Southern blots, where hybridization was done at 37°C (see Fig. 7B, lane 1), and not present in later mapping experiments where hybridization was done at 42°C (Fig. 8A), indicating that it is probably due to cross-hybridization with another gene (Neuman et al., submitted). Again, similar to the situation in the vegetative nuclei, these bands were present even when no DNase I was added to the nuclei, suggesting that this hypersensitive site is also sensitive to digestion by endogenous endonucleases.

To see whether the appearance of this hypersensitive site correlates with the transcriptional activity of this gene, nuclei from vegetative cells were treated with DNase I and probed with SC253 DNA (Fig. 6C). Even when more DNase I was used, there was no preferential digestion at the hypersensitive site such as that which gave rise to bands c and d in Fig. 6D. The overall intensity of the bands in Fig. 6C is lighter than those in Fig. 6D because more DNase I was used for panel C. It should be noted that the amount of DNase I used in lane 2 of panel C was equivalent to that used in lane 4 of panel D of the same figure. Thus, the appearance of this hypersensitive site is under developmental control and correlates with the transcriptional activity of the gene with which it is associated.

The hypersensitive sites in the SC29 region of the chromatin in the postaggregation cells were also analyzed (Fig. 6B). All the genes in SC29 were active at this stage, and, as expected, the same set of bands generated by DNase I cuts were present in the postaggregation cells as well.

Mapping of the hypersensitive sites. The experiments described above show that the appearance of the DNase I-hypersensitive sites in SC29 and SC253 correlates with the expression of these genes. Before a regulatory function of these sites can be postulated, it is necessary to see whether they are located near the transcription start sites of these genes.

We used the indirect labeling method of Wu et al. to determine the location of these hypersensitive sites (31). Nuclei were digested with a fixed amount of DNase I, deemed the optimal concentration from the previous experiments. The DNA was purified and digested with *Eco*RI and analyzed in a Southern blot hybridization with labeled DNA from both ends of the clone as probes (Fig. 8). In Fig. 8A, where the *Alu*I-*Eco*RI fragment of SC253 was used as a probe, the hypersensitive band is 4.2 kilobases (kb), placing the hypersensitive site 4.2 kb from the right-hand *Eco*RI site. This was confirmed by using a DNA fragment from the other

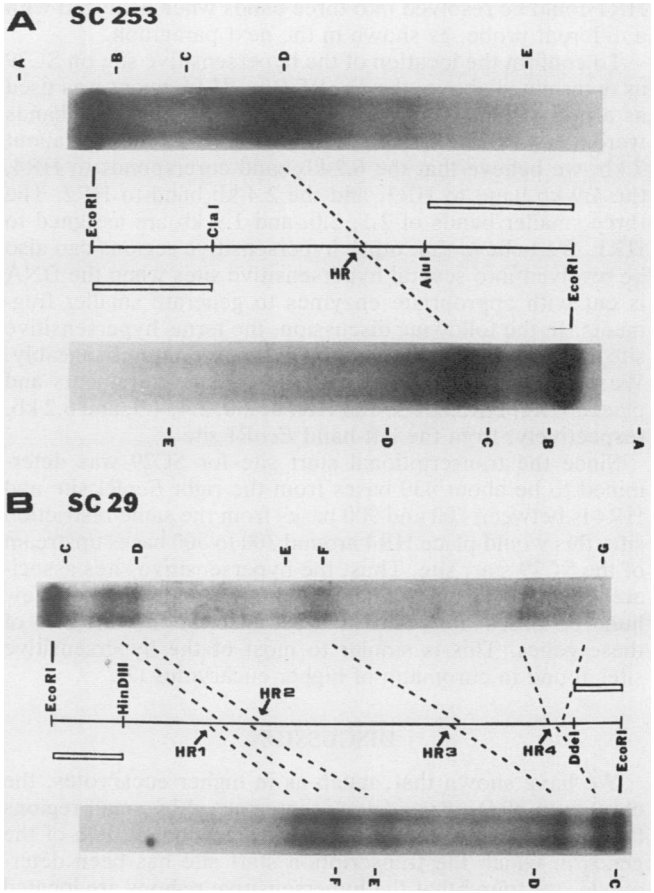

FIG. 8. Mapping of hypersensitive sites. (A) Nuclei from postaggregation cells were digested with 112 U of DNase I per ml. The DNA was purified, digested with *Eco*RI, and analyzed by Southern blot hybridization. The labeled probes used in each hybridization are indicated by the open bars. The dotted lines indicate the locations on the restriction map corresponding to the bands. Panel B is similar to panel A except that the DNA is from vegetative nuclei digested with 56 U of DNase I per ml. In both panels HR stands for hypersensitive region, A, B, C, D, E, F, and G indicate the positions of molecular size markers of 23.7, 9.46, 6.67, 4.26, 2.25, 1.96, and 0.59 kb, respectively.

end of the clone as a probe. Hybridization with the *Eco*RI-*Cla*I fragment placed the hypersensitive site 5.5 kb from the left-hand *Eco*RI site. Since SC253 is about 10 kb long, this placed the hypersensitive site 4.5 kb from the right-hand *Eco*RI site, in close agreement with the 4.2-kb distance measured from the other end. Since smaller fragments are usually more accurately measured in these gels, we placed the hypersensitive site 4.2 kb from the right-hand *Eco*RI site. Therefore, this hypersensitive site is about 400 bases upstream of the transcriptional start site of gene 253. We labeled the site HR, for hypersensitive region, for reasons that will become clear in the following paragraphs.

The mapping of the hypersensitive sites on SC29 is shown in Fig. 8B. When the *Dde*I-*Eco*RI fragment was used as a hybridization probe, the hypersensitive bands were 4.9, 4.4, and 2.0 kb and a smear ranging in size from 0.9 to 0.7 kb. The first three bands represent HR1, HR2, and HR3, respectively. The smear around 0.7 to 0.9 kb corresponds to HR4. We called these hypersensitive regions because some of them consist of several hypersensitive sites. The smear representing HR4 could sometimes be resolved into three or four distinct bands. Similarily, the band corresponding to HR1 could be resolved into three bands when analyzed with a different probe, as shown in the next paragraph.

To confirm the location of the hypersensitive site on SC29 as determined above, the *Eco*RI-*Hin*dIII fragment was used as a probe. In this hybridization, the hypersensitive bands were 6.2, 4.9, 2.4, 2.1, 2.0, and 1.9 kb. Since SC29 is about 7 kb, we believe that the 6.2-kb band corersponds to HR4, the 4.9-kb band to HR3, and the 2.4-kb band to HR2. The three smaller bands of 2.1, 2.0, and 1.9 kb are assigned to HR1. We believe that other hypersensitive regions can also be resolved into several hypersensitive sites when the DNA is cut with appropriate enzymes to generate smaller fragments. In the following discussion, the terms hypersensitive site and hypersensitive region will be used interchangeably. We used the average value of the two measurements and placed HR1, HR2, HR3, and HR4 at 2.0, 2.4, 4.9, and 6.2 kb, respectively, from the left-hand *Eco*RI site.

Since the transcriptional start site for SC29 was determined to be about 430 bases from the right *Eco*RI site and HR4 is between 700 and 900 bases from the same restriction site, this would place HR4 around 300 to 500 bases upstream of the SC29 start site. Thus, the hypersensitive sites associated with genes 29C and 253 are located within a few hundred bases upstream of the transcription start site of these genes. This is similar to most of the hypersensitive sites found in chromatin of higher eucaryotes (7).

DISCUSSION

We have shown that, much as in higher eucaryotes, the chromatin of *D. discoideum* is interrupted by small regions that are hypersensitive to DNase I digestion. In two of the genes in which the transcription start site has been determined, we found that the hypersensitive regions are located 300 to 500 bases upstream of the start sites. The appearance of these hypersensitive sites correlates with the transcriptional state of these genes, suggesting that they may play a role in activating these genes.

The other hypersensitive sites that we have identified are located at the flanking regions of genes 29A and 29B, whose direction of transcription has not been determined. Therefore, we do not know whether they are located at the 3′ end or 5′ end of these genes. Clone SC29 does not contain the 3′ end of 29C. In principle, we could use the end piece of the cloned DNA as a hybridization probe to search for hypersensitive sites beyond the cloned region. However, since the genes in *D. discoideum* are so close to each other, as evidenced by the three genes on SC29, we would not be sure that the hypersensitive sites we found do not belong to another gene. There is a hypersensitive site about 300 bases downstream of the polyadendylation site of gene 253, just beyond the *Eco*RI site (data not shown). Again, because we have no way to determine whether there is another gene nearby, we do not know whether this hypersensitive site is in fact associated with 253. Therefore, at this point we are not sure whether there may be hypersensitive sites associated with the 3′ end of *D. discoideum* genes.

There is now a large body of evidence to support the idea that hypersensitive sites are involved in the regulation of gene expression (7). Besides the fact that the appearance of hypersensitive sites closely correlates with gene induction, these sites are located at or near the enhancer sequences (6, 21), suggesting that the appearance of hypersensitive sites may reflect the enhancer function. This is supported by the fact that insertion of the 72-bp simian virus 40 enhancer element induces the appearance of a hypersensitive site (12) and that a naturally occurring deletion of hypersensitive sequence leads to loss of gene activity (23, 38). However, at this point, the mechanism that activates hypersensitive sites and its relationship to induction of transcription are not entirely clear. *D. discoideum* may provide a convenient system to study these problems because it is relatively easy to prepare material for chromatin studies, and also cloned genes that are introduced back into the cells are properly regulated (24, 26). In this report, we demonstrated the presence of hypersensitive sites in *D. discoideum* and showed that the appearance of these hypersensitive sites correlates with gene expression, indicating that the folding and unfolding of chromatin during gene induction and repression in *D. discoideum* is similar to that in higher eucaryotes.

Our main goal is to study how cell-cell communication regulates gene expression in *D. discoideum*. Gene 253 is one of the genes that are induced at the multicellular stage. Like most other postaggregation stage genes, continuous cell-cell contact is required for its transcription. It differs from these genes, however, in that cyclic AMP cannot replace multicellularity in maintaining its transcription (5). This indicates that besides cyclic AMP, other signals are involved in cell-cell communication during development. We are in the process of searching for the extracellular factor(s) that induces this gene. Identification of hypersensitive sites associated with this gene is the first step towards unraveling the nuclear event that activates transcription of this gene. In this report, we have identified the approximate location of the hypersensitive region associated with this gene. It will be interesting to determine the precise locations of the hypersensitive sites within this region and see whether they correspond to the protein-binding sites that can be determined by in vivo footprint analysis.

While this paper was under revision, Pavlovic et al. reported the identification of hypersensitive sites in the 5′ and 3′ flanking regions of the *D. discoideum* cysteine proteinase I gene (25). Similar to the situation reported here, the hypersensitive sites associated with the 5′ end of this gene are present only when the gene is activated. Interestingly, these hypersensitive sites persist at the later stages of development when the gene is no longer transcribed, suggesting that hypersensitive sites are necessary but not sufficient to activate transcription.

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