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Superhelicity induces hypersensitivity of a human polypyrimidine · polypurine DNA sequence in the human α2-α1 globin intergenic region to S1 nuclease digestion – high resolution mapping of the clustered cleavage sites

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ABSTRACT

Supercoiled recombinant DNAs containing the human adult α-globin gene region have been probed with nuclease S1 *in vitro*. While agarose gel electrophoresis showed only one predominant, double-stranded cleavage generated by S1 within 6 kb of human DNA and 4 kb of pBR322 sequence, a high resolution gel analysis reveals that the unique S1-hypersensitive locus in the human adult α-globin gene region actually contains more than 15 authentic S1 cleavage sites closely spaced together. The mapping approach used here locates the specific S1 cleavage sites on both DNA strands at the nucleotide sequence level. Interestingly, most of these sites are mapped within a 90 bp stretch of GC-rich (66%) polypyrimidine•polypurine DNA that is located 1060 to 1150 bp upstream from the α1-globin gene. These results provide the first high resolution map of double-stranded S1-cleavage sites induced within a specific DNA sequence under supercoil strain. The distribution and relative cutting frequencies of these sites mapped are consistent with a slippage mechanism in which the simple repeating sequences are organized into base-mismatched duplex on supercoiled DNA.

INTRODUCTION

S1 nuclease is a single-strand DNA-specific enzyme (1) useful for probing the structure of purified DNA (2-9) or DNA in chromatin (8). Supercoiled plasmid DNAs are cut into linear molecules by S1 within regions containing two-fold symmetry (2-5), AT-rich sequences (2,3), simple homo-copolymer repeating sequences (7), or junctions between Z- and B-forming DNA (6). Promoter sequences of certain viral and eukaryotic genes are also sensitive to S1 digestion *in vitro* (5,8). Probably due to the complex nature of the DNA folding recognized by S1, it has not been possible to map the multiple, heterogeneous S1-cleavage sites to the resolution of nucleotide sequences (for example, see 9). I report here the high resolution map of two clusters of double-stranded breaks generated by S1 within the human adult α-like globin gene region.

MATERIALS AND METHODS

DNA Samples

The subcloning of the 3.2 kb HindIII-EcoRI fragment containing the human adult α2-α1 intergenic DNA and the 3.8 kb BglII-EcoRI fragment containing the α1 globin gene into the appropriate restriction sites of pBR322 have been described previously (10). Plasmid DNA was prepared from E. coli using an ethidium bromide-CsCl density gradient.

S1 Nuclease Reaction

For the S1 reaction, 20 µg of DNA was precipitated by ethanol. The pellet was resuspended at 4°C overnight in 22 µl of either one of the following two buffers: an aqueous buffer containing 40 mM Na acetate, pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$, a formamide buffer containing 27 mM Na acetate, pH 4.6, 290 mM NaCl, 4 mM Zn acetate, 4 mM PIPES (Sigma), 0.1 mM EDTA, 20 µg/ml calf thymus DNA, and 8% formamide (deionized, MCB). Throughout the plasmid isolation procedures and the treatments described above, the DNA samples were never heated above 37°C. The resuspended DNA samples, pRHα2 or pRBα1, were preincubated at 37°C for 10 minutes and 1 µl of 33 units of S1 nuclease (Sigma) was added. The incubation at 37°C was continued for another 1 hr. The samples were adjusted to 10 mM EDTA and extracted with phenol, ether and precipitated by ethanol. The samples were then resuspended in 50 µl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and analyzed by restriction enzyme digestion and gel electrophoresis.

^{32}P-labelling of DNA Ends Generated by S1 Cleavage

Linear DNA molecules results from S1 cleavage of supercoiled plasmid DNA (see text) were labeled with ^{32}P at the presumably blunt ends generated by S1 by incubating at room temperature with 5 units of Klenow fragment of DNA polymerase I (11) (Biotech) and 0.2 µM of the four α-^{32}P-labeled dATP, dCTP, dGTP and dTTP (Amersham, 3000 Ci/mmole, 10 mCi/ml) in a total volume of 20 λ 10 mM Tris•HCl, pH 7.5, 10 mM $MgCl_2$ and 50 mM NaCl. After 15 minutes, 1 µl of 10 mM each of unlabeled dATP, dGTP, dCTP and dTTP were added and the chasing reaction was continued for another 15 minutes. The end-labeled DNA molecules were then purified by phenol, ether extraction, and resuspended in TE buffer.

RESULTS

One Major S1 Nuclease-sensitive Region in the Human α2-α1 Globin Intergenic DNA

Recombinant clones pRBα1 and pRHα2 contain inserts that together span a major portion of the human adult α globin gene region and overlap each other

for approximately 1.3 kb (Fig. 1a). Under the experimental conditions described in Materials and Methods and in Fig. 1a, each plasmid DNA is cut only once by S1 in a specific region. As shown in Fig. 1b, the S1-sensitive region in supercoiled pRBα1 is located 1.5 kb from the HindIII restriction site and 2.0 kb from each of the two PvuII restriction sites. A similar study of pRHα2 showed that the S1-sensitive region is located 1.0 kb from the EcoRI restriction site and 2.2 kb from the HindIII restriction site (Fig. 1c). The combined data indicate that there is only one major S1-sensitive region in the human α2-α1 intergenic DNA and it is located 1 kb on the 5' side of the α1-gene, approximately 200-300 bp downstream from the BglII restriction site (Fig. 1a).

Salt Effect and Superhelicity Requirement

Two buffers with different salt conditions and formamide concentrations have been used for the S1 digestion reaction. The aqueous buffer (see Materials and Methods for details) has a relatively low concentration of Na ion while the formamide buffer contains 8% of formamide. As shown in Fig. 1c, there is no apparent differences in the gel patterns of pRHα2 digested by S1 in the aqueous and the formamide conditions except in the latter buffer, S1 also cuts pRHα2 at several other places with very low frequencies. The same thing is true for pRBα1 (results not shown). Furthermore, superhelicity is required for the unique, double-stranded DNA cleavage to occur. When EcoRI-linearized pRHα2 DNA was used as the substrate for S1 reaction and the products analyzed on 1% agarose gel (Fig. 1d), there are no unique DNA bands generated by the S1 cleavage.

High Resolution Mapping of S1 Nuclease Cleavage Sites

Most of the previous studies of S1 cleavage of double-stranded DNA have only mapped the cleavage sites by agarose gel or native polyacrylamide gel analysis. To gain more insight into the nature of the S1 reaction, and the actual DNA sequences it preferentially cuts within the human α-globin gene region, it is essential to map the double-stranded breaks to the resolution of nucleotide sequence. This mapping requires consideration of the mechanism of the S1 nuclease reaction.

A resonable working model of double-stranded DNA cleavage by S1 from the previous studies (1-9) can be outlined as follows. Under supercoil strain , specific DNA sequences (inverted repeats, AT-rich DNA, simple repeating sequences, Z-DNA forming sequence, etc.) fold into a unique DNA conformation containing single-stranded structures (cruciforms, loops, B-Z DNA junctions, etc.). After recognizing these single-stranded structures,

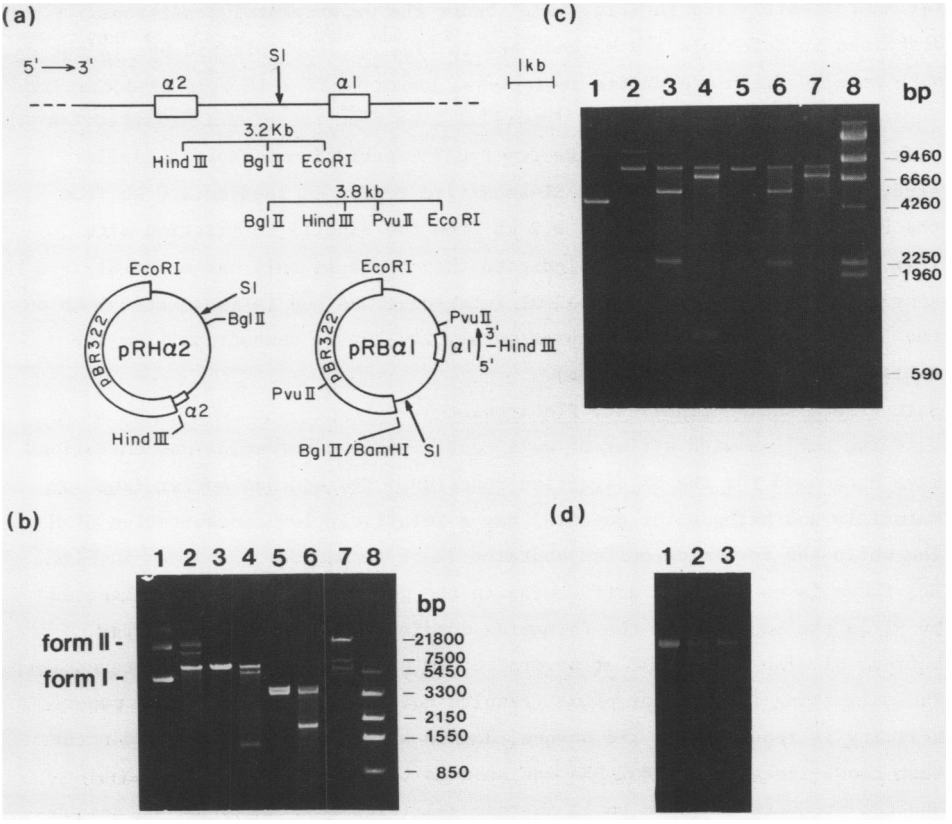

Figure 1. Nuclease S1 cleavage of recombinant DNA clones containing human adult globin gene region. (a) Restriction map and the location of S1 cleavage sites of plasmids pRHα2 and pRBα1. The results shown in (b) and (c) indicate that there is only one predominant S1 cleavage site (or a cluster of sites) mapped at approximately 300 bp downstream (with respect to the transcription direction of the two globin genes) from the BglII restriction sites (the arrows on the maps of genomic DNA, pRBα1 and pRHα2). (b) Restriction mapping of the S1 cleavage sites in pRBα1 in the formamide buffer. The plasmid DNA pRBα1 was cleaved by S1 in the formamide buffer as described in Materials and Methods, digested with different restriction enzymes and analyzed on a 1% agarose gel. Lane 1, pRBα1; lane 2, S1 cleaved pRBα1 (S1-pRBα1); lane 3, HindIII digested pRBα1; lane 4, HindIII digested S1-pRBα1; lane 5, PvuII digested pRBα1; lane 6, PvuII digested S1-pRBα1; lane 7, EcoRI digested λ DNA as size marker; lane 8, partial digest of pRBα1 by HincII as size marker. (c) Restriction analysis of pRHα2 cleaved by S1 in two different buffers. Supercoiled pRHα2 isolated from the ethidium bromide-CsCl density gradient was further purified by agarose gel elution. The plasmid DNA was then treated with S1 nuclease in the aqeuous buffer (S1(aq)-pRHα2) or formamide buffer (S1(FA)-pRHα2). The S1-treated pRHα2 DNA was cut either with HindIII or with EcoRI and analyzed on a 1% agarose gel. Lane 1, supercoiled pRHα2; lane 2, S1(aq)-pRHα2; lane 3, HindIII digested S1(aq)-pRHα2; lane 4, EcoRI digested S1(aq)-pRHα2; lane 5, S1(FA)-pRHα2; lane 6, HindIII

digested S1(FA)-pRHα2; lane 7, EcoRI digested S1(FA)-pRHα2; lane 8, HindIII digested λ DNA as size marker. The numbers indicate the lengths of DNA fragments in kb. (d) S1 nuclease cleavage of linearized pRHα2 in the aqueous and formamide buffers. Supercoiled pRHα2 is digested with EcoRI and then subjected to S1 nuclease cleavage in either the aqueous or the formamide buffer. Lane 1, EcoRI-pRHα2; lane 2, EcoRI-pRHα2 cleaved by S1 in aqueous buffer; lane 3, Eco RI-pRHα2 cleaved by S1 in formamide buffer.

S1 nuclease introduces a unique single-stranded break (nick). It then cuts these nicked and relaxed circles on the complementary DNA strand across from the nick--thus generating linear DNA molecules with two blunt ends. In principle, these unique blunt ends (authentic S1 cleavage sites) should be easily mapped by DNA sequencing of either one of the ends. However, as shown by the results below, the double-stranded DNA cleavage by S1 is much more complex. First, different nicks can be introduced by S1 onto different DNA molecules depending on which single-stranded part within a specific DNA conformation is recognized first. This will give many authentic S1 cleavage sites for a particular DNA sequence. Furthermore, the second nick might not be introduced across from the first one but very close to it. In this case, DNA molecules with short "tails" are first generated and they become blunt-ended after the "tails" are digested by S1. Last, if the DNA sequences near the nicks are AT rich, the "nibbling" activity of S1 (12) can proceed on the nicked circles and/or the blunt-ended DNA molecules. Because of these complications, it is not possible to map the authentic S1 cleavage sites by sequencing the blunt ends after ^{32}P-labeling. Mapping of the breaks on both DNA strands is also necessary to get the precise positions of the authentic S1 cleavage sites.

Our high resolution mapping strategy of double-stranded cleavage sites within the S1-hypersensitive region of α2-α1 globin intergenic DNA is outlined and described in detail in Fig. 2a. Briefly, the linear pRHα2, generated by S1 cleavage in either the aqueous or formamide buffer, was labeled with ^{32}P at both 3'-ends by the exchange-chasing reaction of Klenow DNA polymerase. The end-labeled DNA was then cut with HindIII and EcoRI, and the resulting 2.2 kb and 1.0 kb fragments were eluted from agarose gel. The 2.2 kb fragment was further cut with BglII and analyzed on a 5% polyacrylamide gel (Fig. 2b). Two clusters of radioactive bands, with average lengths of 300 bp and 250 bp, respectively, were released after BglII digestion of the 2.2 kb fragment obtained from S1(FA)-pRHα2 (Lane 2, Fig. 2b); but the BglII digested 2.2 kb fragment of S1(aq)-pRHα2 gave mainly the 300 bp cluster (Lane 3, Fig. 2b).

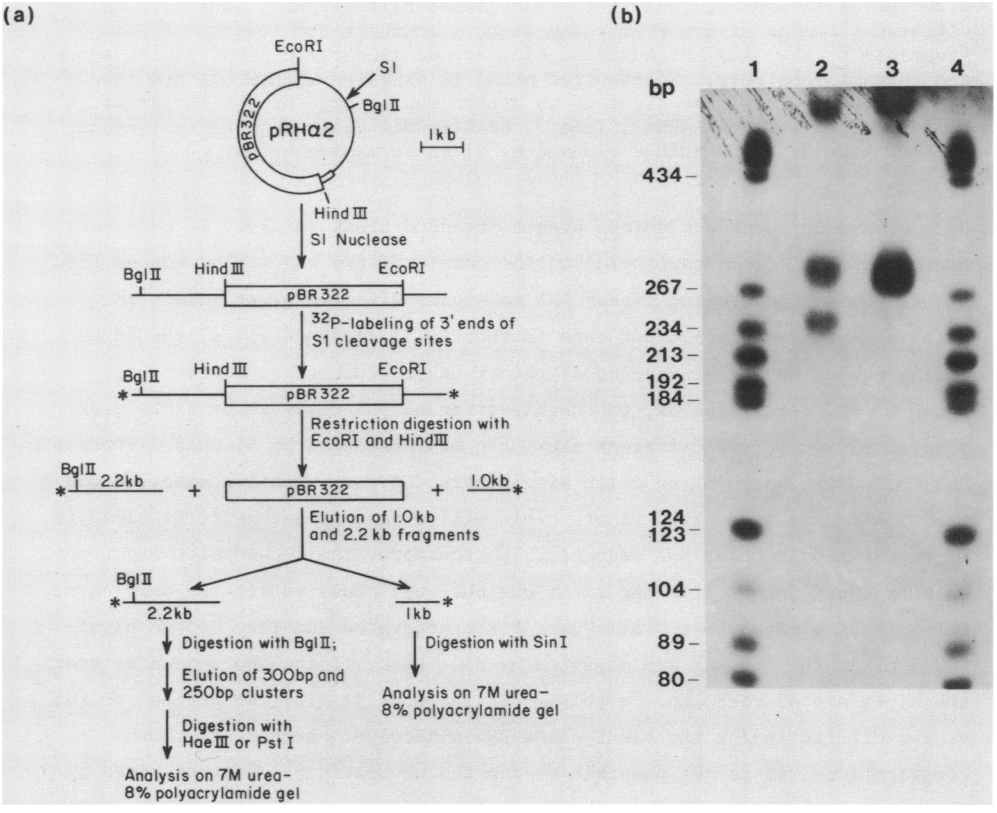

Figure 2. (a) Strategy of high resolution mapping of double-stranded cleavage sites of pRHα2 by nuclease S1. Twenty µg of S1-pRHα2 were prepared and end-labeled with ^{32}P as described in Materials and Methods. The ^{32}P-3' end-labeled DNA were then digested with HindIII or EcoRI and the released 2.2 kb and 1.0 kb fragments, respectively, were purified by agarose gel elution. The 1.0 kb EcoRI fragment was further digested with SinI, denatured, and analyzed by electrophoresis on a 7 M urea-8% polyacrylamide gel and autoradiography (see Figure 3d). The 2.2 kb HindIII fragment was first cut with BglII and then electrophoresed on a 5% polyacrylamide gel (see the autoradiograph in b). The two radioactive clusters with average sizes of 250 bp and 300 bp, respectively, were eluted from the gel and further digested with PstI or HaeIII. The products were then denatured and analyzed by electrophoresis in 7M urea-8% polyacrylamide gel and autoradiography (see Figure 3b and 3c). (b) Autoradiograph of the BglII digests of the 2.2kb fragment prepared as described in (a). Lane 1, marker, HaeIII digested pBR322 DNA fragments labeled with ^{32}P at their 3' ends; lane 2, BglII digest of the 2.2 kb fragment prepared from S1(FA)-pRHα2; lane 3, BglII digest of the 2.2 kb fragment prepared from S1(aq)-pRHα2; lane 4, marker, HaeIII digest of pBR322 DNA.

The approximate locations of these two clusters of S1 sites are indicated by "*" in the map of Fig. 3a. Each of the three clusters of DNA bands in the gel of Fig. 2b was eluted, redigested with PstI or HaeIII, and analyzed on denaturing gel using DNA sequencing markers made from pBR322 (Fig. 3b, 3c). The sizes of the bands seen on these DNA sequencing gels correspond to the precise distances between the S1 cleavage sites on the sense strand (with respect to α-globin gene transcription) and the restriction sites (PstI or HaeIII). The sites mapped in this way are indicated on the upper DNA strand of Fig. 4 by the arrows "↓" (for the BglII-250 bp cluster) and "↓" (for the BglII-300 bp cluster). Note that very similar patterns of S1 cleavage sites are obtained for the two different S1 buffer conditions (for example, compare lanes 1 and 2 of Fig. 3b or Fig. 3c). The general patterns are reproducible in three independent experiments.

To map the S1 cleavage sites on the other DNA strand, the eluted 1.0 kb fragment (Fig. 2a) was cut with SinI and analyzed on a denaturing gel with DNA sequencing markers (Fig. 3d). The sites mapped are indicated on the lower strand of Fig. 4 by the arrows "↑".

DISCUSSION

The S1 cleavage sites mapped as described above are summarized in Fig. 4. Several interesting conclusions can be obtained from the summary map of Fig. 4. (1) At least 15 of the major double-stranded S1 cleavage sites mapped are probably authentic ones based on the criterion that corresponding cuts on both DNA strands can be mapped. These authentic sites are located to the 5' side of the base pairs -1083, -1086, -1087, -1089, -1090, -1092, -1093, -1096, -1098, -1099, -1100, -1103, -1134, -1135, and -1137, respectively, upstream from the α1-globin gene. I have not identified which DNA strand is "nicked" first by S1 at each one of these 15 positions. (2) All the authentic S1 cleavage sites are located within DNA rich in polypyrimidine•polypurine sequences. In particular, twelve of these sites are mapped in a simple repeating $(CCT)_n$ sequence occasionally interupted by CCA. As noted in Fig. 4, S1 does not cut in this region at every possible base. Instead, some of the bonds are preferentially cut while others are not cut at all. This specific cleavage pattern may depend on the folding conformation when the DNA segment is under torsional strain . (3) Some of the cleavage sites can only be mapped on one of the two DNA strands. It may have resulted from the "nibbling" activity of the S1 nuclease. This is most likely to be the case for those cuts on the 5' side of the bases -1064, -1066, -1067, -1070,

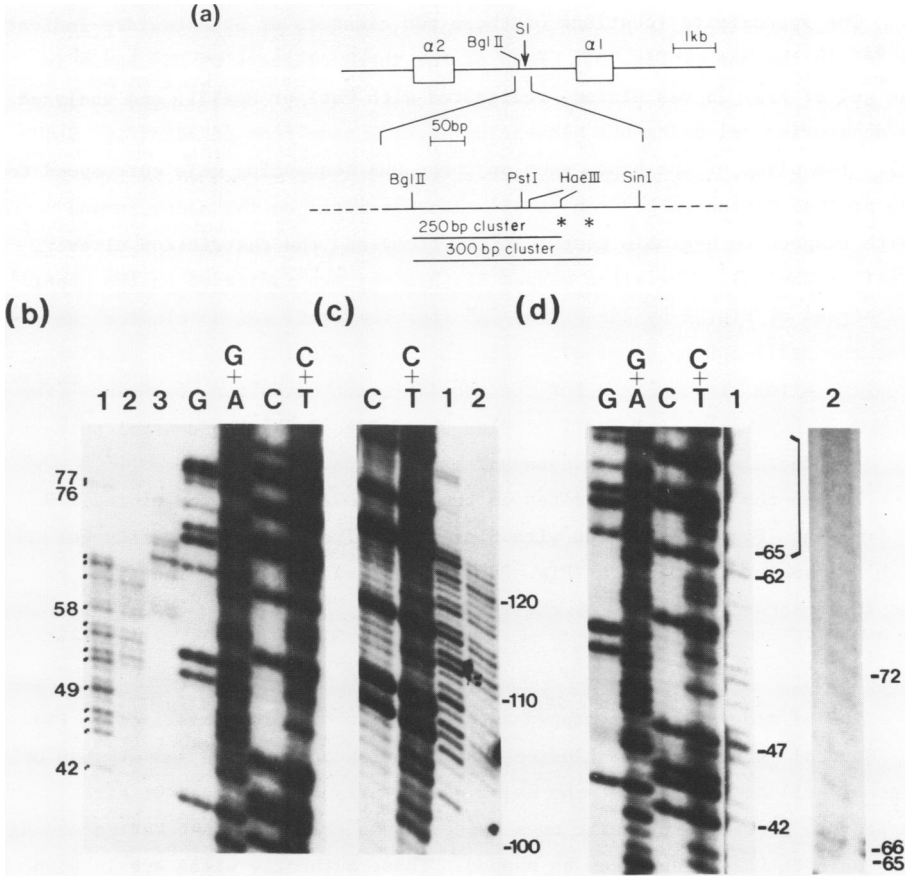

Figure 3. High resolution mapping of S1-nuclease cleavage sites in pRHα2. (a) Restriction maps of BglII, HaeIII, PstI and SinI sites surrounding the two clusters (*) of double-strand cleavage sites generated by S1. The locations of these restriction sites have been determined by both nucleotide sequencing and restriction mapping (13). For their exact positions relative to the cap site of α1 globin gene, see Figure 4. (b) and (c). High resolution gel analysis of restriction digests of the 300 bp and 250 bp clusters eluted from 2b. The 300 bp clusters isolated from both S1(aq)-pRHα2 and S1(FA)-pRHα2 as described in Figure 2 were digested with either HaeIII or PstI, purified by phenol and ether extraction, resuspended in deionized formamide, heated at 90°C for 3 minutes, and analyzed in 7M urea-8% polyacrylamide gel. A 631 bp EcoRI-Hinf fragment derived from pBR322 was labeled at the 3' end of the EcoRI site by filling in with (α-^{32}P) dATP, dTTP and Klenow fragment, subjected to Maxam-Gilbert chemical cleavage reactions, and used as size markers after heat denaturation. The numbers on the side of the bands in the autoradiographs indicate their sizes in nucleotides. (b), lane 1, HaeIII digest of the 300 bp cluster isolated from S1(aq)-pRHα2. The dots on the side denote the major bands on the gel; lane 2, HaeIII digest of 300 bp cluster isolated from S1(FA)-pRHα2; lane 3, PstI digest of the 250 bp cluster isolated from S1(FA)-pRHα2. c, lane 1, PstI digest of the 300 bp cluster

from S1(aq)-pRHα2; lane 2, PstI digest of the 300 bp cluster from S1(FA)-pRHα2. (d). High resolution gel analysis of the SinI digest of 1.0 kb fragment obtained from S1(aq)-pRHα2. The 1.0 kb fragment obtained as described in Figure 2a was digested with SinI, purified and denatured as described above, and electrophoresed on 7M urea-8% polyacrylamide gel (lane 1). The bands longer than 65 nucleotides were separated better on a longer run and the ones having lengths between 65 and 80 nt are shown in lane 2. Note that although the DNA sequencing markers and the samples were loaded on the same gel, they have been exposed to X-ray film for different periods of time in order to get even intensity. The bands corresponding to the 3 S1-cleavage sites on the 5' sides of bases -1134, -1135, and -1137 (see Fig. 4) are not shown.

-1071, -1072, -1074, -1076, and -1077 because they are located within an AT-rich region. This also explains the absence of mappable S1 sites on the lower DNA strand on the 5' side of base pairs -1081, -1080, -1069, and -1068, respectively. The AT-rich region in between bases -1065 and -1080 may also have caused the relatively low frequencies of detection of the S1 sites mapped on the lower strand in between bases -1089 and -1104.

The S1-mapping results described strongly suggest that double-stranded, simple repeating polypyrimidine•polypurine sequences may adopt a specific modified B or non-B DNA conformation in solution. X-ray data have shown that several simple repeating DNA sequences, including poly[d(A-G)]•poly[d(T-C)] can adopt modified B or non-B conformation in fiber (14,15). Johnson and Morgan (16) have demonstrated that poly[d(T-C)]•poly[d(A-G)], poly(d(T-T-C)]•poly[d(A-A-G)], and poly[d(C-C-T)]•poly[d(G-G-A)] can form tetrastrand complexes in solutions of low pH. More recently, two simple repeating sequences, $(dC\text{-}dG)_n \cdot (dG\text{-}dC)_n$ and $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$, have been demonstrated to adopt left-handed or Z-DNA conformation in physiological ionic conditions under negative supercoil strain (6,17-19). In particular, the junctions between $(dC\text{-}dG)_n \cdot (dG\text{-}dC)_n$ sequence and its flanking DNA, when present on a circular DNA molecule with appropriate supercoil turns, is sensitive to S1 digestion (6). The other more likely explanation for the S1 nuclease sensitivity of this region is that, due to the repetitiveness of the sequences CCT, a helix with mismatched, single-stranded loops could form (slippage mechanism) under supercoil torsion. These DNA mismatches would be recognized and cleaved by the nuclease S1. This model is consistent with the finding that, as shown in Fig. 4, the nuclease S1 preferentially cuts the sugar-phosphate bonds within a CCT sequence but not those connecting two adjacent CCT repeating units. The slippage mechanism has previously been proposed for the S1 nuclease sensitivity of another simple repeating sequence (AG)n (7).

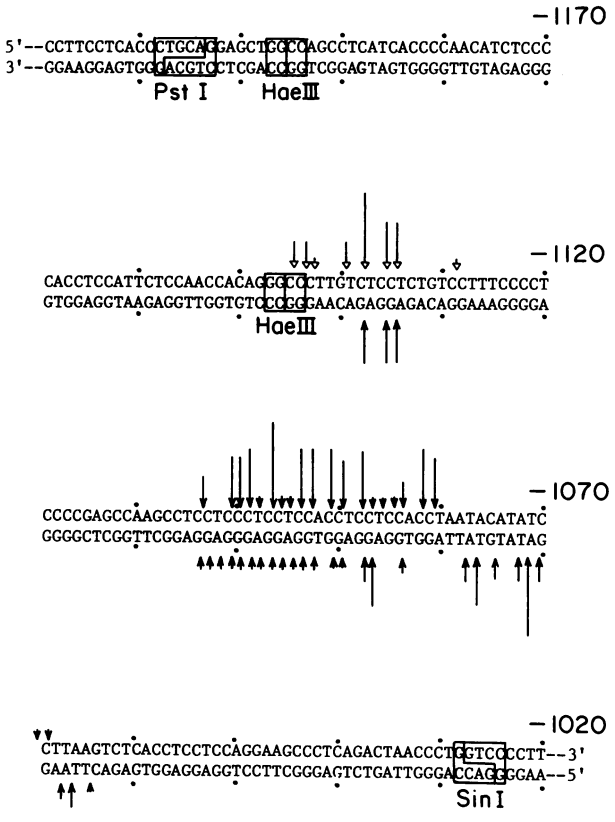

Figure 4. Distribution of the S1-cleavage sites in the human α2-α1 globin intergenic region. The mapping results of S1-cleavage sites on either DNA strand from experiments described in Figures 1-3 are summarized here in the double-stranded DNA sequences from 1020 bp to 1220 bp upstream from the cap site of α1-globin gene (13). Also shown in the figure are the positions and cutting sites of the restriction enzymes PstI, HaeIII, and SinI. ↓: sites mapped by experiments described in Figure 3b (lanes 1 and 2) and in Figure 3c. ⇓: Sites mapped by experiment described in Figure 3b, lane 3. ↑: Sites mapped by experiments described in Figure 3d. The relative sizes of these marks indicated their approximate relative cutting frequency within each of the two clusters of sites as judged from the band intensities shown in Figure 3.

Polypyrimidine•polypurine sequences occur widely in eukaryotes including human, mouse, Drosophila and yeast at intervals of 6-8 kb throughout the genomes (20). They have been proposed to play a significant role in chromosome organization and genome rearrangement. It is interesting to note that the S1-hypersensitive region described in this study is one of the

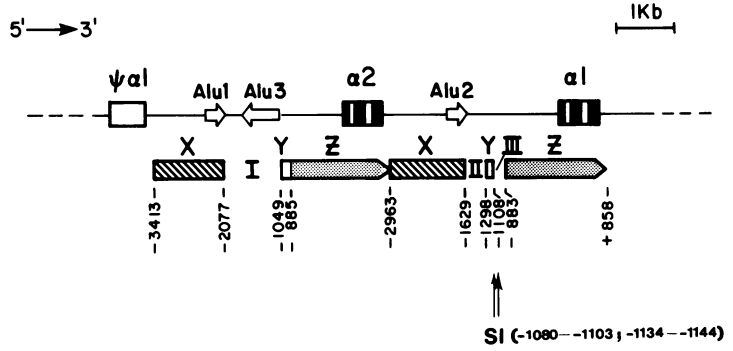

Figure 5. Precise positions of the clustered S1-cleavage sites in the human α1 globin gene-containing duplication unit. The top map shows the locations of the two homologous, duplicated DNA units with the three non-homology blocks (I, II, and III) resulted from DNA rearrangement during evolution (ref. 13). The numbers on the boundaries of these non-homology blocks and the three homology blocks (X, Y, Z) show their positions relative to the 5' end of α2 or α1 mRNA. A minus sign indicates the position is upstream, and a plus sign indicates it is downstream from the capping site of the mRNA. The arrows show the locations of the major (large arrow) and minor (small arrow) clusters of S1-cleavage sites mapped as described in Fig. 1-4.

three places in human adult α-globin gene region where gross DNA rearrangements have occurred during evolution (Fig. 5; ref. 13). At the moment, we do not know whether the S1-hypersensitivity described above exists in vivo and whether it correlates with expression of the α-globin genes.

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