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DEMONSTRATION OF AN S1-NUCLEASE SENSITIVE SITE NEAR THE HUMAN β-GLOBIN GENE, AND ITS PROTECTION BY HMG 1 AND 2

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SUMMARY. An S1-nuclease sensitive site exists within supercoiled plasmids containing the 5'-flanking sequences of the human β-globin gene. This site is located approximately 540 base pairs upstream from the start of the gene within a region of 52 alternating purine-pyrimidine residues which has the potential to adopt either cruciform structures or Z-form DNA. This site is protected from specific cleavage by S1-nuclease by the high-mobility-group chromosomal proteins HMG1 and 2, which may be specifically acting to protect short sequences of single-stranded DNA.

There has been considerable interest in recent years in modifications in the primary and secondary structure of DNA, which may play a role in the regulation of gene expression. Actively transcribed genes have been shown to be associated with altered regions of chromatin structure which differ from bulk chromatin in their accessability to nucleases [recent reviews (2,24)]. Larsen and Weintraub (11) have shown that the single-stranded DNA-specific S1-nuclease can cleave chick chromatin in the 5' flanking sequences of the β-globin genes in cells expressing these genes. For the chick adult β-globin gene, the S1-nuclease-sensitive region of chromatin corresponded to the DNAase I hypersensitive region (15) and similar S1-nuclease sensitive sites were found within supercoiled plasmid DNA containing the chick adult β-globin gene (11). We have now demonstrated that an S1-nuclease sensitive site exists in a supercoiled plasmid containing the 5' portion and flanking sequences of the human β-globin gene, and that the S1-nuclease sensitivity of this site can be abolished by the presence of the chromosomal high mobility group proteins HMG1 and HMG2 (5). This S1-nuclease sensitive site exists within a sequence of 52 alternating purine-pyrimidine residues (17), which may be a region having altered secondary structure *in vivo*.

MATERIALS AND METHODS

Plasmids were the gift of R.M. Freund. The plasmid hβ1S was cloned by Maniatis (3), and contains the 4.4 kbPstI fragment of the human β-globin gene

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inserted into pBR322. The plasmid βF5 was subcloned by Freund (9), and contains the 1.9 kb Bam HI fragment of the human β-globin gene inserted into PAT 153. Plasmids were grown in HB101 cells, and the supercoiled plasmid fraction was isolated from CsCl gradients. HMG1 and 2 were prepared from pig thymus by the method of Isackson et al. (8). Aspergillus oryzae S1-nuclease digestions were performed in 50 mM NaCl, 30 mM Sodium acetate, pH 4.6, 1 mM Zinc acetate, 50 ug/ml BSA. DNA was digested at 0.1 mg/ml and phenol extracted after S1-nuclease digestion and ethanol precipitated. 5'-Polynucleotide kinase labelling was performed by the method of Maxam and Gilbert (14).

Non-denaturing DNA gels were performed in 1% agarose gels in 40 mM sodium acetate, 20 mM Tris, 2 mM EDTA, pH 7.7 Denaturing DNA gels were performed by the method of McMaster and Carmichael (16), by electrophoresis of glyoxylated DNA in composite 3% acrylamide/0.5 agarose gels in 10 mM sodium phosphate, pH 7.0.

RESULTS

S1-nuclease sensitivity of the human β-globin 5'-flanking sequences. S1-nuclease relaxes the supercoiled plasmid hβ1S containing the human β-globin gene Pst I fragment. There is an initial relatively rapid single stranded nicking reaction (Fig. 1B, lanes 1 and 6) followed by a slower linearization

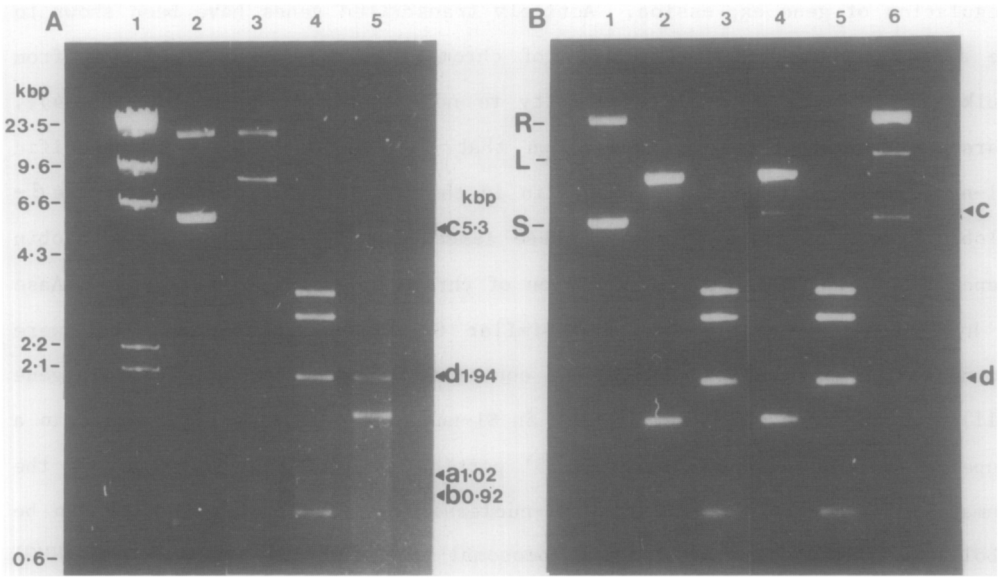

Fig. 1 Gel electrophoresis in 1% agarose showing S1-nuclease cleavage of supercoiled hβ1S. (A) Lanes 3-5, 3¼ h digest of hβ1S with 500 U/ml S1-nuclease at 30°C, followed by Bam HI digesion (lane 4) or Eco RI digestion (lane 5). Lane 2, hβ1S incubated in the absence of S1-nuclease. Lane 1, λ - Hind III marker. (B) Lanes 4-6, 2½ h digest of hβ1S with 50 U/ml S1-nuclease at 30°C, followed by Bam HI digestion (lane 5) or Eco RI digestion (lane 4). Lanes 1-3, Incubation of hβ1S in the absence of S1-nuclease, followed by Bam HI digestion (lane 3) or EcoRI digestion (lane 2). Symbols: R, relaxed circular form; L, linear form; S, supercoiled form; bands a, b, c and d are subfragments of DNA produced after restriction endonuclease digestion only after prior incubation with S1-nuclease, as mapped in Fig. 2.

reaction (Fig. 1A, lanes 2 and 3). The linearization of the plasmid occurs at a discrete site 1.02 kb upstream from the internal Bam HI site (Fig. 2), as demonstrated by the generation of two new subfragments after either a subsequent Bam HI digest (Fig. 1A, lane 4, 1.02 kb and 0.92 kb) or EcoRI digest (Fig. 1A, lane 5, 5.3 kb and 1.94 kb). These subfragments are absent when hβ1S is digested with EcoRI or Bam HI without prior S1-nuclease digestion (Fig. 1B, lanes 2 and 3). The long S1-nuclease digestions necessary for linearization of the plasmid result in the loss of some of the high molecular weight bands (Fig. 1A, lane 5) which can be seen after shorter S1-nuclease digestions (Fig. 1B, lane 4) followed by EcoRI digestion. The S1-nuclease cleavage at a site 1.02 kb upstream of the internal β-globin gene Bam H1 site has been confirmed by digesting the supercoiled plasmid βF5, which contains a 1.94 kb Bam H1 fragment of the β-globin gene 5' sequence (Fig. 3B, lane 1). The two subfragments (a,b) of the 1.94 kb sequence produced after a Bam H1 digest of the S1-nuclease cleaved material are only seen after long S1-nuclease digests at 30°C (these bands may not be visible on the reproduction of the photograph). The S1-nuclease sensitive site has been further mapped by kinase labelling of the free 5' DNA termini generated after a lower temperature digest (22°C), where most of the DNA has been converted to the relaxed circular form (Fig. 3A, lane 2). Subsequent restriction endonuclease digestion of this material shows the presence of only two discreet bands on electrophoresis using denaturing conditions after either a Hae III digest (Fig. 4, lane 1) or an Mbo II digest (Fig. 4, line 3). The β-globin 5' flanking sequences have been sequenced (17), and from the data in Fig. 4, the S1-nuclease-sensitive site can be located to 920 ± 10 bp from the upstream Bam HI site (Fig. 2).

Influence of HMG1 and 2 on the S1-nuclease digestion of βf5. S1-nuclease digestion of the supercoiled plasmid βf5 in the presence of 25 ug/ml HMG1 and 2 at 30°C results in a lower rate of digestion (compare Fig. 3A, lanes 1 and 3). An equimolar mixture of HMG1 and 2 was used, and under the conditions used there are about 35 molecules of HMG1 and 2 per plasmid, or one HMG1 or 2 molecule per 170 bp of DNA. The extent of relaxation and linearization of the supercoiled plasmid is reduced to the extent found for an equivalent digestion of the uncomplexed plasmid at the lower temperature of 22°C (Fig. 3A, lane 2). Analysis of the denatured products of Hae III and Mbo II digests (Fig. 4,

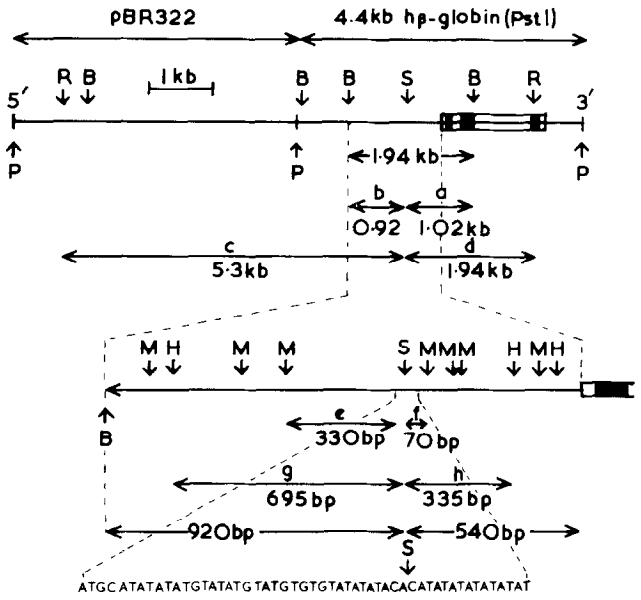

Fig. 2 Map of restriction endonuclease sites found within hβ1S (and β F5) compiled from published data (3,12,17). The sequence of 52 alternating purine-pyrimidine residues is illustrated at the bottom of the figure. The boxed region marks the β-globin gene. Symbols: B, Bam HI; H, Hae III; M , Mbo II; P, Pst I; R, Eco RI; S, S1-nuclease sensitive sites. DNA fragments marked a, b, c, d, e, f, g and h refer to subfragments produced from restriction endonuclease digestions only after prior incubation with S1 nuclease, and refer to bands marked on Figs. 1, 3 and 4.

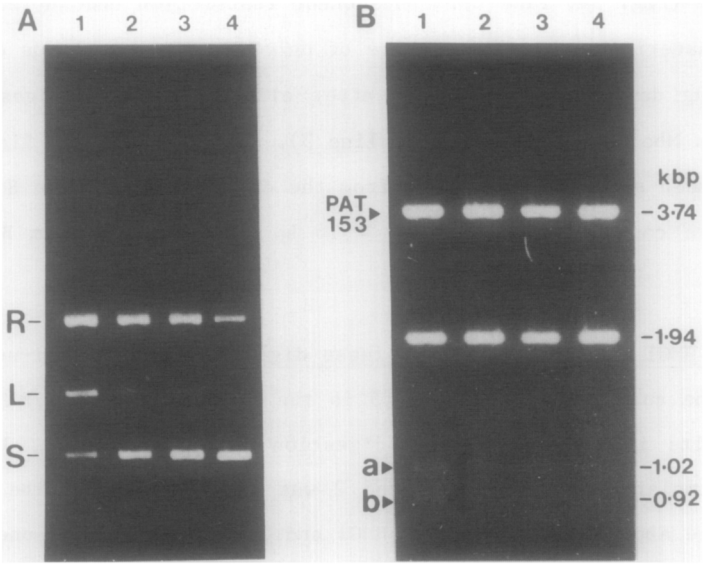

Fig. 3 Gel electrophoresis in 1% agarose showing S1-nuclease cleavage of β F5 in the presence and absence of HMG1 and 2. Digestions performed for 2½ h in 500 U/ml S1-nuclease. (A) Plasmid DNA following S1-nuclease digestion. (B) As in (A), after digestion with Bam HI. Lanes: 1, digestion at 30°C; 2, digestion at 22°C; 3, digestion at 30°C, plus 0.025 mg/ml HMG1 and 2; 4, no S1-nuclease added, 30°C. Symbols as in Fig. 1.

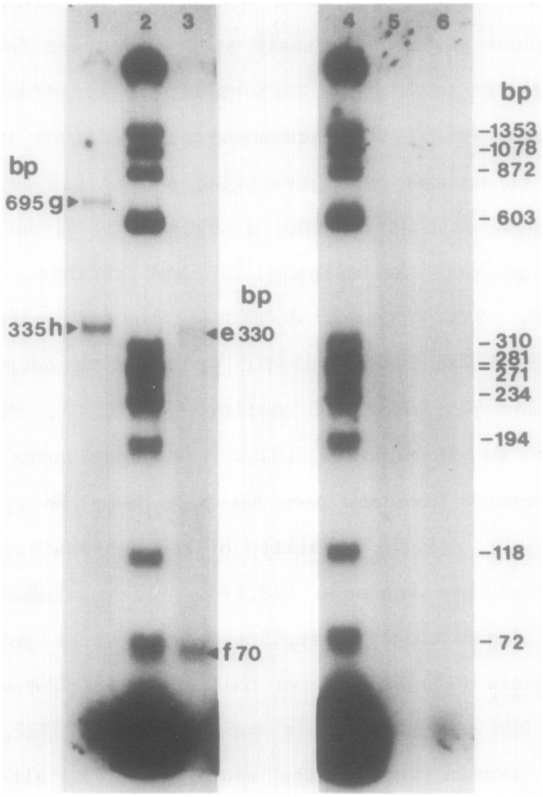

Fig. 4 Autoradiograph of gel electrophoresis of glyoxylated DNA in 3% acrylamide/0.5% agarose, showing S1-nuclease nicking of βF5 in the presence and absence of HMG1 and 2 as in Fig. 3. S1-nuclease digestions performed for 2½ h in 500 U/ml S1-nuclease, followed by 5' polynucleotide kinase end labelling, and restriction endonuclease digestion. Lanes: 1 and 3, S1-nuclease digestion at 22°C; 5 and 6, S1-nuclease digestion at 30°C plus 0.025 mg/ml HMG1 and 2; 1 and 5, Hae III digestion; 3 and 6, Mbo II digestion; 2 and 4, φX174 - Hae III marker. Bands e, f, g and h are fragments of DNA produced from restriction endonuclease digestions, only after prior S1-nuclease digestion, as mapped in Fig. 2.

lanes 5 and 6) of the S1-nuclease digested plasmid reveals that the presence of HMG1 and 2 abolishes nicking of the DNA at any discrete site. This is in sharp contrast to the discrete nicking seen within the uncomplexed plasmid digested at 22°C, which shows the same pattern of relaxed and linear forms before restriction endonuclease digestion as the complexed plasmid.

DISCUSSION

We have shown that S1-nuclease cuts the 5' flanking sequence of the human β-globin gene at a discrete site within the supercoiled plasmids hβ1S and βf5. This site is located at approximately 540 bp upstream from the start of the gene and it exists within a sequence of 52 alternating purine-pyrimidine

residues. It has been previously shown that there are several types of DNA sequence which can adopt altered secondary structure under favourable conditions which are associated with short regions of single stranded DNA (1,7,13, 19,23). Short inverted repeat DNA sequences can be driven into S1-nuclease-sensitive cruciform structures by supercoiling energy (13,19). The cleavage of a histone gene repeat unit at an $d(AC)_{16}$ sequence by S1-nuclease (7) may be the result of out of register slippage of DNA strands. Wang (23) has demonstrated that the DNA fragment d(CGCGCG) can be converted to Z-DNA. Supercoiling of plasmids can induce polyd(GC) inserts to adopt a localised Z-DNA conformation under physiological conditions (20,22), the bounderies of which are sensitive to S1-nuclease (22). Various forms of alternating purine-pyrimidine sequence have now been shown to have the potential to adopt a Z-DNA conformation (1), and the stability of these transient states increases with the length of the sequence (18,22). Large numbers of tracts of polyd(TG) have been found widely distributed in diverse eukaryotic genomes (6), and Nordheim *et al*. (18) have shown that an anti-Z-DNA antibody can bind to a CACGGGTGCGCATG DNA sequence within supercoiled pBR 322, in addition to polyd(CG) inserts. Examination of the sequence of 52 alternating purine-pyrimidine residues present around the S1-nuclease site 5' to the human β-globin gene (see bottom Fig. 2) shows that this sequence has the potential of adopting any of the three secondary structure modifications discussed above. Several regions of inverted repeat sequence can be aligned, which ought to be stable by the criteria previously demonstrated (13,19), and these structures would be S1-nuclease-sensitive. The sequence $d(TA)_7$ is unlikely to be long enough for out of register slipping to occur, and is not the most highly favoured Z-DNA forming structure, but the presence of several d(TG) and d(AC) sequences within this relatively long unit make it more likely that Z-DNA regions may transiently exist within this unit. Nordheim's results indicate that randomly arranged naturally occurring alternating purine-pyrimidine sequences can adopt a Z-DNA conformation (18). It seems unlikely that S1-nuclease cutting at this site is due to localised melting of d(A,T) sequences as another longer d(A,T) sequence exists further upstream and no S1-nuclease cutting is seen at this site.

We have shown that HMG1 and 2 are able to protect a discrete site from nicking by S1-nuclease within a negatively supercoiled plasmid. Javaherian

and Sadeghi (10) have shown that HMG1 and 2 are able to reduce the linking number (4) of DNA by about 24° per molecule bound, but for one molecule of HMG1 or 2 bound per 170 bp of DNA, the superhelical density would only be altered by -0.004. The plasmids have been grown in E. coli, and thus should have a superhelical density of about -0.06 (4). Therefore the DNA unwinding effect of HMG1 and 2 should be insufficient to significantly affect the torsional stress within the supercoiled plasmid. It would seem more likely that the greater affinity of HMG1 and 2 for single stranded DNA rather than double stranded DNA (10) was responsible for the protection of the S1-nuclease-sensitive site. Our results are in agreement with Shastri et al. (21), who has shown that HMG1 inhibits the digestion of single stranded DNA by S1-nuclease.

It seems likely that the secondary structure of DNA plays some role in gene regulation, and we have demonstrated a sequence near the 5' regulatory sequences of a gene which can be induced to adopt an altered conformation. The protection of this site from S1-nuclease by HMG1 and 2 suggests that these proteins may function in vivo by protecting regions of altered DNA conformation. It remains to be shown whether the S1-nuclease sensitive site is present in transcriptionally active human β-globin chromatin, as has been found with the chick adult β-globin gene.

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