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The histone H5 gene is flanked by S1 hypersensitive structures

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ABSTRACT

The potential of the cloned histone H5 gene to form altered DNA structures has been examined by S1 nuclease digestion of supercoiled recombinant plasmids containing up to 8.8 kbp of chicken DNA. The three main nicking sites map at the upstream and downstream sequences flanking the structural gene. The cleavage sites share sequence homology, strand specificity, and do not seem to be single-stranded. The sequence of the S1-sensitive sites does not suggest that the fragments can adopt any of the known DNA secondary structures .

INTRODUCTION

An early event in the process of gene activation is a change in chromatin structure that renders the activated gene more susceptible to nucleases (for reviews see refs. 1-3). The general increase in sensitivity is hyphenated by nuclease hypersensitive sites that usually map in the regions flanking the genes, generally at or close to promoter and possible regulatory regions. The alteration of the chromatin structure at these sites could be the result of binding of regulatory and/or transcriptional factors that may exclude and/or affect the structure of a nearby nucleosome(s) (4-7). Besides the specific DNA sequences necessary to bring about recognition by the factors involved in transcription, it is reasonable to think that these or other sequences may contain structural information that may aid in the process of sequence recognition.

In the recent years, S1 nuclease has been used as a probe for altered secondary structure of cloned DNA. Under certain conditions, S1 nuclease has been shown to cleave at the junction between right- and left-handed DNA (8, 9), at palindromic sequences that have the potential to form cruciform structures

(10, 11) , at homo- and simple co-polymeric sequences (12-14) that may slip out of register, at AT-rich regions which have a higher tendency to melt (11), and at other types of sequences (13-17) whose potential to form altered secondary structure is not yet known.

In this article I describe the S1 nuclease hypersensitivity of the cloned chicken histone H5 gene and flanking regions (18), and the identification of the sequences cleaved. In addition, I show how the conditions of digestion influence the S1 nuclease cleavage pattern, and compare these patterns with those produced by micrococcal nuclease and DNase I.

MATERIALS AND METHODS

Materials

All enzymes used were purchased from Boehringer, BRL, Biolabs, and New England Nuclear. [α-^{32}P]dNTPs {$4x10^3$ to $6x10^3$ Ci/mmol} and [γ-^{32}P]-ATP {$5x10^3$ to $7x10^3$ Ci/mmol} were purchased from Amersham and New England Nuclear.

Plasmid DNA

The construction of pchV2.5B/H has been described (18). Plasmid pchV8.8HR was constructed by ligation of the Hind III 8.8 kbp DNA fragment of λchV (18) into the Hind III site of pAT 153 (19). The cloned DNA of pchV8.8HR begins at the same Hind III site that pchV2.5B/H but extends an additional 6.3 kbp downstream of the H5 gene. The two cloned DNA fragments have the same orientation with respect to the vector.

Endonuclease digestions

Nuclease digestions were carried out at a DNA concentration of 0.2 mg/ml. The buffers used were: 30mM sodium acetate [pH 4.6-6.0], 1mM $ZnCl_2$ for S1; 10 mM Hepes [pH 7.5], 0.4 mM $MgCl_2$, 0.004 mM $CaCl_2$ for DNase I; 10 mM Hepes [pH 7.5], 0.4 mM $CaCl_2$ for micrococcal nuclease [MNase]. Restriction endonuclease digestions were carried out as recommended by the manufacturers.

Mapping procedures

S1 cleavage sites in plasmid DNA were mapped by indirect end-labeling (20). Southern blots (21) were hybridized with cloned DNA fragments labeled by nick-translation (22). Prior to electrophoresis, the samples [25-50 ng] were diluted with salmon

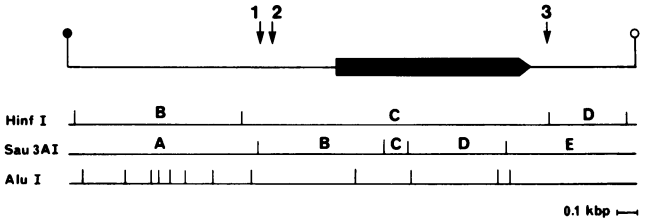

Figure 1. Physical map of the chicken histone H5 gene cloned in plasmid pchV2.5B/H. The position of the structural sequences and the polarity of transcription is indicated by a pointed black bar. Sequences flanking the gene are indicated by a thin line . The arrows point to the S1 nuclease hypersensitive sites. Hind III (●); Bam HI (○). The cleavage sites of the restriction nucleases used for the mapping the S1 hypersensitive sequences are indicated.

sperm DNA [5 µg] to prevent artifacts due to the dependency of the electrophoretic mobility on DNA concentration. DNA was separated in Ø.8-Ø.9% agarose gels or in 5% polyacrylamide gels depending on the length of the fragments analyzed. DNA fragments were labeled at the 5'-end by T_4 polynicleotidyl kinase and [y-^{32}P]-ATP after alkaline phosphatase treatment (23). Fine mapping of the S1 nuclease cleavage sites was carried out in 6-12% polyacrylamide DNA sequencing gels (24). The rest of the methods used have been previously described (18).

RESULTS

S1 nuclease hypersensitive sites flanking the H5 gene.

The sensitivity to S1 nuclease of the histone H5 gene region was analyzed by using the recombinant plasmid pchV2.5B/H [Fig.1]. This plasmid contains a 2.6 kbp fragment of chicken DNA, encompassing the entire mRNA coding region [884 bp] and 1.7 kbp of flanking sequences, inserted in the Hind III- Bam HI sites of pAT 153 (18). When this plasmid is digested with S1 nuclease, its DNA is converted from form I [supercoiled] to nicked form II [nicked circles] and eventually to form III [linear] . In the experiments dealing with the mapping of nuclease hypersensitive sites of molecules cleaved at the two strands, the digestion was allowed to proceed until approximately 2Ø-3Ø% of the DNA was converted to form III [not shown]. After the digestion, the DNA was cut with Hind III,

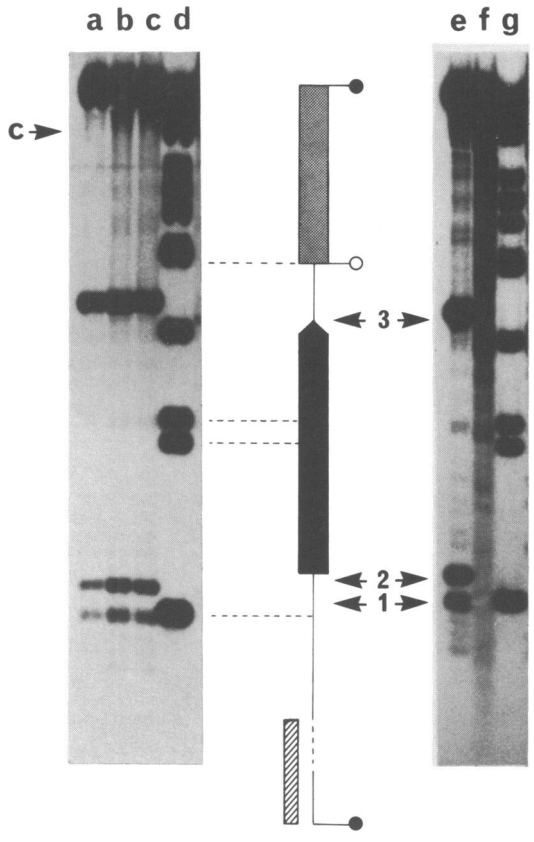

Figure 2. Mapping of the cleavage sites of S1 nuclease in molecules cut at the two strands. Autoradiography of a Southern blot hybridized with fragment Hinf I B [cf Fig.1], indicated by the cross-hatched bar. Supercoiled [a,b,c,e] and Hind III linearized [f] pchV2.5B/H DNA was digested with 50 units/ml of S1 at 37°C in S1 buffer [pH 4.6] containing 30 mM NaCl. Digestion times were [a,e] 1, [b] 3, [c] 5, and [f] 10 minutes,respectively. [d,g] Sau 3AI partial digestion of pchV2.5B/H. DNA samples were digested with Hind III before electrophoresis. pAT 153 sequences are indicated by the stippled bar. Other symbols as in Fig. 1.

fractionated by electrophoresis, and blotted to nitrocellulose filters . The S1 cleavage sites were identified by indirect end-labeling (20) using fragment Hinf I-B [Fig.1] as a probe .

Under low salt conditions [30 mM NaCl], S1 cleaves pchV2.5B/H DNA in a remarkably specific manner [Fig.2, a-c, e]. The three prominent sites map approximately 290 and 370 bp upstream of the

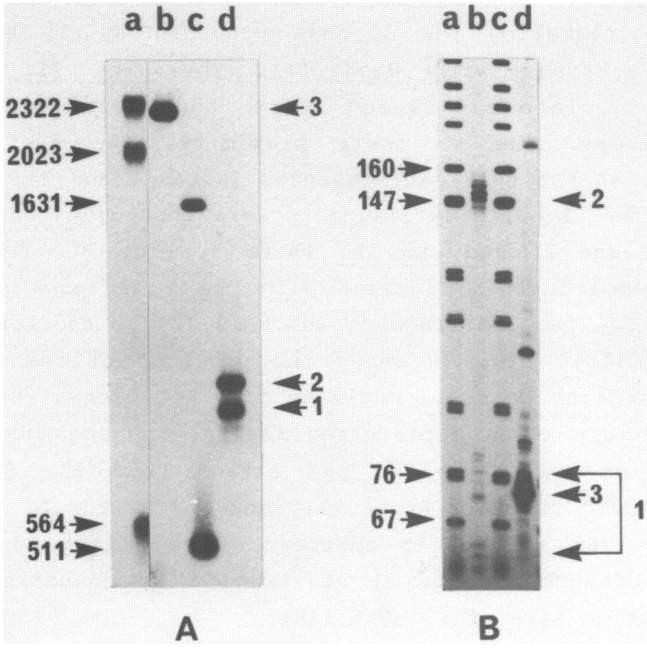

Figure 3. Fine mapping of the cleavage sites of S1 nuclease in molecules cut at the two strands. [A]: Autoradiography of purified 5'-end labeled Hind III-S1 nuclease fragments originating at the upstream [d, sites 1 and 2], and downstream [b, site 3] cleavage sites of pchV2.5B/H. [a] 5'-end labeled Hind III fragments of 1; [c] 5'-end labeled Hinf I fragments of pBR 322. DNA was run in a 0.8% agarose gel. [B]: Autoradiography of a 8% polyacrylamide DNA sequencing gel; [a, c] 5'-end labeled Hpa II fragments of pBR 322, [b] Hinf I digest of the purified Hind III-S1 fragments [sites 1 and 2], [d] Sau 3AI + Dde I double digest of the purified Hind III- S1 fragment [site 3]. Only the relevant part of the gel and lengths of the markers is shown.

capping site [sites 1 and 2] and 80 bp downstream of the site of polyadenylation of H5 mRNA (18) [site 3]. The intensity of the autoradiography signals is a measure of the relative frequency at which S1 cleaves each site, since once the DNA has been relaxed by the first nick the nuclease does not recognize the same sites [Fig.2, f]. Thus, it is noteworthy that the frequency of cutting at site 3 is five- to eight-fold higher than that at the two upstream sites. Note also that under the conditions of digestion, S1 does not cleave at the possible cruciforms of pBR 322 (10, 11) [Fig.2, c-->].

A more precise determination of the hypersensitive sites was achieved by labeling the 5'-ends produced by S1 before the secondary cleavage with Hind III. The Hind III fragments containig a labeled S1-end were then purified by gel electrophoresis. Due to their proximity, the two fragments originating at the upstream sequences [sites 1 and 2] were taken together [Fig. 3A]. These fragments were then digested with Hinf I [sites 1 and 2] and Sau 3AI [site 3, Fig. 1]. The Sau 3AI fragments were further digested with Dde I because this enzyme produces a S1- labeled fragment which is 105 bp shorter than the former one (18). Fig. 3B shows the products of the digestions run in denaturing gels. S1 nuclease does not cleave the DNA at a single site but along a preferred region. The sequence of these sites reading the mRNA-like DNA strand from the 5'-end is: CCTGCACATTACCTCCTTGTCTC [site 1] and ATCACATCTCTC [site 2] starting at 365 and 287 bp upstream of the first capping site [+1], and GTAAGGAGG [site 3] starting 75 bp downstream of the polyadenylation site of H5 mRNA (18).

Fine mapping of the S1 hypersensitive structures

Although the mapping protocol employed above allows to locate with some precision the sequences cut by the nuclease, it has two main limitations. First, it does not yield any information about the strand specificity of the cleavage . Secondly, it is possible that, even under the mild conditions of digestion employed, S1 nuclease may nibble the ragged ends, thus displacing artifactually the site of the original cleavage. To verify these questions, I have devised a simple experimental approach schematized in Fig. 4. The S1 digestion of the supercoiled plasmid DNA is stopped when nicked circles are the major product of the digestion; that is, before S1 cuts through the first nick. The nicked circles are then cut with restriction nucleases choosen such that the nicked strand is close to one of the restriction site(s) [H, Fig. 4]. The products of the restriction nuclease digestion are then labeled at the 5'-ends and the fragment(s) of interest is purified by preparative gel electrophoresis. The nicked DNA fragment(s), labeled at both 5'-ends, is then analyzed in denaturing DNA sequencing gels. If nicking by S1 nuclease is strand specific, then either fragment

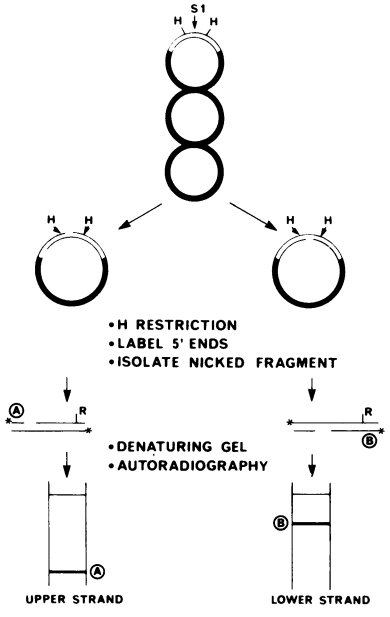

Figure 4. General experimental approach to map the sites and strands cleaved by S1 nuclease in supercoiled DNA molecules.

A or B will be visualized, together with the full-length complementary strand and non-nicked molecules. In any other situation, a mixture of fragments A and B ,as well as non-nicked molecules, will be obtained depending on the relative frequency of cutting at each strand. The labeling efficiencies of each 5'-end can be controlled for by digestion with a restriction nuclease that cuts the fragment asymmetrically [R, Fig.4] and analysis of the fragments in a non denaturing gel. Although 5'-end labeling of internal nicks is not efficient, this eventuality can be controlled for by analyzing, in a denaturing gel, the fragments produced by restriction nucleases that cut at either side of the nick.

This approach was applied to determine the strand and sequences nicked at sites 2 and 3. A mixture of nicked and supercoiled circles, resulting from a mild S1 digestion of pchV2.5B/H DNA, were cleaved with Hinf I and fragment Hinf I-C [Fig. 1] was isolated. The products of a Sau 3AI digestion of fragment Hinf I-C were labeled at the 5'-ends with T_4

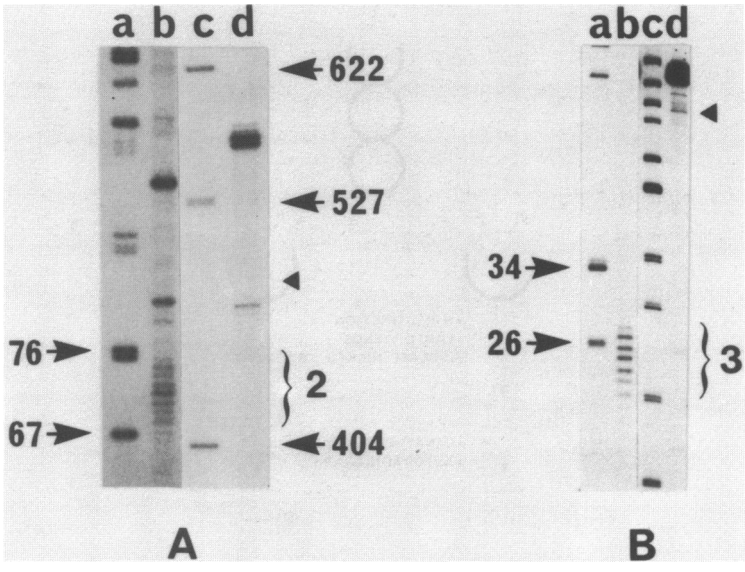

Figure 5. Fine mapping of the sites and DNA strands cleaved by S1 nuclease. S1-nicked, fragment Hinf I-C from pchV2.5B/H DNA was digested with Sau 3AI . The purified fragments Sau 3AI-B [A], and Sau 3AI-E/Hinf I [B] were labeled at the 5'-ends and electrophorized in 6% and 10% polyacrylamide DNA sequencing gels, respectively. [A]: Autoradiography of fragment Sau 3AI- B run for 2 [b, the additional bands are of unknown origen, but they are not related to S1 cleavage since they were not seen in a previous autoradiogram, accidentally destroyed, of the freshly labeled fragments], and 4 hours [d]; [a, c] 5'-end labeled Hpa II fragments of pBR 322 run for 2, and 4 hours, respectively. [B]: Autoradiography of fragment Sau 3AI-E/Hinf I run for 1.5 [a], and 2.5 hours [d]; [a,c] 5'-end labeled Hpa II fragments of pBR 322 run for 1.5, and 2.5 hours, respectively. The relevant lengths of the markers is indicated.

polynucleotide kinase and separated by low melting point agarose gel electrophoresis. The 561 bp long Sau 3AI-B fragment [harboring site 2] and the 209 bp long Sau 3AI -E/Hinf I fragment [harboring site 3, Fig. 1] were run in DNA sequencing gels [Fig. 5]. At each site, S1 nuclease nicked the DNA at a stretch of a few nucleotides [Fig. 5A, b and 5B, b], some of them being more frequently cleaved than others. Since the average size of the fragments containing sites 2 and 3 is small [i.e.: 72 and 27 nucleotides, respectively], the sequence of the cutting sites can be determined with a maximal error of one or two nucleotides [the error may be due to small anomalies in the

mobility of DNA marker fragments of equal length but different nucleotide composition; this effect makes that some of the marker bands and the full length fragments appear as close doublets]. The results shown in Fig. 5 also demonstrate that S1 nuclease nicks preferentially only one of the two DNA strands. The arrowheads [Fig. 5] point to the position in the autoradiogram where the other S1- nicked strand would be if cleavage occurred at the two DNA strands. No such band(s) [average 489 bp; i.e.: 561 bp-72 bp] is observed for the upstream site [Fig. 5A, d; the band observed at 473 bp correspondonds to a contamination with fragment Sau 3AI-D, Fig. 1, carried over during purification], and a band of low intensity is observed for the downstream site [Fig. 5B, d]. However, in the latter case the region of this band is obscured by the unspecific single strand cuts introduced during the manipulation of the fragments, and it is not clear whether this band is, indeed, the result of S1 digestion. In any event, if S1 nicks this strand it does so at a much lower frequency than the complementary one, as measured by the relative intensities of the bands in lanes b and d [Fig. 5B]. Control experiments indicated that the single stranded cuts at sites 2 and 3 were not present in the small population of nicked circles of the initial plasmid preparation; that the efficiency of labeling was comparable for each 5'-end of a given fragment , and that the internal nicks were not labeled to a detectable level by T_4 polynucleotide kinase [not shown].

DNA sequence of the S1 hypersensitive structures

Fig. 6 shows the DNA sequence, strand specificity, and nicking frequencies of S1 nuclease at sites 2 and 3. The S1 nicking sites are shifted a few nucleotides downstream of those mapped in molecules cleaved at the two strands, confirming that some nibbling had occurred, and that the first mapping was not precise. Although in each case a different strand is cut, the cleaved sequence is similar and shares a $CA(P_y)_5$ motif in opposite polarity. In spite of the fact that the strand composition of site 1 is similar to that of sites 2 and 3, an identical element is not found at or nearby the sequence mapped in molecules cleaved in the two strands [see above]. Because of

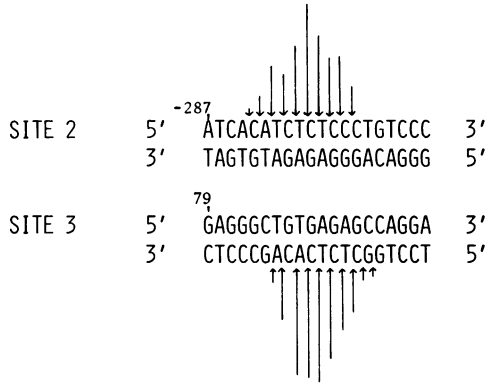

Figure 6. DNA sequence of the nicking sites of S1 nuclease in pchV2.5B/H. The sequence of site 2 is numbered from the first capping site of H5mRNA [+1] (18), and that of site 3 from the site of polyadenylation (35,36). The arrows point to the strand preferentially cleaved. The frequency of S1-nicking at each site, represented by the magnitude of the arrows, was independently measured and, therefore, the intensities between the two sites are not directly comparable.

this, and since such short motifs are found a few times scattered over the entire portion of the sequenced chicken DNA [2,133 bp (18), M. Affolter and A. Ruiz-Carrillo, unpublished], I looked for other regions of homology around the three S1 sites. The rationale being that perhaps other sequences, not the ones actually cut, are responsible for the S1 hypersensitivity. A computer search-out revealed the presence of three related pyrimidine-rich stretches [consensus $CTCCP_yTGTCP_y$, Fig. 7] close to the three sites. However, an identical consensus sequence

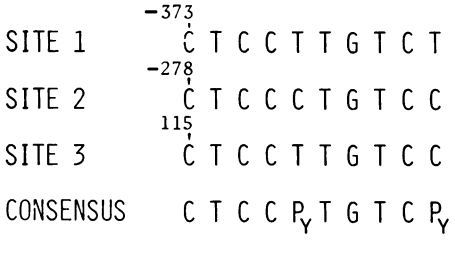

Figure 7. Blocks of DNA sequence homology near the major S1 nuclease cleavage sites flanking the H5 gene.

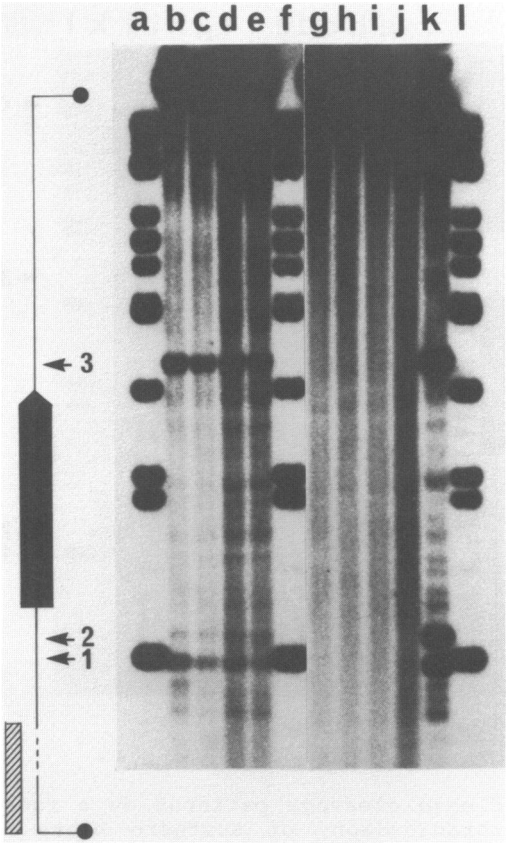

Figure 8. S1 nuclease cleavage patterns as a function of ionic strength and insert length. Autoradiography of a Southern blot hybridized with fragment Hinf I-B labeled by nick-translation. Supercoiled [b-e], and Hind III linearized [g-j] pchV8.8HR DNA was digested with S1 nuclease in S1 buffer [pH 4.6] at 37°C for 1 minute under the following conditions : [b, g] 25 units/ml, 0 mM NaCl; [c,h] 50 units/ml, 30 mM NaCl; [d, i] 250 units/ml, 60 mM NaCl; [e, j] 600 units/ml, 90 mM NaCl. [a, f, l] Sau 3AI partial digestion of pchV2.5B/H DNA. [k] pchV2.5B/H DNA digested with S1 nuclease under the conditions described for [c, h]. DNA samples were cleaved with Hind III before electrophoresis. Other symbols as in fig. 2.

[-483]CTCCTTGTCT[-474] is found in a region not cut by the nuclease. Whether these sequences have any influence on the structure of the hypersensitive sites is not yet known, but it is clear that they are not the sole determinants of S1 nuclease sensitivity.

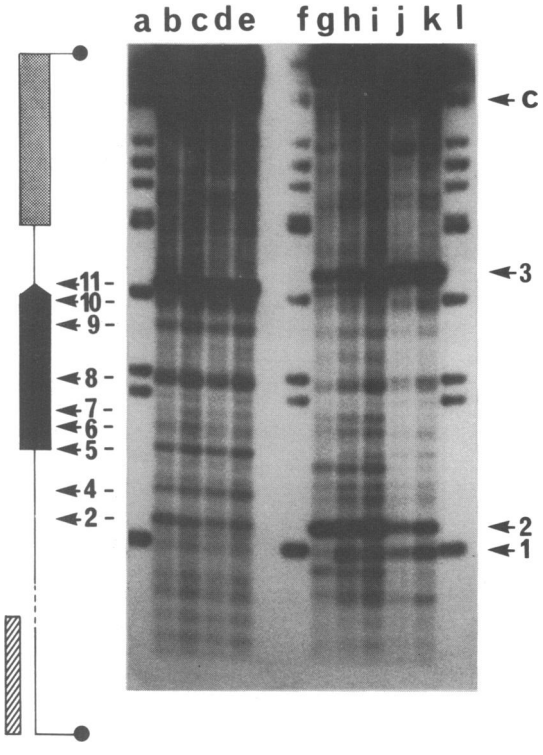

Figure 9. S1 nuclease cleavage patterns as a function of pH and temperature. Autoradiography of Southern blots hybridized with fragment Hinf I-B labeled by nick-translation. Supercoiled pchV2.5B/H DNA was digested with S1 nuclease in S1 buffer containing 30 mM NaCl under the following conditions: [b, c] 400 units/ml at 37°C, pH 5.3, for 3 and 5 minutes, respectively; [d, e] 1,200 units/ml at 37°C, pH 6.0, for 3 and 5 minutes, respectively; [g] 800 units/ml at 0°C, pH 4.6, for 20 minutes; [h, i] 200 units/ml at 20°C, pH 4.6, for 3 and 5 minutes, respectively; [j, k] 5 units/ml at 50°C, pH 4.6, for 3 and 5 minutes, respectively. [a, f, and l] Sau 3AI partial digestion of pchV2.5B/H. DNA samples were digested with Hind III before electrophoresis. Other symbols as in fig. 2.

Fig. 8 shows that the S1 hypersensitive sequences are in a significant way "associated" to the H5 gene and not randomly distributed. In this case pchV8.8HR was digested with S1 under standard conditions. This recombinant plasmid contains an extension of 6.2 kbp downstream of the sequences of pchV2.5B/H , inserted in the same orientation with respect to the vector . The sites cleaved by S1 are the same ones mapped before [Fig. 8,

c], although the relative sensitivity of the two upstream sites 1 and 2 is changed.

Influence of the conditions of digestion.

The asymmetric cutting pattern of S1 nuclease argues against the possibility that the cleaved sequences have a single stranded character. However, it is possible that whatever the structure is, its formation is dependent on thermal energy. This was examined by performing the S1 digestion at several temperatures [Fig. 9, g-k]. Even though the three sites are always recognized, temperature appears to play a role in determining the hierarchy of S1 sensitivity. At lower temperatures [0°-20°C], the relative frequency of cutting at site 2 [Fig. 9, g-i] is increased, whereas at higher temperature [37°-50°C] cutting at sites 1 and 3 is favored [Fig. 2, a-c; Fig. 9, j, k]. Clearly, the stability of these structures is not thermodynamically equivalent. The fact that S1 nuclease at 50°C does not cleave preferentially at the AT-rich regions downstream of the polyadenylation site of H5 mRNA suggests that site 3 acts as a primary sink of the torsional free energy of the supercoiled molecule.

The stability of the structures recognized by S1 appears to be exquisitly sensitive to changes in pH [Fig. 9, b-e]. At pH 5.3-6.0, the pattern of cleavage becomes more complex and the main sites observed at pH 4.6 are no longer prefferentially recognized. Under these conditions cleavage at the main pBR 322 cruciform [<--c] (10,11) and at a new site on the chicken DNA [site 11, Fig. 9] is favored . This site maps close to the the site of H5 mRNA polyadenylation and is the same one recognized, although at lower frequency, at pH 4.6 and lower temperatures [Fig. 9, g-i]. Although this site has not been mapped with the same precision as the previous ones, its sequence 5'-GCAACTTCTCTGAGTGTTTA-3' (18) contains a P_y-rich stretch with alternating (TC) similar to the sequence being cut at pH 4.6. Despite the results obtained at higher pH, it is unlikely that, at pH 4.6, C protonation plays a major role in determining the S1 cleavage pattern by inducing local denaturation of the duplex DNA. Thus, there are two stretches $(C)_7$ and $(C)_6$ in the sequenced portion of the chicken DNA which are not cleaved at pH

4.6. For the same reasons, depurination can not be responsible for the hypersensitivity observed at the lower pH.

Fig. 8 [b-d] also shows that an increase in the salt concentration during the reaction results in a decreased frequency of cutting at the specific sites [see also Fig. 10, d].

Sequence preference versus sequence sensitivity.

The results presented in the last section could be due either to a change in the mode of action of S1, depending on the conditions of digestion which in turn affect the activity of the enzyme, or to conformational changes of the substrate resulting in the loss of the hypersensitive structures. In addition, structural effects induced by interaction of the nuclease with the substrate (25) may also influence the cleavage pattern. This last is important in view of the different enzyme to substrate ratios used at higher pH's and ionic strengths and at low temperature [from 10 to 40-fold that used in the standard conditions of 30 mM NaCl, pH 4.6]. Under optimal conditions of S1 activity, the enzyme may more easily recognize important alterations [hypersensitive sites], if they exist, than subtle differences on the secondary structure of the DNA. However, under suboptimal conditions, either the hypersensitive regions are modified by the changing conditions of digestion, or due to the saturating amounts of S1, the nuclease may also cleave at those sites that present slight variations of the B structure of DNA [sequence preference] as frequently as at the hypersensitive sites . If the first were correct, the S1 cleavage pattern of supercoiled and linear plasmid DNA should resemble more to each other under suboptimal than under optimal conditions. Moreover, if the hypersensitive sites are specifically recognized only by S1 whereas more subtle differences in DNA structure are less specific, the patterns produced by different nucleases may be not too dissimilar to those produced by S1 under suboptimal conditions.

I tested this possibility by examining the digestion patterns of linear and supercoiled pchV2.5B/H DNA produced by MNase, DNase I and S1 , the last one under suboptimal conditions of activity [0.14 M NaCl]. The results in Fig. 10 show that whereas

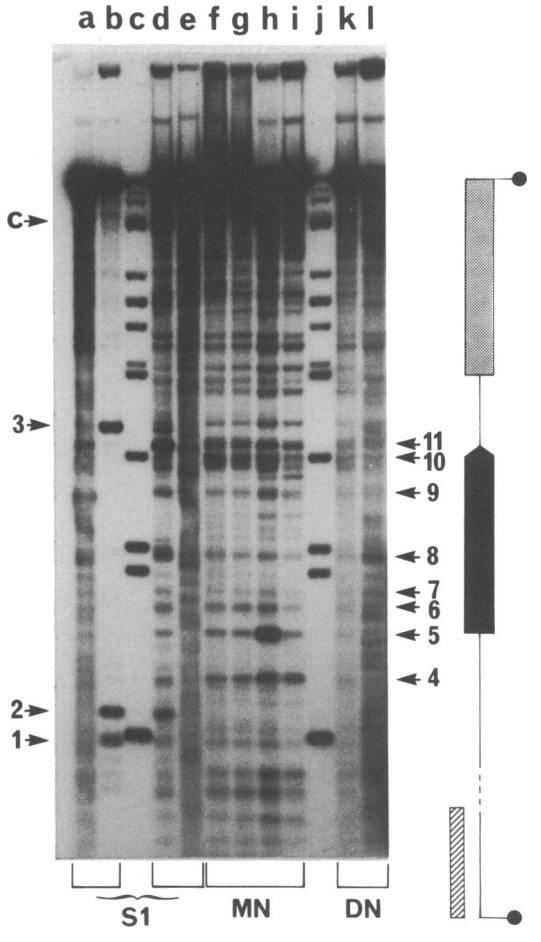

Figure 10. Cleavage patterns of pchV2.5B/H DNA by several nucleases. Autoradiography of a Southern blot hybridized with fragment Hinf I-B labeled by nick-translation. Supercoiled [b, d, g, h, i, l], and Hind III linearized [a, e, f, k] pchV2.5B/H DNA was digested under the following conditions: [a, b] 50 units/ml of S1 nuclease in S1 buffer [pH 4.6], 30 mM NaCl at 37°C for 10 and 3 minutes; [d, e] 2000 units/ml of S1 nuclease in S1 buffer [pH 4.6], 140 mM NaCl, at 37°C for 5 and 10 minutes; [f, g] 0.01 unit/ml of MNase in MNase buffer, 30 mM NaCl at 37°C for 10 and 3 minutes; [h] 0.5 units/ml of MNase in MNase buffer, 30 mM NaCl at 0°C for 3 minutes; [i] 5 units/ml of MNase in MNase buffer, 140 mM NaCl at 0°C for 5 minutes; [k, l] 10 ug/ml of DNase I in DNase I buffer, 140 mM NaCl at 0°C for 60 minutes. [c, j] Sau 3AI partial digestion of pchV2.5B/H DNA. DNA samples were digested with Hind III prior to electrophoresis. MN: MNase, DN: DNase I. Other symbols as in fig. 2.

the patterns of S1 digestion at 30 and 140 mM NaCl are dramatically different for supercoiled DNA [b, d], they are similar for linear DNA. Furthermore, the digestion patterns of supercoiled and linear DNA at 140 mM NaCl are, in fact, not too different [d, e] both in the chicken and in plasmid regions, allowing for some differences in the cutting frequencies of certain sites [site 11, Fig. 7]. More importantly, the S1 patterns at 140 mM NaCl are very similar to those of MNase on supercoiled and linear DNA at different salt concentrations and temperatures [f-i]. Even DNase I, an enzyme that has not been previously found to display sequence preference , recognizes a subset of the sites cleaved by the other two nucleases [k, l]. The majority of the sites cleaved by MNase and S1 at 0.14 M NaCl are the same ones observed with S1 at higher pH or low temperatures [Fig. 9, sites 4-11].

These results support the view that the S1 nuclease hypersensitive structures are either lost or drastically disfavoured at a pH between 4.6 and 5.3, and at salt concentrations higher than 90-100 mM NaCl.

DISCUSSION

I have characterized the S1 nuclease hypersensitive sites of supercoiled recombinant plasmids carrying the histone H5 locus, and found that they flank the structural sequences of the gene. The cleavage sites are not the result of S1 sequence preference but may reflect anomalies in the secondary structure of the DNA. The sites nicked by S1 share in common a short alternating (TC) sequence flanked by a (CA) motif, and a pyrimidine-rich stretch close to the cutting sites. Although the contribution of the latter sequences to the sensitive structures is not known, it is clear that they are not sufficient in determining the site of cleavage.

The sequences cleaved neither have the potential to form cruciform structures nor are they flanked by sequences known to be able to adopt a left- handed configuration (26, 27). In fact, under the conditions that favor cleavage at the specific sites , the cruciforms of pBR 322 (10, 11) are cut at very low relative frequencies. Recent work from other laboratories has shown that

polypyrimidine/polypurine tracts can constitute the primary site of S1 nuclease cleavage. Examples of this include the $(G)_{18}/(C)_{18}$ box of the cloned chicken β^A-globin gene (13,14),the elements $(CA)_{10}(CT)_{22}/(GT)_{10}(GA)_{22}$ and $(GA)_{16}/(CT)_{16}$ flanking the histone H1 gene of the h22 histone gene repeat of the sea urchin P.miliaris (12), a $(GA)_{10}/(CT)_{10}$ box in the intergenic spacer between the genes of histones H3 and H4 of Drosophila (28), and a chicken repetitive DNA sequence consisting of $(AGAGG)_{32}/(TCTCC)_{32}$ and a box TCTCTCA/AGAGAGT (17). Except the major S1 hypersensitive site of the B^A-globin gene, all the other hypersensitive sequences have alternating (TC) in one of the two strands, as is the case of the H5 gene described here. In addition, there are two other examples of S1 hypersensitivity that may conform to the sequence composition cited above. In the case of Adenovirus 12 DNA, the cleavage site was mapped roughly at the Goldberg-Hogness box of the major late promoter (16). However, since double stranded cut molecules were used to determine the site of cleavage, it is very likely that the actual nicking site was artifactually displaced [see results section] . Interestingly, a sequence CACTCTCT/GTGAGAGA is found 24 bp downstream of the site determined by the authors which may, most likely, be the real cleavage site. The same kind of correction can be applied in the case of the S1 hypersensitive sites of the Drosophila heat-shock genes (15). In every instance, the sites determined by the authors map a few nucleotides 'upstream' of sequences containing alternating (TC) of the general type $(CT)_nCA/(GA)_nGT$, which are probably the actual cleavage sites.

Despite their sequence homology,the members of the family of hypersensitive sites discussed above display a hierarchy of sensitivity towards S1 nuclease nicking. Thus, some of the sequences are cut at higher frequencies than others, and the majority, but not all (12), require the torsional energy of supercoiling to be manifested. Some of them appear to be strongly pH-dependent [(17), this work] whereas others are not (14), and the same can be said of their dependency on the ionic strength. It appears as though the length of the sequence may be an important factor in determining its relative sensitivity.

Although, as proposed by Hentschel (12), slippage could explain the S1 sensitivity of some of these sequences, it is clear that this mechanism can not explain the sensitivity of all of them. Moreover, when the question has been examined, it has been found that the polypyrimidine strand is selectively nicked [(14, see however ref. 13 for a different result), this work], observations which suggest that the sequences do not have a single stranded character; view which is supported by the result that sites 2 and 3 are cleaved at 0°C. It is now recognized that DNA has a potential for considerable conformational flexibility (29, 30) and it is clear that this feature is used to form unique structures like the ones described here. The asymmetry of the S1 nuclease action may, in fact, reflect different conformations of the polypyrimidine and polypurine strands.

The non-random location of the S1 hypersensitive sites in the regions flanking the H5 gene argues for a biological role of such structures. An attractive possibility is whether the ability of the S1 hypersensitive sequences to adopt an altered DNA conformation is used in gene transcription. Interestingly, the sequences cleaved by S1 usually map close to DNase I hypersensitive sites of active genes (32, 33). In the case of the histone H5 gene, the S1 sites 1 and 2 are located 150-200 bp upstream of a DNase I hypersensitive site, whereas the S1 site 3 maps in the same region recognized by DNase I [in preparation]. Despite these correlations, some of the sequences discussed here are, however, not likely to be involved in transcription, since they are located either in regions not known to have a direct effect on the expression of the genes (31) or they can be deleted without affecting gene transcription in a noticeable way (15). On the other hand, these sequences could be involved in recombinational or transpositional events. In this regard it is relevant that the H5 gene, which can be considered as a highly specific H1 variant that probably evolved from a primitive H1 gene, is found in a single copy outside of the clusters of the other histone genes (18, 34).

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