Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 2296–2300, April 1985
Biochemistry

A method for mapping intranuclear protein–DNA interactions and its application to a nuclease hypersensitive site

(chicken adult β-globin gene/DNA/promoter/nucleus)

P. DAVID JACKSON AND GARY FELSENFELD

Laboratory of Molecular Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205

Contributed by Gary Felsenfeld, December 10, 1984

ABSTRACT **We have devised a method for mapping sites on DNA within the nucleus that are protected against nuclease attack by interaction with bound protein or other factors. This "footprinting" method uses an end-labeled sequence-specific DNA probe, which is annealed to the DNA from nuclear digests under carefully controlled conditions. The annealed complexes are treated with single-strand-specific nuclease, the resulting duplex molecules are electrophoresed on gels, and the gels are autoradiographed. The high sensitivity and resolution of the method have made it possible to obtain a detailed map of DNase I cutting patterns in the 5′ flanking sequence of the chicken adult β (β^A)-globin gene within nuclei from various tissues. In nuclei from adult erythrocytes, this domain is hypersensitive to nucleases. However, we detect within the domain two well-defined regions that are protected against attack, indicating the presence of one or more bound factors. Nuclei from oviduct or 5-day-old embryonic erythrocytes, in which the domain is not hypersensitive, show limited and different patterns of protection.**

Specific interactions *in vitro* between nuclear proteins and defined DNA sequences have now been detected in many eukaryotic systems (1–7). To understand how these proteins function in the nucleus, it is necessary to determine whether the pattern of protein binding observed *in vitro* is present *in vivo*. The most precise information about such patterns is obtained from "footprinting" experiments in which the differential resistance to attack by nucleases, or other reagents that cleave DNA, is used to define the domain of binding. It would obviously be desirable to compare the altered cleavage pattern obtained from complexes formed *in vitro* with the pattern of factors bound to the same DNA domain within the nucleus.

One technique capable of providing such information is the genomic sequencing method described recently by Church and Gilbert (8). In this method, fragmented genomic DNA is electrophoresed, transferred to an inert support, and indirectly end-labeled by hybridization to appropriate short radioactive DNA probes. A second technique, which identifies the outer boundaries of protein protection by digestion with exonuclease III, has been described by Wu (9, 10).

In this paper, we present a different method for defining cleavage patterns on native nuclear structures related to the S1 nuclease mapping technique developed by Berk and Sharp (11), but modified to give the greatly increased sensitivity and specificity necessary for the detection of short DNA sequences, each of which may be present at <0.01 times the genomic abundance. The procedure makes use of long single-stranded terminally labeled DNA probes, which are annealed to DNA fragments derived from nuclear digests. The resulting duplexes are heated to eliminate nonspecific hybridization, digested with a single-strand-specific nuclease, electrophoresed, and the gel is autoradiographed. Cleavages in genomic DNA are thereby mapped relative to the probe end label in hybrid molecules.

With this procedure, we have mapped the DNase I digestion pattern in the 5′ flanking region of the chicken adult β (β^A)-globin gene in the nuclei of adult erythrocytes. This region has been shown to contain a domain, about 200 base pairs (bp) long, that is hypersensitive to nucleases (12). Comparison with patterns obtained on protein-free DNA, or in nuclei from other tissues, reveals within the hypersensitive domain defined regions that are protected against DNase I attack and that probably reflect the presence of bound nonhistone proteins.

METHODS

Probe Preparation. Cloning in M13 mp18 (P-L Biochemicals) vector and preparation of single-stranded phage DNA were carried out by established procedures (13). Appropriate fragments from the neighborhood of the chicken β^A-globin gene were subcloned into these vectors from the plasmid pCAβG1 (14). The probe (see Fig. 3*B*) was prepared by primed synthesis using the large fragment of *Escherichia coli* DNA polymerase I, with single-stranded m18BX phage DNA as template. Approximately 4 μg of phage DNA is required for each 250 μg of genomic DNA analyzed (5000-fold molar excess of cloned sequence over its genomic complement). Primed template was prepared by mixing a 2.5-fold molar excess of 17-nucleotide sequencing primer (New England Biolabs) with template at a DNA concentration of 0.167 g/liter in 0.083× PBC (1× PBC is 0.5 M NaCl/70 mM Tris·HCl, pH 7.5/70 mM $MgCl_2$). The mixture was incubated at 90°C for 5 min, then at 37°C for 1 hr, frozen on dry ice, and stored frozen until use. The primer is chosen so that its 3′ end is 70–100 nucleotides from the site of interest in the genome, and outside any nuclease-hypersensitive region.

Labeling of probe within the genomic sequence was confined to 10–20 nucleotides at the 5′ terminus (for m18BX: 17-base unlabeled primer, 20-base ^{32}P-labeled M13 sequence, and 11- to 13-base ^{32}P genomic end label), by incubating first with limiting amounts of [α-^{32}P]dNTPs, and taking advantage of secondary structure in the template to provide stopping points in synthesis (see *Results* and Fig. 2). We found it necessary to adjust labeled dNTP concentrations over a range of ≈2-fold for various templates to obtain suitable end labeling. The probe used here was synthesized by adding primed template (4 μg in 24 μl) to 89.3 μl of a mixture containing 24.8 pmol each of α-^{32}P-labeled dATP, dCTP, dTTP, and dGTP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear), 0.102× PBC, 0.15 mM 2-mercaptoethanol, and 6.5

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: bp, base pair(s).

units of large fragment DNA polymerase I (Bethesda Research Laboratories). After incubation at 20°C for 1 hr, the probe strand was extended through the remaining genomic sequence and into adjacent M13 vector sequence with greatly reduced label incorporation by adding 20 μl of a mixture containing 10.6 nmol each of the four unlabeled dNTPs in 0.08× PBC/0.13 mM 2-mercaptoethanol/0.33 unit of DNA polymerase I large fragment per μl. Incubation was continued at 20°C for 20 min, at 37°C for 10 min, and, finally, at 68°C for 10 min, to denature polymerase.

The probe–template complex was digested with a restriction endonuclease chosen to create a large size difference between the newly synthesized probe and its M13 template. For the m18BX probe, 50 units of *Eco*RI endonuclease, 20 μl of buffer [0.5 M NaCl/1 M Tris·HCl, pH 7.5/50 mM $MgCl_2$/1 g of bovine serum albumin per liter (Bethesda Research Laboratories)], and H_2O to 200 μl were added to the above reaction mixture and incubated for 1 hr at 37°C. The reaction was stopped, and the DNA was purified by extraction with neutralized phenol and chloroform, concentrated with *n*-butanol, and precipitated with an equal volume of isopropanol at room temperature. The pellet, after washing with 70% aqueous ethanol, was dried and resuspended in 7 μl of formamide, heated at 68°C for 10 min, and the strands were separated by electrophoresis on a 1.5-mm thick gel (5% acrylamide/0.25% bis-acrylamide) containing 45 mM tris borate, pH 8.3/1.25 mM Na_2EDTA/9 M urea. The sample was electrophoresed at 30–40 V/cm (the gel is hot) for 2 hr. The gel was stained with ethidium bromide, and the probe band was cut out and electroeluted onto DEAE paper (Whatman DE81) as described (15), except that the gel slice containing the probe band was isolated and completely wrapped in the paper. After eluting the DNA from the paper at 50°C with two 2-ml aliquots of 2.5 M NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA/50 μg of sonicated calf thymus DNA per ml, the eluate was centrifuged through a 0.45-μm pore size cellulose acetate filter (Centrex, Schleicher & Schuell) concentrated to 0.4 ml by filtration (Centricon 30, Amicon), adjusted to a NaCl concentration of 1.5 M, and isopropanol precipitated. The probe was pure enough at this point to examine restriction digests containing the chicken globin sequences at 0.1× genomic abundance.

The probe was purified further to remove double-stranded molecules. The probe was dissolved in 120 μl of 10 mM Tris·HCl, pH 8/1 mM EDTA (TE buffer), and 13.33 μl of 10× hybridization buffer was added (1× hybridization buffer = 0.318 M NaCl/20 mM Na Hepes, pH 7.5/0.1 mM EDTA). This was sealed in a glass capillary, heated at 107°C for 5 min, then transferred directly to a 68°C bath for 8–10 hr. The mixture was diluted into 0.2 ml of sonicated calf thymus DNA (0.2 g/liter) and applied to a 0.5-ml column (0.86 × 0.86 cm) of hydroxyapatite (Bio-Rad) equilibrated with 50 mM sodium phosphate (pH 7) and maintained at 68°C. The column was washed with 15–20 ml of the equilibration buffer, then eluted with 150 mM sodium phosphate (pH 7). The probe was eluted in ≈1 ml. The sample was desalted and concentrated to a final volume of 100 μl (Centricon 30 filter). The probe was precipitated, again denatured in formamide, and repurified on a denaturing gel as described above. The faintly stained band could be localized with a Geiger counter. The band was eluted and purified as described above, but was taken up in a final volume of 65 μl of TE buffer. This step served to remove accumulated radiolytic degradation products; the probe was used immediately, because storage degraded the signal/noise ratio.

Genomic Analysis. Nuclei were prepared from adult and 5-day-old embryonic erythrocytes according to previously used methods (12, 16) and from adult oviduct by a modification of the methods of Palmiter (17). Nuclei were digested with DNase I, and the DNA was purified (16) and treated with RNase A (Worthington; 0.1 g per g of DNA) that had been incubated at 100°C to inactivate DNase. Samples were incubated in TE buffer (1 hr, 37°C), then the solvent was adjusted to 60 mM NaCl/6 mM Tris·HCl, pH 7.5/6 mM $MgCl_2$/6 mM 2-mercaptoethanol/0.1 g of bovine serum albumin per liter, and 2.5 units of the restriction endonuclease *Pvu* II was added per μg of DNA; incubation was continued for 2 hr at 37°C. The DNA was repurified as described above. This restriction reduces the size of the genomic sequence, limiting the ability of the cold genomic strand to compete with the probe strand, which is in 15- to 20-fold excess, for hybridization to their genomic complement.

Hybridization of single-stranded 5′-end-labeled probe with genomic DNA was carried out by mixing 0.05 ng ($1.5–2.0 \times 10^5$ dpm) of probe with 25 μg of genomic DNA in 16 μl of 1× hybridization buffer, sealing the mixture in a glass capillary, heat denaturing (5 min, 107°C), and promptly transferring to a 68°C bath for a 10- to 12-hr hybridization. Genomic DNA is 60%–70% renatured, and hybridization to probe is complete in this time. The reaction was stopped by expelling the mixture into 0.7 vol of TE buffer. After isopropanol precipitation, 70% ethanol wash and drying, the pellet was dissolved in 15.6 μl of 15 mM NaCl/1.5 mM Na Hepes, pH 7.5. Nonspecific hybridization of the probe to related sequences in the genome was eliminated by incubation of the low ionic strength solution of DNA at 70°C for 30 min. This step is essential (see *Results*).

The hybridized samples were digested with mung bean nuclease to remove single-stranded regions from the hybrids, as well as unhybridized probe. To the sample was added 6.5 units of mung bean nuclease (P-L Biochemicals) in 10.4 μl of 125 mM sodium acetate, pH 5.3/2.5 mM magnesium acetate/2.5 mM L-serine/2.5 mM 2-mercaptoethanol/0.0125% Triton X-100 (Sigma)/0.25 mM zinc acetate (18). The sample was incubated at 40°C for 1 hr, and the reaction was stopped by addition of 2.9 μl of 2.5 M NaCl/0.5 M Tris·HCl, pH 8.5, followed by isopropanol precipitation. The dried sample was dissolved in 3 μl of 10 mM EDTA, pH 8.0/6% Ficoll/0.06% xylene cyanol FF (Fisher), loaded in a 0.8 × 5 mm well on a 0.8 × 330 × 380 mm gel (8% acrylamide/0.4% bis-acrylamide), and electrophoresed in a buffer containing 89 mM Tris borate, pH 8.3/2.5 mM Na_2EDTA, at 18 V/cm for 7 hr. The gel was dried onto DE81 paper and autoradiographed for 7–14 days at −80°C, using Kodak XAR-5 film and a DuPont Cronex Lightning Plus intensifying screen (19).

Protein-Free DNA and Restriction Standards. Ten aliquots of purified chicken erythrocyte DNA were digested separately to completion with the enzymes *Apa* I, *Sma* I, *Dde* I, *Ban* II, *Mbo* I, *Hin*fI, *Alu* I, *Hha* I, *Fnu*DII, and partially with *Apa* I. The DNA samples were purified, combined, and treated with RNase A and *Pvu* II as described above. This mixture was hybridized with probe and treated with mung bean nuclease as described above to provide size standards and internal controls.

DNase I-treated protein-free DNA for the region being examined was obtained by digestion of plasmid pSBD4 (3), which contains the region of interest. The digest was treated with RNase A and *Pvu* II as described above, and mixed with chicken DNA to give a plasmid globin sequence concentration 4 times the genomic abundance. The mixed samples were hybridized at $7.5–10 \times 10^5$ dpm of probe per 25 μg of chicken DNA. Stringency wash and mung bean nuclease digestion conditions were the same as for genomic samples. Only 10 μg of DNA per lane was loaded on the gel to equalize plasmid and genomic globin sequence concentrations. Plasmid DNA was used for these controls because the size distribution of bulk DNA in the mixture of chicken DNA and DNase I-digested plasmid is not radically different from the size of the DNase I digests from erythrocyte or oviduct nuclei (average, $5–100 \times 10^3$ nucleotides). On the other hand,

DNase I digests of protein-free chicken DNA having the appropriate number of cuts in the neighborhood of the globin gene would have an average size of only 100–300 nucleotides, inappropriate for use as a control, because in the latter case much more single-stranded DNA would be present during the mung bean nuclease digestion.

RESULTS

Observation of DNase I protection at the genomic level requires the ability to map cuts in DNA uniquely, and with the high sensitivity necessary to detect low concentrations of DNA fragments. The method described here achieves these ends by using a 5′-end-labeled single-stranded probe hybridized to genomic DNA, obtained in the examples described below by partial digestion of nuclei with DNase I.

The single-stranded probe is prepared by primed synthesis on an M13 single-stranded template containing the cloned genomic sequence of interest. The synthesis is carried out as a pulse–chase with the goal of incorporating 10–20 α-^{32}P-labeled nucleotides of high specific activity at the 5′ end of the probe, followed by unlabeled nucleotides in the remaining probe sequence (Fig. 1). We accentuate existing polymerase pause sites by adjustment of limiting [α-^{32}P]dNTP concentrations, making it easy to confine the label to the 5′ terminus (Fig. 2). (Palindromes are an effective but not a necessary element of pause sites.) The end label is extended with unlabeled dNTP, and the resultant probe is purified to remove the M13 template as well as degraded probe.

The genomic DNA is digested with a restriction endonuclease to reduce its size before hybridization, carried out in considerable probe excess. After hybridization, the samples are incubated at increased temperature to denature nonspecific hybrids. This step is analogous to the "high stringency wash" step in Southern blotting (20), and the considerations governing the choice of conditions are the same for both: Higher G+C content (m18BX probe is ≈70% G+C) and greater homology to repeated sequences dictate incubations at higher temperature. Omission of this step is a potential source of artifact.

After heating, the complex is digested with mung bean nuclease under conditions that eliminate single-stranded DNA while preserving hybrids. The pattern of resistant duplexes is insensitive to a small change in enzyme activity (increase by a factor of <4 or decrease by a factor of 2; unpublished data). A mixture of chicken DNA restriction fragments assayed in parallel with the sample provides both size standards and an internal control for such changes.

These standards (Fig. 3*A*, lanes d, k, and r) show that the radioactively labeled bands generated by our procedures correspond as expected to duplexes extending from the 5′ end label of the probe to the first restriction endonuclease cut in the complementary genomic strand. More than 95% of the radioactivity in these controls is in bands of size corresponding to the known restriction sites on chicken DNA. As

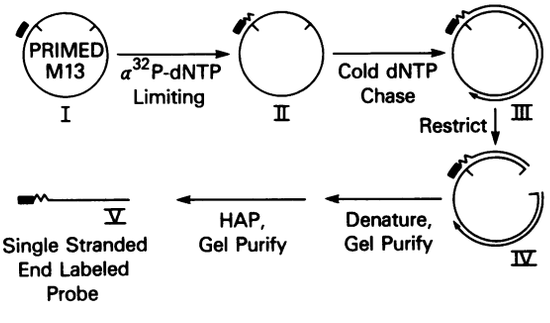

FIG. 1. Preparation of 5′-end-labeled single-stranded probe.

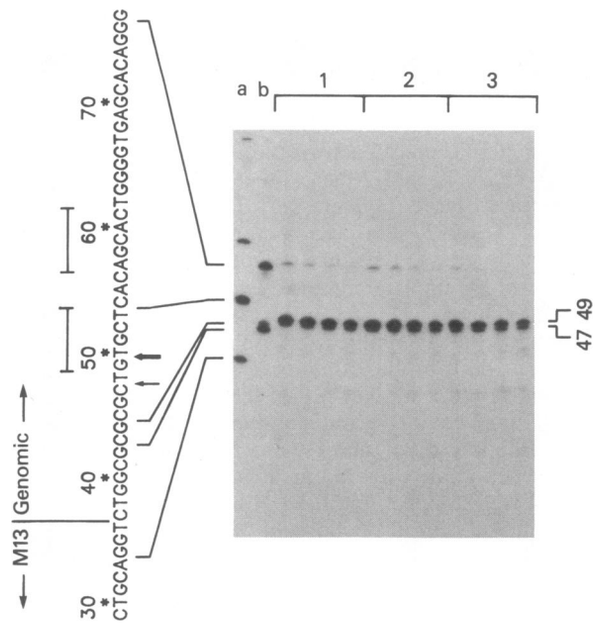

FIG. 2. Extent of 5′ end label is controlled by template structure and limited availability of [α-^{32}P]dNTP. Primed synthesis reactions on template m18BX were performed as described, except that incubation time and amount of [α-^{32}P]dNTP were varied, and a chase with unlabeled excess dNTP was omitted except in lanes a and b. Reaction mixtures 1, 2, and 3 contained 27.9, 26.4, and 22.7 pmol, respectively, of each [α-^{32}P]dNTP. Incubation time for each reaction was (from right to left) 25, 40, 60, and 90 min, at which time an aliquot was taken and denatured in formamide for gel analysis. Lanes a and b represent separate restriction digests of reaction 1 chased with unlabeled dNTP (lane a, separate *Pst* I and *Hgi*AI digests; lane b, separate *Hha* I and *Ban* I digests) and denatured in formamide for gel analysis on 12.5% acrylamide/0.625% bisacrylamide containing Tris borate buffer and 9 M urea. Note that the 47 and 49 base markers at the right of the gel and the arrows on the sequence correspond to stop points that represent labeled synthesis extending 11 and 13 bases, respectively, into the genomic sequence. Bases are numbered starting from the 5′ terminus of the primer. Lines above the sequence mark a palindrome.

expected, genomic fragments with a 3′ terminus that is 3′ of the labeled region of the probe (e.g., predicted bands at 118, 138, and 174 bp) are not seen. [Minor bands, seen in the absence of DNase I digestion (e.g., lane 1), arise from the action of endogenous nucleases.] Thus, the technique faithfully maps the distance from the 5′ end of the probe to the nearest internal cut in the 3′ direction.

To demonstrate the resolution of this technique, we have analyzed the region extending from −6 to −220 nucleotides upstream from the start of transcription in the chicken β^A-globin gene. This lies within the domain found (12) to be hypersensitive to nucleases in nuclei isolated from erythrocytes (9-day-old embryo to adult) with a history of β^A-globin gene expression; no hypersensitivity is seen in cells in which the gene has never been expressed, such as oviduct or 5-day-old embryonic erythrocytes.

A partial sequence of the region and the relative positions of the probe and genomic sequences are shown in Fig. 3 (12). The probe will detect cuts in the poly(dC)-containing strand. The pattern of DNase I cutting in adult erythrocyte nuclei, mapped by our technique, is shown in Fig. 3*A* (lanes m–q). It must be compared with the pattern of cuts obtained on protein-free DNA (lanes t–w). There is no evidence of protection against DNase I action in most of the region between nucleotides −6 and −220, but strong protection is observed within the region bounded by nucleotides −136 and −163, and less clearly in the region bounded by nucleotides −175 to −200. The latter region contains the poly(dC)-string; pro-

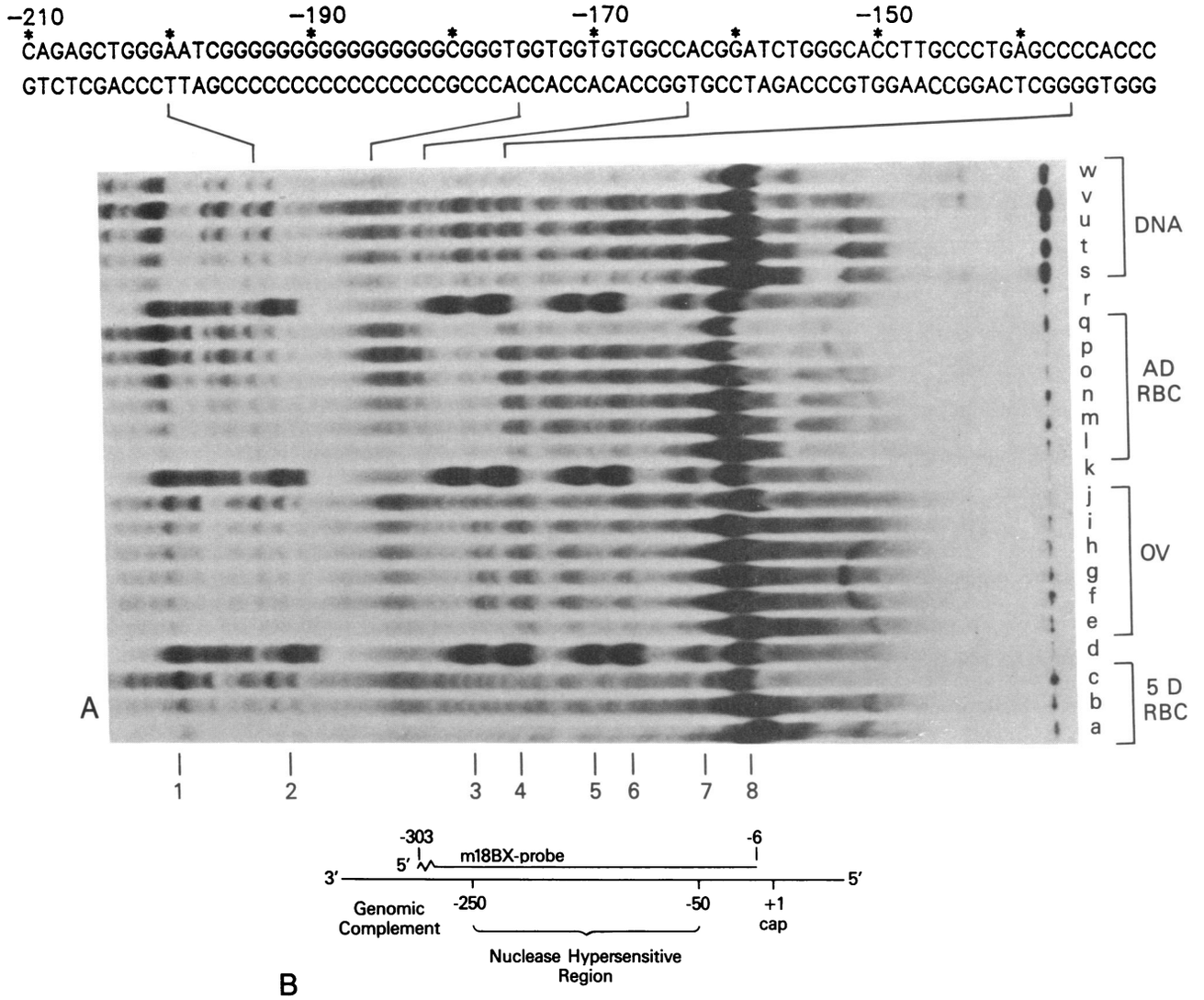

FIG. 3. (*A*) Determination of DNase I cutting patterns near the 5′ end of the chicken β^A-globin gene in protein-free DNA and in nuclei both from adult erythrocytes (AD RBC) (in which the gene is expressed) and from oviduct (OV) and 5-day-old embryonic erythrocytes (5 D RBC) (in which the gene is transcriptionally inactive). Digestion with DNase I was in 2.5-fold concentration increments, except for lanes e, l, and s, which are controls omitting DNase I for protein-free DNA, adult erythrocyte, and oviduct, respectively. Protein-free DNA (at 0.06 g/liter) and nuclei (at 1 g of DNA per liter) were digested 30 min at 20°C in 50 mM NaCl/10 mM Na Hepes, pH 7.5/5 mM sodium butyrate/5 mM $MgCl_2$/1 mM $CaCl_2$ at ranges of DNase I as follows: lanes t–w, 4.9–77 ng of DNase I per mg of pBD102 (see *Methods*); lanes m–q, 4.6–180 ng of DNase I per mg of adult erythrocyte DNA; lanes f–j, 71–2780 ng of DNase I per mg of oviduct DNA; and lanes a–c, 445–2780 ng of DNase I per mg of 5-day-old erythrocyte DNA. Reactions were stopped and the DNA was purified and analyzed for nuclease cuts in the genomic complement of the m18BX probe. Size standards (lanes d, k, and r) represent mixed restriction enzyme digests of chicken DNA analyzed as described above with the m18BX probe. Sizes (and position of cut relative to mRNA cap site) of bands numbered 1–8 are as follows: 90 bp (−213), 106 bp (−197), 147 bp (−156), 163 and 164 bp (−140 and −139), 194 bp (−109), 211 bp (−92), 260 bp (−43), and 299 bp (−6). Electrophoresis of DNA was from right to left. Exposure time was 10 days. β^A-globin mRNA *in vivo* displays more than one initiation start site (21). One of these is at the cap site position reported earlier (12). We have continued to use this site to define position +1 in the sequence shown. (*B*) Relationship of m18BX probe to its complementary genomic sequence.

tection can be seen at its edges (−178 and −197), especially in relation to the strong unprotected pattern immediately outside those points.

When the same analysis is applied to DNase I digestion products of oviduct nuclei, a different result is obtained. A comparison of lanes o and p (adult erythrocytes) with lane j (oviduct) shows that, consistent with its nuclease hypersensitivity, erythrocyte chromatin is 40 to 100 times more sensitive to DNase I than is oviduct chromatin, despite the presence in erythrocyte chromatin of the two protected regions, described above, within the hypersensitive domain. Although the pattern of protection found in erythrocytes is absent in oviduct chromatin, several areas of weak protection, overlaid on the background of reduced sensitivity, are found at −101, −151, and −205. This additional weak protection is eliminated at the highest level of DNase I digestion of oviduct chromatin (lane j), with the exception of the protection at −205, which remains.

The small number of experiments carried out with 5-day-old embryonic erythrocyte nuclei (Fig. 3*A*, lanes a–c) show overall resistance to nuclease attack, and little if any specific protection of the kinds seen either in adult erythrocyte or oviduct nuclei.

DISCUSSION

The procedure we have described permits the mapping at high resolution of cleavages induced in specific DNA sequences within the nucleus by nucleases or other agents. The method is related to the procedure of Berk and Sharp

(11) for locating sites of RNA chain initiation, and to techniques for determining the size distribution of genomic DNA fragments in nuclear digests that involve hybridization of genomic DNA to uniformly labeled probes, followed by S1 nuclease digestion (22, 23). However, in our procedure, we obtain greatly increased sensitivity and specificity by the use of end-labeled probes, a high temperature incubation step to eliminate nonspecific hybridization, and then careful control of both hybridization and mung bean nuclease digestion.

We estimate that the resolution of the technique is approximately ±2 nucleotides in the most highly resolved portion of the gel; the limiting factors are the precision of action of the single-strand-specific nuclease, and the extent to which degradation of the multiply end-labeled probe by radioactive decay broadens the bands. Although this resolution is not sufficient to permit complete genomic sequence analysis (8), it is quite satisfactory for analysis of altered genomic nuclease digestion patterns and other applications in which single base resolution is unimportant. The method we have described has the advantage that the genomic DNA is not transferred from the gel to another support, so that the problems of poor transfer and solid-phase hybridization efficiency of small DNA are avoided, and none of the special equipment used for such transfers is required. Furthermore, the limitation of radioactive labeling to only a small number of nucleotides at the 5′ end of the probe assures correct assignment of cutting sites, even at levels of digestion that result in multiple cuts in the region being probed.

As a first application of this procedure, we have mapped the nuclease protection provided by factors bound *in vivo* to the 5′ flanking region of the chicken β^A-globin gene. Previous work in this laboratory has shown that in nuclei from adult erythrocytes the domain extending from about −70 to −270 bp from the origin of transcription is unusually sensitive to nucleases and does not have a normal nucleosome bound to it (12). Furthermore, reconstitution and filter binding studies suggest that protein factors present in chicken erythrocyte nuclei bind to this domain and may function to maintain it in a state of hypersensitivity to nucleases (3).

The pattern in Fig. 3*A* (lanes m–q) shows that within the adult erythrocyte nucleus, a portion of the β^A-globin hypersensitive domain is resistant to attack by DNase I. One protected sequence (region 1) is within the region bounded by nucleotides −136 and −163; it includes a long palindromic sequence (9 bp in 25 nucleotides). With somewhat less certainty we detect protection of a second sequence (region 2) bounded by nucleotides −175 and −200, which contains the string of 16 dC residues. As will be reported elsewhere, DNase I protection experiments with reconstituted complexes of cloned DNA and partially purified chicken erythrocyte nuclear factors reveal that both of these regions are strongly protected.

A different pattern is obtained in nuclei isolated from oviduct or 5-day-old embryonic erythrocytes. In the latter case, the DNase I cutting pattern is quite similar to that of naked DNA; no special protection is seen. In oviduct, it may be that the 5′ portion of region I is protected (Fig. 3*A*, lanes f–i), but at the most extensive DNase I digestion all of the potential DNase I cutting sites are accessible (lane j), making that conclusion equivocal. A detailed analysis of the structure of the 5′ hypersensitive region in oviduct and other tissues will be presented elsewhere. We conclude that if there is a pattern of protection in oviduct, it is quite different from that in adult erythrocytes.

In interpreting these results, it should be kept in mind that the amount of enzyme necessary to achieve comparable extents of digestion is at least 40 times greater for nuclei from oviduct or 5-day-old embryonic erythrocytes than it is for nuclei from adult erythrocytes, consistent with the association of nuclease hypersensitivity only with 9-day-old to adult erythrocytes (12). It is not paradoxical that a pattern of protected sequences exists within a hypersensitive domain: Hypersensitivity is measured relative to the reduced sensitivity of adjacent nucleosome-covered DNA, while the pattern measures protection of discrete sequences within the hypersensitive domain.

There is evidence for the presence of nonhistone proteins in other hypersensitive domains (9, 24). In a different kind of experiment, Wu has used exonuclease III to map the boundaries of protein complexed to the hypersensitive domain in the 5′ flanking region of *Drosophila* heat shock genes and has shown that protein factors present in heat shocked cells can be bound *in vitro* to this domain (9, 10).

The technique we describe here should provide a quite general method for mapping the binding sites of nonhistone proteins to DNA within the nucleus. It should also prove useful in detecting nucleosome phasing *in vivo* and in locating sites of S1 nuclease cleavage. An approach similar to ours has, in fact, been used (H. Weintraub, personal communication) for this purpose.

Our initial result obtained with this method reveals that specific sequences within the hypersensitive domain of β^A-globin gene are covered, and that this pattern of protection is absent or highly modified in nuclei in which the gene is not expressed, and in which hypersensitivity is absent. The information provided by such an analysis, particularly when accompanied by *in vitro* studies with partially purified DNA binding factors, should help us to understand how hypersensitive sites are generated and how their structure is related to the function of expressed genes.

We thank Dr. Takeshi Kimura for help in preparation of 5-day-old embryonic erythrocytes, and Ms. Betty Canning for expert preparation of the manuscript.

1. Delucia, A. L., Lewton, B. A., Tjian, R. & Tegtmeyer, P. (1983) *J. Virol.* **46**, 143–150.
2. Tegtmeyer, P., Lewton, B. A., DeLucia, A. L., Wilson, V. G. & Ryder, K. (1983) *J. Virol.* **46**, 151–161.
3. Emerson, B. M. & Felsenfeld, G. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 95–99.
4. Parker, C. S. & Topol, J. (1984) *Cell* **37**, 273–283.
5. Payvar, F., DeFranco, D., Firestone, G. L., Edgar, B., Wrange, O., Okret, S., Gustafsson, J.-A. & Yamamoto, K. R. (1983) *Cell* **35**, 381–392.
6. Rawlins, D., Rosenfeld, P. J., Wides, R. J., Challberg, M. D. & Kelly, T. J., Jr. (1984) *Cell* **37**, 309–319.
7. Dynan, W. S. & Tjian, R. (1983) *Cell* **35**, 79–87.
8. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1991–1995.
9. Wu, C. (1984) *Nature (London)* **309**, 229–234.
10. Wu, C. (1984) *Nature (London)* **311**, 81–84.
11. Berk, A. J. & Sharp, P. A. (1977) *Cell* **12**, 721–732.
12. McGhee, J. D., Wood, W. I., Dolan, M., Engel, J. D. & Felsenfeld, G. (1981) *Cell* **27**, 45–55.
13. Messing, J. (1983) *Methods Enzymol.* **101**, 20–78.
14. Wood, W. I., Nickol, J. & Felsenfeld, G. (1981) *J. Biol. Chem.* **256**, 1502–1505.
15. Dretzen, G., Bellard, M., Sassone-Corsi, P. & Chambon, P. (1981) *Anal. Biochem.* **112**, 295–298.
16. Wood, W. I. & Felsenfeld, G. (1982) *J. Biol. Chem.* **257**, 7730–7736.
17. Mulvihill, E. R. & Palmiter, R. D. (1977) *J. Biol. Chem.* **252**, 2060–2068.
18. Laskowski, M., Sr. (1980) *Methods Enzymol.* **65**, 263–275.
19. Swanstrom, R. & Shank, P. R. (1978) *Anal. Biochem.* **86**, 184–192.
20. Southern, E. (1979) *Methods Enzymol.* **68**, 152–176.
21. Dolan, M., Dodgson, J. B. & Engel, J. D. (1983) *J. Biol. Chem.* **258**, 3983–3990.
22. Camerini-Otero, R. D., Sollner-Webb, B., Simon, R. H., Williamson, P., Zasloff, M. & Felsenfeld, G. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 57–75.
23. Bloom, K. S. & Anderson, J. N. (1982) *J. Biol. Chem.* **257**, 13018–13027.
24. Borgmeyer, U., Nowack, J. & Sippel, A. E. (1984) *Nucleic Acids Res.* **12**, 4295–4311.