Native genomic blotting: High-resolution mapping of DNase I-hypersensitive sites and protein–DNA interactions

(chromatin structure/histone genes/HeLa S3 cells)

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ABSTRACT   DNase I-hypersensitive sites are observed in the promoter regions of actively expressed genes, potentially active genes, and genes that were once active. We have developed an approach that greatly increases the resolution for mapping these sites by electrophoresing genomic DNA on native polyacrylamide gels prior to electroblotting and hybridization. This improved method has been used to scan the promoter and coding region of a cell-cycle-dependent human histone H4 gene with an accuracy of ±5–10 base pairs. Protein–DNA interactions can be seen in the autoradiograph as light areas and DNase I-hypersensitive sites as dark bands. Therefore, this method provides a rapid and relatively simple means to accurately localize protein–DNA interactions as well as DNase I-hypersensitive sites, thus directly displaying DNase I hypersensitivity and protein–DNA complexes on one autoradiograph. It also potentially allows the analysis of small changes in DNase I-hypersensitive sites under various biological conditions. With this technique rather large regions of DNA can be screened to determine areas that should be analyzed by more sophisticated methods, such as genomic sequencing or gel retardation assays.

Structural features of chromatin are analyzed in nuclei using a variety of nucleases, among them micrococcal nuclease (1), DNase I (2), DNase II (3), S1 nuclease (4), mung bean nuclease (5), exonuclease III (6), and restriction endonucleases (7). DNase I is widely used due to its low sequence specificity and to the discovery of DNase I-hypersensitive sites that can be correlated with active chromatin (8). Until now, these DNase I-hypersensitive sites have always been mapped using agarose gels, which do not allow very high resolution of DNase I-generated subbands. We have improved on this approach by separating genomic DNA on native polyacrylamide gels and then electrotransferring the DNA to nylon membranes prior to hybridization. Our results indicate that we can accurately localize DNase I-hypersensitive sites [±5–10 base pairs (bp)] as well as display protein–DNA interactions as light areas on the same autoradiograph. Therefore, this method can be used to scan an area of several hundred base pairs for DNase I-hypersensitive sites at high resolution, while also localizing possible protein–DNA interactions in that same region.

The data we present are for a cell-cycle-dependent human H4 histone gene, F0108 (9), which has been shown (10) to contain a nuclease sensitive site in the 5'-flanking region. This broad sensitive site, which was recognized by both DNase I and S1 nuclease, showed differential accessibility during the cell cycle and contained an unchanging hypersensitive core that mapped from −150 to −50 bp. The cell cycle changes in the DNase I and S1 nuclease patterns were characterized by an overall expansion of the large region of

sensitivity (500–1000 bp) that surrounds the core. Two sites of protein–DNA interaction in this gene have also been established with the genomic sequencing technique at single-nucleotide resolution (11). Site I extends from −150 to −117 bp on the upper strand and from −146 to −113 bp on the lower strand, whereas site II is from −91 to −50 bp on the upper strand and −97 to −47 bp on the lower strand. Both sites appear to contain binding sites for more than one protein and seem to be functionally related to the transcription of this gene throughout the cell cycle. No differences in the protein–DNA complexes at single-nucleotide resolution were observed during the 3- to 5-fold enhanced transcription of the gene in early S phase as compared to the basal level in mitosis/G1 phase. But since cell-cycle alterations in DNase I sensitivity could be observed in DNA separated on a low-resolution agarose gel, we attempted to localize these differences at the intermediate resolution of the native genomic blotting method we have developed.

MATERIALS AND METHODS

Isolation of Nuclei and Purification of the DNA. Nuclei were isolated from exponentially growing or synchronized HeLa S3 cells (12), then treated with DNase I, and the DNA was isolated as described (10). Aliquots of purified DNA (12–15 μg) were digested with *Pvu* II (1% agarose gels) or *Hinc*II (4% polyacrylamide gels). DNase I digestion of deproteinized DNA and the salt wash of the nuclei were done according to Pauli *et al.* (11).

Gel Electrophoresis and Blotting. Agarose gels were electrophoresed overnight in 1× TBE buffer (1× TBE = 100 mM Tris·HCl, pH 8.3/100 mM boric acid/2 mM EDTA, pH 8.3), transferred to Zetabind by the alkaline Southern transfer method (15, 16), and hybridized with a random oligo primer extended probe (13) (specific activity, 5×10^8 cpm/μg). The polyacrylamide gels [polyacrylamide/bisacrylamide, 40:1 (wt/wt)] were electrophoresed at 100 V for 3 hr in 0.5× TBE buffer. The gel was then removed from the glass plates and sealed without any buffer in a plastic bag. The bag was placed in boiling water for 10 min and then electrotransferred for 90 min, UV crosslinked, and hybridized with an M13 probe according to the procedure of genomic sequencing (14). Exposures were overnight at −70°C with an intensifying screen.

RESULTS AND DISCUSSION

To compare the traditional approach for assaying DNase I hypersensitivity on agarose gels with our native genomic system (Fig. 1), nuclei from exponentially growing HeLa cells were treated with DNase I, and the DNA was isolated and digested with the appropriate restriction enzymes. Aliquots of digested DNA were separated in an agarose gel, transferred by the alkaline Southern blot procedure (15, 16), and then hybridized with a random oligo primer extended probe, which was derived from the 3' end of the F0108 human
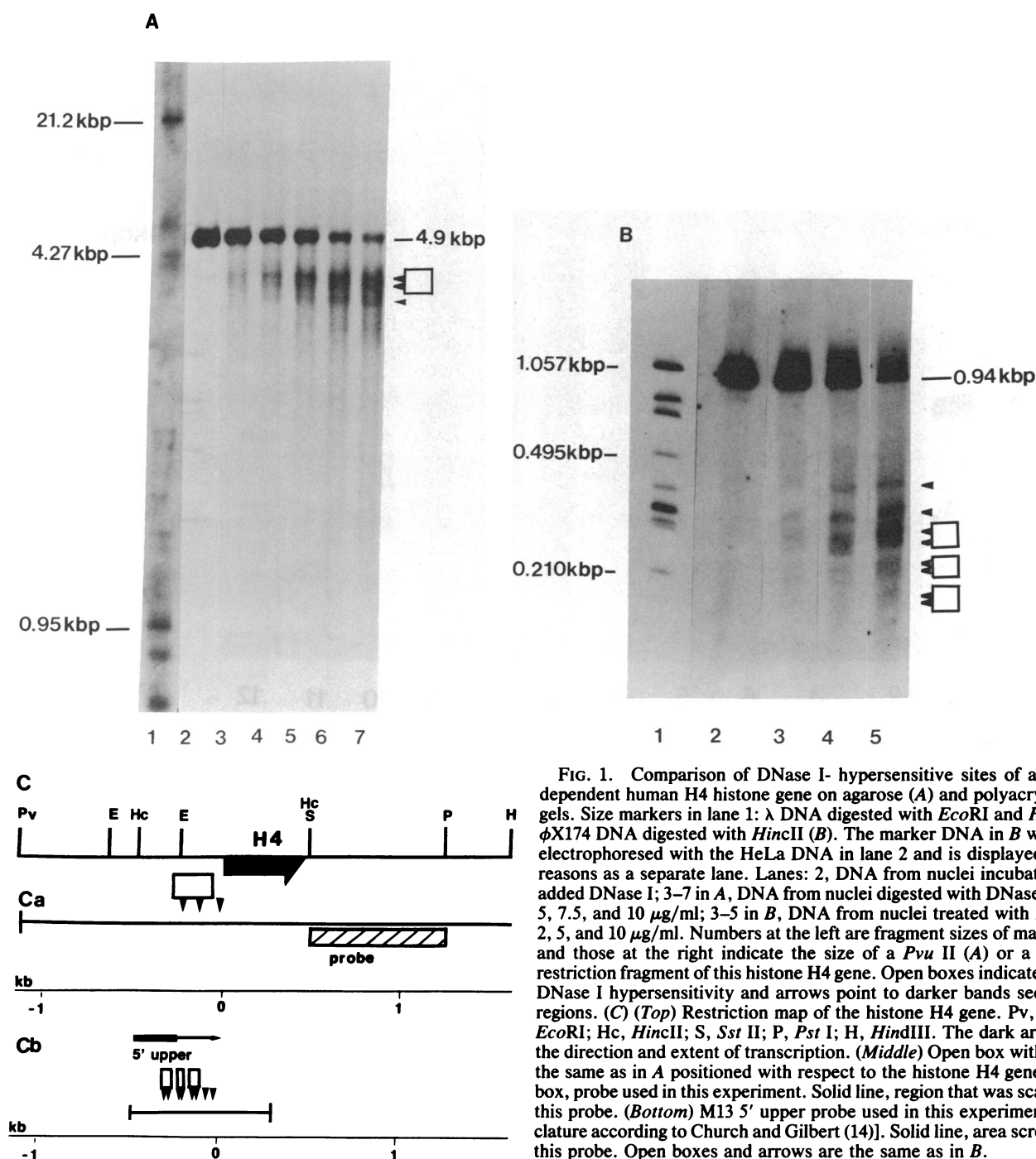
A

B

C

FIG. 1.   Comparison of DNase I- hypersensitive sites of a cell-cycle-dependent human H4 histone gene on agarose (*A*) and polyacrylamide (*B*) gels. Size markers in lane 1: λ DNA digested with *Eco*RI and *Hind*III (*A*), φX174 DNA digested with *Hinc*II (*B*). The marker DNA in *B* was actually electrophoresed with the HeLa DNA in lane 2 and is displayed for visual reasons as a separate lane. Lanes: 2, DNA from nuclei incubated with no added DNase I; 3–7 in *A*, DNA from nuclei digested with DNase I at 2, 3.5, 5, 7.5, and 10 μg/ml; 3–5 in *B*, DNA from nuclei treated with DNase I at 2, 5, and 10 μg/ml. Numbers at the left are fragment sizes of marker DNA, and those at the right indicate the size of a *Pvu* II (*A*) or a *Hinc*II (*B*) restriction fragment of this histone H4 gene. Open boxes indicate regions of DNase I hypersensitivity and arrows point to darker bands seen in these regions. (*C*) (*Top*) Restriction map of the histone H4 gene. Pv, *Pvu* II; E, *Eco*RI; Hc, *Hinc*II; S, *Sst* II; P, *Pst* I; H, *Hind*III. The dark arrow marks the direction and extent of transcription. (*Middle*) Open box with arrows is the same as in *A* positioned with respect to the histone H4 gene. Hatched box, probe used in this experiment. Solid line, region that was scanned with this probe. (*Bottom*) M13 5′ upper probe used in this experiment [nomenclature according to Church and Gilbert (14)]. Solid line, area screened with this probe. Open boxes and arrows are the same as in *B*.

histone H4 gene (Fig. 1*C Middle*). A main band of 4.9 kbp was generated (Fig. 1*A*), which is a typical fragment size that is used for mapping DNase I-hypersensitive sites. A DNase I generated subband was visible early in digestion, and the pattern did not change even after extensive digestion. This subband can be resolved into two or possibly three slightly darker bands.

In our native genomic blotting method, the digested HeLa DNA samples were separated on a native polyacrylamide gel. The gel was then boiled to denature the DNA and subjected to electroblotting. A single-stranded M13 probe was used for hybridization (Fig. 1*C Bottom*). The main band generated in this case was 940 bp, and the subband could now be resolved into a series of bands and light areas. Our calculations indicate that the subband from Fig. 1*A* was expanded three times in length, which is even more remarkable considering

the fact that the polyacrylamide gel was considerably shorter than the agarose gel and was electrophoresed in a fraction of the time. The pattern of DNase I digestion was established very early in digestion and did not alter with increasing amounts of DNase I (lanes 3–5). As a control, nuclei were incubated with no DNase I added (Fig. 1 *A* and *B*, lanes 2), demonstrating the absence of endogenous nucleases. The plasmid marker DNA in lane 1 was actually electrophoresed with the genomic DNA shown in lane 2. However, since it is known that the migration of small pieces of DNA in polyacrylamide gels can be greatly affected by their sequence (17), this was not a reliable way to make accurate measurements of our genomic DNA. Indeed, we found that the marker DNA fragments were shifted by ≈30 bp compared to the HeLa sequences we examined. Therefore, to obtain accurate sizing it was necessary to make partial restriction
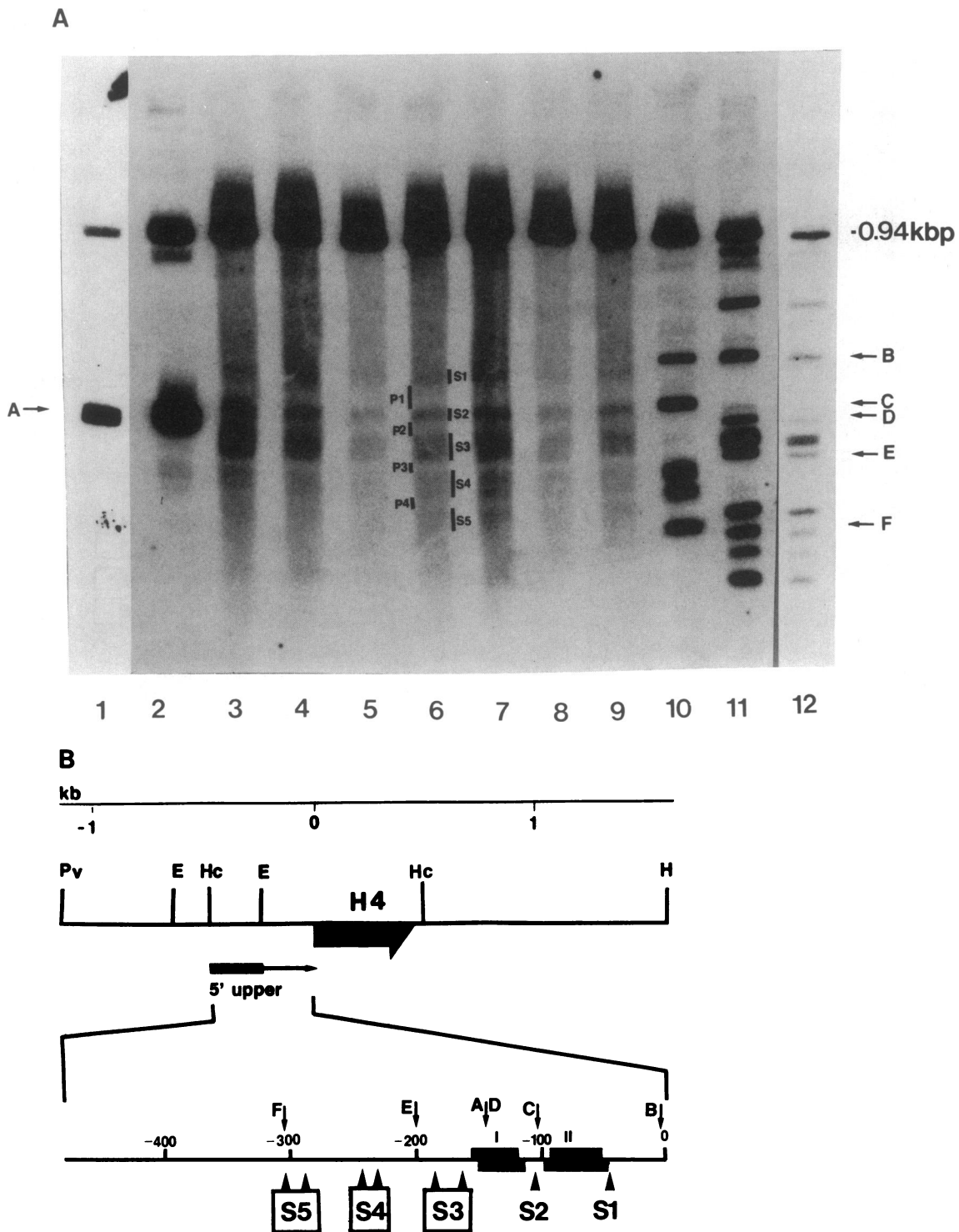
FIG. 2.   High-resolution mapping of DNase I-hypersensitive sites throughout the cell cycle. (*A*) Lanes: 2 and 10, *Hinc*II-digested HeLa DNA from nuclei treated with *Taq* I and *Msp* I, respectively; 1, lighter exposure of lane 2; 3–9, *Hinc*II-digested DNA from nuclei of thymidine-blocked cells, early S phase (1 and 3 hr after release of the thymidine block), late S phase (6 and 8 hr after release), and mitosis/G_1 phase (10 and 12 hr after release), respectively, that were treated with DNase I at concentrations between 5 and 10 μg/ml; 11, deproteinized HeLa DNA digested with *Hinc*II and partially digested with *Mnl* I; 12, lighter exposure of lane 11. Number on the right indicates the size of the *Hinc*II fragment of this histone H4 gene. Letters indicate cleavage of the DNA by restriction enzymes at known sequences as follows: A, *Taq* I site at −132 bp; B, *Mnl* I site at +3 bp; C, *Msp* I site at −97 bp; D, *Mnl* I site at −136 bp; E, *Mnl* I site at −198 bp; and F, *Msp* I site at −307 bp, with respect to the ATG codon. The areas of hypersensitivity are labeled S1–S5, and the areas of protection are labeled P1–P4. Conditions for electrophoresis, transfer, and hybridization are as in Fig. 1*B*. (*B*) (*Upper*) Restriction map of the human H4 histone gene. Black arrow shows restriction sites and probe as in Fig. 1. (*Lower*) Expansion of the promoter region from the *Hinc*II site at −472 bp to the ATG codon. Open boxes and arrows, same sites as in Fig. 1*B*. Letters A–F are the same as in *A* and are displayed at their position in the sequence. Solid bars show protection from DNase I digestion on the upper strand (above line) and lower strand (below line) as determined by the genomic sequencing technique (11).

digests of genomic HeLa DNA and electrophorese these fragments alongside the DNase I samples.

Fig. 1*C* shows a summary of our comparison of these methods. With a traditional agarose gel we can detect one

region of hypersensitivity that may consist of two or three bands (Fig. 1*C Middle*), whereas with our native genomic blotting method we can resolve the hypersensitive region into a series of many sensitive and protected bands (*Bottom*).

Since we obtained such high resolution we wished to determine if any small changes in DNase I sensitivity in this region could be observed during the cell cycle using this method. Therefore, we electrophoresed DNA from DNase I-treated nuclei from various times during the cell cycle of synchronized HeLa cells on a polyacrylamide gel (Fig. 2*A*). Our markers were restriction digests of HeLa DNA, which would generate several specific small fragments. From sequence data (18) the exact positions of these restriction sites are known. As shown in Fig. 2 we were able to accurately map the sites of DNase I hypersensitivity in the 5'-flanking region of this H4 histone gene. In total we observed five regions that were hypersensitive to DNase I and four protected areas. Their positions relative to the ATG codon are as follows: S1 (hypersensitive region 1), −10 to −40 bp; S2, −90 to −115 bp; S3, −150 to −200 bp; S4, −220 to −260 bp; and S5, −275 to −305 bp, with S2 and S3 constituting the core of hypersensitivity. The light areas between the bands that are protected from DNase I map as follows: P1 (protected region 1), −40 to −90 bp; P2, −115 to −150 bp; P3, −200 to −220 bp; and P4, −260 to −275 bp. The sequences upstream and downstream of this 300-bp region show some sensitivity to DNase I but lack a distinct pattern of bands and light areas. At no time during the cell cycle were there any obvious differences in the DNase I pattern at this resolution, in agreement with our finding that the central core of DNase I hypersensitivity is unchanging. Therefore, the cell-cycle differences seen in DNase I sensitivity must occur beyond the region we have examined or at a higher level of chromatin structure than we can detect with our method.

The protected areas P1 and P2 correlate extremely well with the two sites of protein–DNA interaction (site II and site I, respectively), which we have established *in vivo* with the genomic sequencing method (11). Surrounding these sites are regions of DNase I hypersensitivity (S1, S2, and S3) that are particularly strong. In addition, we found two other small light areas (5–20 bp) that are located farther upstream (P3 and P4) and do not correspond to any identified sites of protein–DNA interaction. As a control we digested deproteinized genomic DNA with DNase I (Fig. 3*A*) and, surprisingly, we found a distinct pattern of many sharp bands, indicating sequence-specific cleavage by DNase I. However, the pattern from −10 to −200 bp was quite different in deproteinized

DNA (lanes 2 and 4) as compared to chromatin digests (lane 1). Unambiguously the light areas P1 and P2 were not observed in this control digestion. In the area upstream of −200 bp, the DNase I pattern in deproteinized DNA is very similar to that from nuclei. Therefore, we concluded that the protected areas P3 and P4 reflect sequence-specific digestion rather than protein–DNA interactions. This correlates well with the fact that this area is in a relatively open configuration as determined by DNase I (10) and restriction digestions of nuclei (19). Therefore, our technique could be used to give an overall indication of the relative accessibility of various regions of chromatin.

Since we had identified a S1 nuclease sensitive site for this H4 histone gene that was located in the area of DNase I hypersensitivity (10), we wanted to determine if S1 nuclease recognizes other features of chromatin structure than DNase I. The S1 pattern generated with our method was rather different than the DNase I pattern, with one large light area covering the region where we could detect two protected sites (P1 and P2) with DNase I (data not shown). S1 nuclease obviously did not cut in the sequence between the two sites but displayed an additional highly accessible region between −65 bp and +65 bp, a result observed as well in micrococcal nuclease digests (20). This fits very well with data obtained by DNase I analysis on our high-resolution gels that showed that the region downstream of −10 bp has the same pattern in deproteinized DNA and nuclei (Fig. 3*A*), thus reflecting an open region. These data demonstrate the ability of this method to detect different cutting patterns of different nucleases, thereby recognizing a variety of features of chromatin structure.

To further confirm the presence of protein–DNA interactions, nuclei were washed with NaCl to remove regulatory proteins prior to digestion with DNase I. We have shown that the regulatory proteins of this H4 gene could be dissociated at 0.2 M NaCl (11). Therefore, we electrophoresed DNA from nuclei that were washed with 0.05, 0.18, or 0.44 M NaCl and then digested with DNase I (Fig. 3*B*). The two light areas P1 and P2 were present at 0.05 M NaCl (lane 2) and started to disappear at 0.18 M NaCl (lane 3), correlating well with data from the genomic sequencing technique where the DNase I protection pattern began to decrease at 0.16 M NaCl. At 0.44 M NaCl (lane 4), the light areas had disappeared, suggesting the dissociation of the protein–DNA complexes. No obvious changes during the salt washes could be observed upstream of −200 bp or downstream of −10 bp. The light areas P3 and P4 were still present even after the high-salt
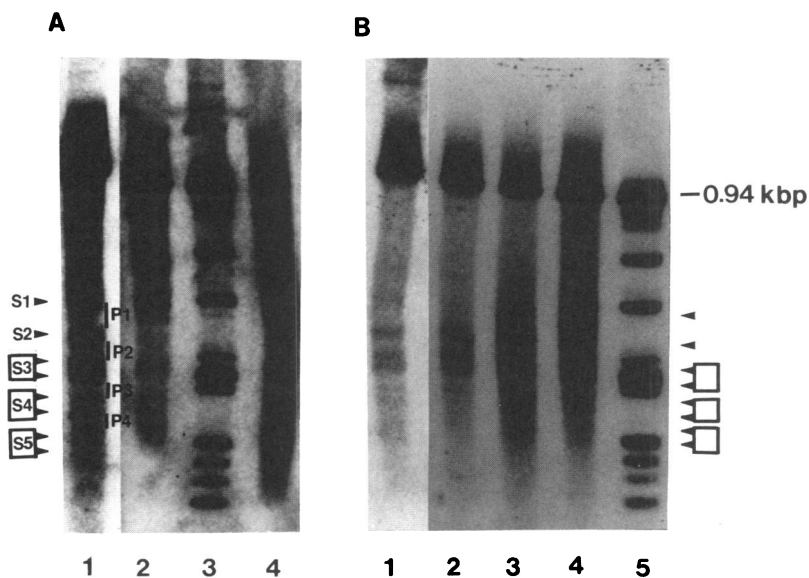

−0.94 kbp

FIG. 3. DNase I pattern of deproteinized DNA (*A*) and salt-washed nuclei (*B*). (*A*) Lanes: 1, *Hinc*II-digested DNA from nuclei digested with DNase I at 10 μg/ml; 2 and 4, deproteinized HeLa DNA digested with *Hinc*II and then with DNase I at 130 pg/μg and 270 pg/μg, respectively; 3, deproteinized DNA digested with *Hinc*II and partially with *Mnl* I. Boxes and arrows are the same as in Fig. 1*B*. (*B*) Lanes: 1–4, *Hinc*II-digested DNA from nuclei in early S phase that were washed with 0.01, 0.05, 0.18, and 0.44 M NaCl, respectively, prior to digestion with DNase I at concentrations of DNase I at 1–2 μg/ml; 5, same as lane 3 in *A*. Numbers and boxes are the same as in Figs. 1*B* and 2*A*. Conditions for electrophoresis, transfer, and hybridization are as in Fig. 1*B*.

wash. This fact and the similarities of the digestion pattern of the regions upstream and downstream to the digestion pattern of naked DNA again suggest that the light and dark areas in the region −10 to −200 bp are due to specific protein–DNA interactions, whereas the upstream and downstream areas probably reflect an open structure that permits DNase I to recognize the sequence.

We are aware that when analyzing the F0108 H4 histone gene we had the advantage of already knowing which region to search for protein–DNA interactions (or light areas). Therefore, we performed similar experiments on a different gene: a cell-cycle-dependent human H3 histone gene (18). Two light areas could be detected on the autoradiograph that were analogous to sites P1 and P2 in the H4 gene (data not shown). The more downstream site seemed to be less protected than the upstream one and certainly less defined than we have observed in the H4 histone gene. The mapping of these sites was confirmed by genomic sequencing including the fact that the downstream site was not easily detectable at single nucleotide resolution (U.P., S.C., H. Nick, G.S., and J.S., unpublished results). These results demonstrate that our approach can be generally applied for mapping protein–DNA interactions in addition to high-resolution mapping of DNase I-hypersensitive sites.

We have described a method for mapping DNase I-hypersensitive sites by electroblotting genomic DNA from native polyacrylamide gels. Our results show that with the native genomic blotting method (*i*) the resolution of DNase I hypersensitive sites is improved by at least 3-fold, (*ii*) we are able to distinguish protein–DNA interactions from sequence-specific digestion, (*iii*) salt-wash studies can be performed to determine a salt concentration at which the protein–DNA complexes dissociate, (*iv*) the pattern of chromatin digests compared to naked DNA can give some indication of the relative accessibility of this region, (*v*) small changes in DNase I-hypersensitive sites may be detected in active and inactive chromatin, and (*vi*) DNase I hypersensitivity can be directly correlated with protein–DNA interactions.

This technique allowed us to scan a region of a human H4 histone gene from −350 bp to +470 bp (roughly 800 bp) for DNase I hypersensitivity and protein–DNA interactions, revealing a complex structure that had not been seen with methods of lower or higher resolution. We believe that the localization of DNase I-hypersensitive sites on agarose gels is an important step in determining the general region where DNase I hypersensitivity occurs. But instead of going directly to a complicated and tedious method like genomic sequencing, one can apply our approach to scan an intermedi-ate area. Thus our native genomic blotting method provides a bridge between low-resolution and single-nucleotide resolution techniques, displaying hypersensitivity and protein–DNA interaction on one autoradiograph.

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