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Characterization of a supercoil-dependent S1 sensitive site 5′ to the *Drosophila melanogaster hsp 26* gene

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We have analyzed the prominent supercoil-dependent S1 nuclease cleavage site 5′ to hsp 26 in the plasmid 88B13, which contains 11.7 kilobases from the Drosophila locus 67B1. The double stranded cleavage product is generated by initial nicking on the purine strand, six preferred sites occurring between positions -96 and -90 (relative to the start of transcription) with weaker ones extending to position -84, followed by cleavage on the pyrimidine strand at positions -86 and -84. A derivative of 88B13, 88B13-X, was generated by insertion of an Xho I linker at position -84; this does not affect the positions or strand specificity of the S1 cleavage in that region. A small deletion, Δ41.1, removes the homopurine/homopyrimidine stretch from positions -86 to -132 and is no longer sensitive to cleavage by S1 nuclease 5′ to hsp 26. Mung bean and P1 nucleases recognize the same site 5′ to hsp 26 and give the same general pattern of cleavage. All three nucleases show an initial cleavage of 88B13 DNA at this site at pH 5.5 but not at pH 6.5, indicating that the DNA structure there may be pH dependent in vitro.

INTRODUCTION

DNA can assume a variety of conformations other than B-form (1). There have been suggestions that these alternative structures may play a role in the regulation of gene expression (see 2 for review). In some cases the formation of these structures is a function of the superhelical density of the DNA, and there is evidence that the degree of superhelicity affects promoter function and hence gene expression in some prokaryotic operons (see 3, 4 for reviews). In eukaryotic systems there are several reports that DNA topology can affect gene expression (5-8).

Selleck et al (9) investigated the presence of novel DNA structures formed in response to supercoiling in the plasmid 88B13 by mapping single strand-specific nuclease cleavage sites. This plasmid contains four genes from the Drosophila small heat shock gene cluster at locus 67B1 (10). A site very sensitive to cleavage by S1 nuclease was detected 5′ to the gene encoding the small heat shock protein of 26 kilodaltons, hsp 26. In addition a weaker site was detected near the 5′ end of another gene, hsp 28. There have been many other reported cases of S1 sensitive sites in the regulatory

regions of other genes; in some cases, there is evidence that these sites exist in vivo. Earlier work (11,12) has indicated an S1 sensitive site 5′ to the β-globin gene in chick red blood cell chromatin; Han et al (13) have reported a DNA structure 5′ to the Drosophila hsp 70 gene sensitive to both S1 and Neurospora crassa nucleases in isolated nuclei.

In this paper we have focused on the S1 sensitive site 5′ to hsp 26. This site is particularly interesting because it lies between two DNase I hypersensitive sites mapped in the chromatin 5′ to this gene, just upstream of the heat shock consensus sequence (14). DNase I hypersensitive sites are nucleosome-free regions that have been correlated with the potential for gene expression. One can hypothesize that the formation of these sites may involve a DNA conformation that is incompatible with nucleosome formation (15, 16 for reviews). The location of the S1 sensitive region in the vicinity of DNase I hypersensitive sites 5′ to hsp 26 suggests the possibility that this DNA structure could affect the formation of the chromatin structure and/or the the expression of the gene. In an attempt to understand what DNA sequences are required to form the S1 sensitive site 5′ to hsp 26, we have mapped the location and strand specificity of nuclease cleavage in plasmid 88B13 at the nucleotide level using S1, mung bean and P1 nucleases. We have also generated a small deletion of 46 base pairs (bp) that removes completely the S1 sensitivity in this region. We find that a long stretch of homopurine/homopyrimidine sequence 5′ to hsp 26 appears to be responsible for the sensitive structure. The results indicate the importance of analyzing cleavage patterns on both strands of DNA in considering potential models of alternative DNA structures.

MATERIALS AND METHODS

Plasmid Constructions.

Supercoiled DNA was purified either by the boiling method (17) or alkaline lysis (18) as described in Maniatis et al (19).

88B13-X was constructed as follows: supercoiled 88B13 plasmid DNA was digested with S1 nuclease (Boehringer Mannheim) as described in Selleck et al (9). Linearized DNA was gel purified, the ends filled using Klenow (Boehringer Mannheim), and Xho I linkers (New England Biolabs) added to the ends as described in Maniatis et al (19). Following removal of excess linker by Xho I digestion the plasmid was recircularized using T4 DNA ligase (Bethesda Research Laboratories). The site of insertion was determined by chemical sequencing (20) of an Pst I-Eco RI fragment spanning this region (Figure 1). This revealed the loss of two nucleotides (TA) at positions -84

and -85 in 88B13 and the loss of one "G" from the Xho I linker (Figure 4; distances are given in base pairs relative to the start of transcription described in reference 21).

Deletions of 88B13-X (designated Δ35, Δ36, Δ41, Δ43 and Δ65) were generated by linearizing 80 µg of 88B13-X with Xho I, incubating with 5 units of exonuclease III (Bethesda Research Laboratories) for 30 minutes at 37°C under the conditions recommended by the manufacturers; purifying the DNA by phenol (plus 0.1% hydroxyquinoline) extraction, chloroform:isoamyl alcohol (24:1) extraction and ethanol precipitation; and incubating the DNA with 2000 units of S1 nuclease at 25°C for 20 minutes. The DNA was purified, the ends filled in with Klenow, Xho I linkers added and the plasmids recircularized all as above. Deletions recovered were mapped to a resolution of ±10 bp by end-labelling at the Xho I site (19), followed by cleavage with either Sac I or Pst I (578 bp 3′ and 640 bp 5′ to the Xho I linker in 88B13-X respectively), and separation on a 10% acrylamide gel run in 1xTBE (90mM Tris, 90mM boric acid, 2.5mM EDTA) which was dried and exposed to Kodak XAR X-ray film. The S1 sensitivity of supercoiled plasmid DNA for each deletion was determined as described in Selleck et al (9).

Δ41.1 was generated by replacing an Xho I-Bgl II fragment of Δ41 with a gel purified Xho I-Bgl II fragment of 88B13-X (containing hsp 26 and its flanking regions, Figure 1) thus restoring the 3′ side of the deletion in Δ41 to its wild type sequence. The desired plasmid was identified by restriction analysis and its sequence determined by dideoxy sequencing (22; Figure 4) of the Pst I-Xho I fragment (containing the deletion) and the Xho I-Eco RI fragment (to confirm the restoration of sequences between the linker and hsp 26). Sequencing across the region of the deletion was repeated during the high resolution analysis of S1 nuclease cleavage (Figure 3b and 3c).

All restriction enzymes purchased either from New England Biolabs or International Biotechnologies Inc. and reactions performed in either 1xRE (50mM Tris-Cl pH 8.0, 10mM $MgCl_2$, 50mM NaCl) or 1xUB (33mM Na acetate, 10mM Mg acetate, 66mM K acetate, 0.5mM DTT, 0.1 mg/ml nuclease-free BSA).

S1 Analysis: Low Resolution.

For analysis at pH 5.0 supercoiled plasmid DNA (4 µg) was incubated with 3 units of S1 nuclease in a 40 µl reaction containing S1 buffer (200mM NaCl, 50mM Na acetate pH 5.0, 1mM $ZnSO_4$, 0.5% glycerol) at 25°C for 15 minutes. The samples were immediately extracted once with phenol (plus 0.1% hydroxyquinoline), once with chloroform:isoamyl alcohol (24:1), then with diethyl ether, and the DNA precipitated with ethanol. The samples were resuspended in 1xRE and digested to completion with Bam HI. As a linear

control, supercoiled plasmid DNA was first restricted with Bam HI, purified and then incubated with S1 nuclease in the conditions described above. For analysis at pH 5.5 and pH 6.5 plasmid DNA (2.5 µg) was incubated with 3 units of S1 nuclease at the relevant pH in single strand nuclease buffer (SSNB- 200 mM NaCl, 50mM 2-[N-morpholino] ethane sulfonic acid (MES) pH 5.5 and 6.5 respectively, 1mM $ZnSO_4$, 0.5% glycerol) at 25°C for 15 minutes and 30 minutes. The DNA was purified as described for samples treated with S1 nuclease at pH 5.0 and digested to completion with Sac I. All samples were then separated electrophoretically in a 1% agarose gel in 1xTAE (40mM Tris-Cl pH 7.5, 20mM Na acetate, 2mM EDTA). The DNA was transferred to nitrocellulose (23) and hybridized to the appropriate nick-translated fragment of 88B13 (bars a,b and c in Figure 1) in an indirect end-labelling experiment (24,25). Nick translation (26) was performed as described in Maniatis et al (19).

S1 Analysis: Fine Structure.

Nicking by S1 at pH 5.0 was carried out in S1 buffer and at pH 5.5 and 6.5 in single strand nuclease buffer as described above. In a typical reaction 40 µg of plasmid DNA in 640 µl of S1 buffer was treated with 480 units of S1 at 25°C. 80 µl (5µg) aliquots were removed at appropriate times and the reaction halted by bringing the samples to 20mM EDTA. 0.25 µg of this was analyzed for the degree of nicking on a 0.7% agarose gel run in 1xTAE (see above) at 1v/cm for 15-20 hours with recirculation of the buffer. The remainder of the samples were extracted once with phenol/chloroform (1:1 plus 0.1% hydroxyquinoline), once with diethyl ether, and passed over a 1 ml Sephadex G50 (Pharmacia- DNA grade) spun column, equilibrated in water, to remove the bulk of the EDTA. 20 µl of the eluate was digested to completion with Hha I. Digests were precipitated with ethanol and resuspended in sequencing gel loading buffer (19) for high resolution indirect end-labelling analysis.

For high resolution analysis ~0.6 µg DNA was denatured and run on a 6% polyacrylamide sequencing gel in 1xTBE plus 8M urea (19) at a constant 30 watts. As markers Hha I digested plasmid DNA which had been subjected to chemical sequencing reactions (20) was run in parallel. Sequencing gels were electroblotted to "Nytran" membrane (Schleicher and Schuell-pore size 0.45 µm) and the DNA immobilized by ultraviolet crosslinking as described by Church and Gilbert (27).

RNA probes were synthesized using phage T7 or T3 RNA polymerase transcripts of an Eco RI-Hha I fragment (see Figure 1d) cloned into the "Bluescribe" vector (Vector Cloning Systems) and linearized at Hind III or Eco RI sites in the polylinker, respectively. The T7 transcripts are specific

for the transcribed strand and the T3 transcripts for the non-transcribed strand. Each transcription reaction contained 1 µg linearized template, 100 µCi of 3000 Ci/mmol α-^{32}P-UTP (ICN Radiochemicals), 40 mM Tris-Cl pH 8.0, 8mM $MgCl_2$, 50mM NaCl, 2mM spermidine, 0.8U/µl RNasin (Promega Biotech), 30mM DTT, 0.4mM ATP, 0.4mM CTP, 0.4mM GTP and 25 units of enzyme (T7 from United States Biochemical, T3 from Vector Cloning Systems). The final volumes of the reactions were 10 µl for T7 RNA polymerase and 3 µl for T3 RNA polymerase. Incubation was at 37°C for 1.5 hours whereupon the products were ethanol precipitated twice and used for probing directly as described by Church and Gilbert (27). Hybridizations of filters, approximately 15x35 cm, were in 3 ml at 65°C for 16-18 hours.

Other Single Strand Nucleases: Fine Structure.

Nicking by mung bean (Pharmacia), P1 (Pharmacia) and N.crassa (Pharmacia) nucleases was carried out in SSNB at pH 5.5 or 6.5 as described above. Phosphodiesterase I from Crotalus adamanteus venom (Sigma) was used in 20mM Tris.HCl pH 9.0, 1mM $MgCl_2$ at 25°C. All reactions were done with 40µg of plasmid DNA in 640 µl at 25°C. Enzyme concentrations and incubation times were chosen to approximately equalize the extent of nicking in any given experiment (judged by ethidium bromide staining of samples analyzed by agarose gel electrophoresis). All reactions were halted with the addition of EDTA to 36mM and the samples treated as described above under S1 Analysis: Fine Structure.

RESULTS

Figure 1 is a partial restriction map of the 11.7 kb of Drosophila DNA in the pBR322 derived plasmid, 88B13, the starting material for most of the studies reported here. Initial mapping of the S1 sensitive site 5′ to hsp 26 is shown in Figure 2 lane 1. This confirms the presence of a strong site of S1 cleavage approximately 100 bp from the start of transcription of hsp 26. The results shown in Figure 2, lane 2 illustrate that the cleavage by S1 occurs only in supercoiled DNA; when the plasmid is first linearized with Bam HI and then incubated with S1 nuclease no cleavage at this site is detected. (The 4.3 kb band that is apparent in all lanes is due to hybridization of contaminating pBR322 sequences present in the preparation of the Eco RI-Bam HI fragment used as a probe.)

We investigated this site in a more detailed fashion, using a higher resolution indirect end-labelling approach (27) to map cleavage at the nucleotide level. This is illustrated in Figure 3. Plasmid DNA was incubated with S1 nuclease for the specified times and restricted with Hha I. The DNA

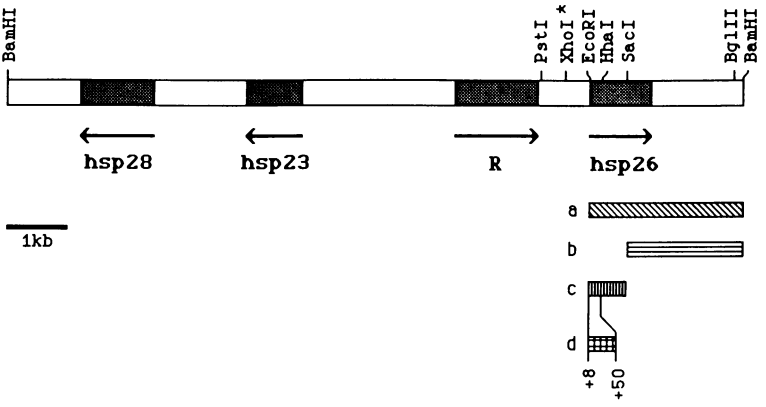


Figure 1: Partial restriction map of the Drosophila DNA insert in the plasmid 88B13. The vector is pBR322 (not shown) and the site of insertion is Bam HI. The positions of the four genes are represented by the shaded segments; the arrows indicate direction of transcription. Bars labelled a, b and c, indicate the 2.3kb Eco RI-Bam HI, 1.85kb Sac I-Bam HI and 480bp Eco RI-SacI fragments respectively, used in the indirect end-labelling for low resolution experiments. The bar labelled d indicates an enlarged representation of the 42 bp Eco RI-Hha I fragment cloned into the 'Bluescribe' riboprobe vector, transcripts of which in each direction are used as strand specific probes in indirect end-labelling experiments at the nucleotide level. The Xho I site, marked by an asterisk, is only present in derivatives of 88B13 described in this work

fragments were then separated on a sequencing gel, alongside plasmid DNA markers described in Materials and Methods. The DNA in the acrylamide gel was transferred to a nylon membrane by electroblotting and probed using a short strand-specific probe abutting the Hha I restriction site (labelled transcripts from the Eco RI-Hha I fragment illustrated in Figure 1). This allows us to map S1 cleavages to the nucleotide on both strands of the DNA. Note that as the sites are mapped indirectly, no labelling of the plasmid DNA itself is needed. This strategy has the additional advantage of being directly applicable to an investigation of the DNA structure in vivo.

Figure 3, panel (a) shows the degree of S1 cleavage of samples used in this study. DNA prior to Hha I digestion was separated on an agarose gel and stained with ethidium bromide. These lanes correspond to the samples used for the similarly numbered lanes in panels (b) and (c). In panel (b), lanes 1-6, 88B13 was incubated with S1 nuclease for 0.5 -20 minutes, respectively, and the S1 cutting sites in the GA strand mapped using radiolabelled transcripts of an Eco RI-Hha I fragment (Figure 1) as a strand specific probe. ('GA' strand refers to the transcribed strand which is predominantly G and A

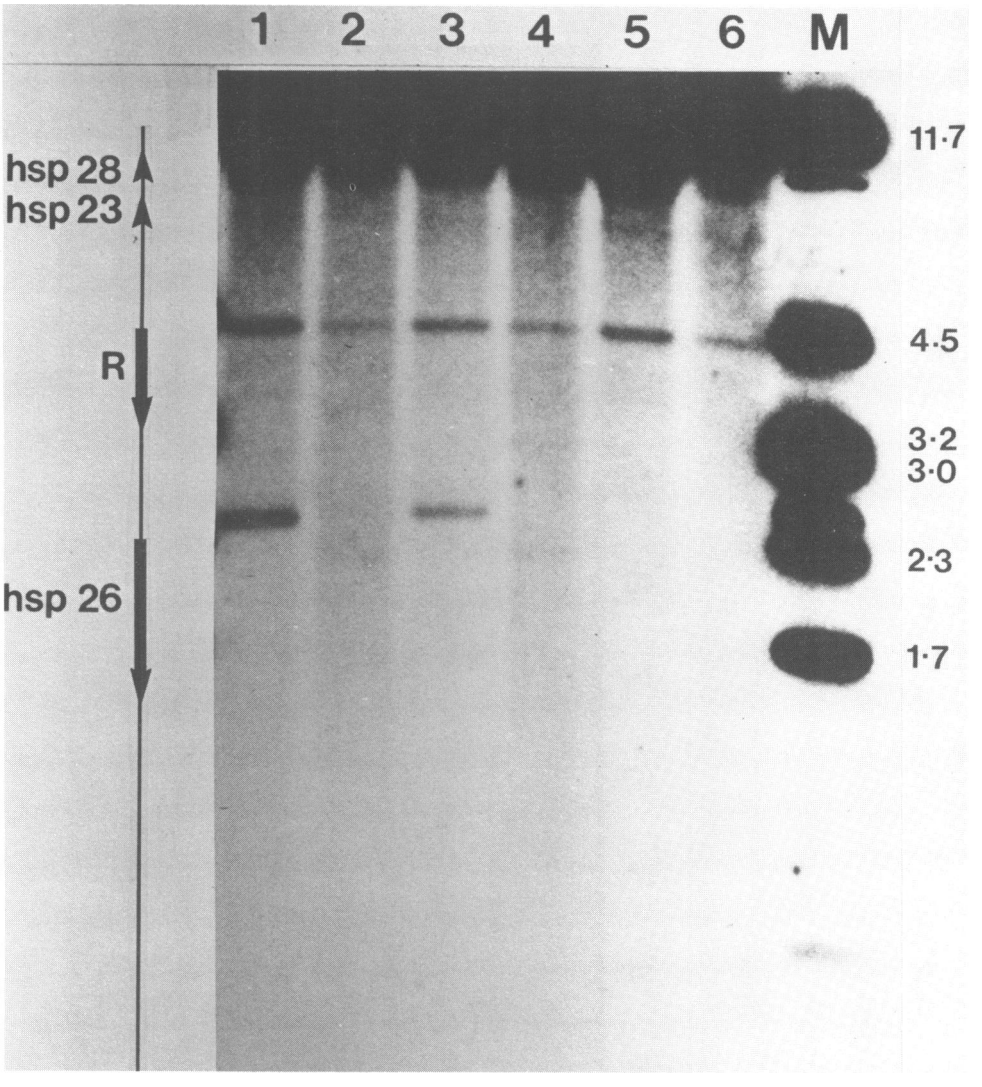
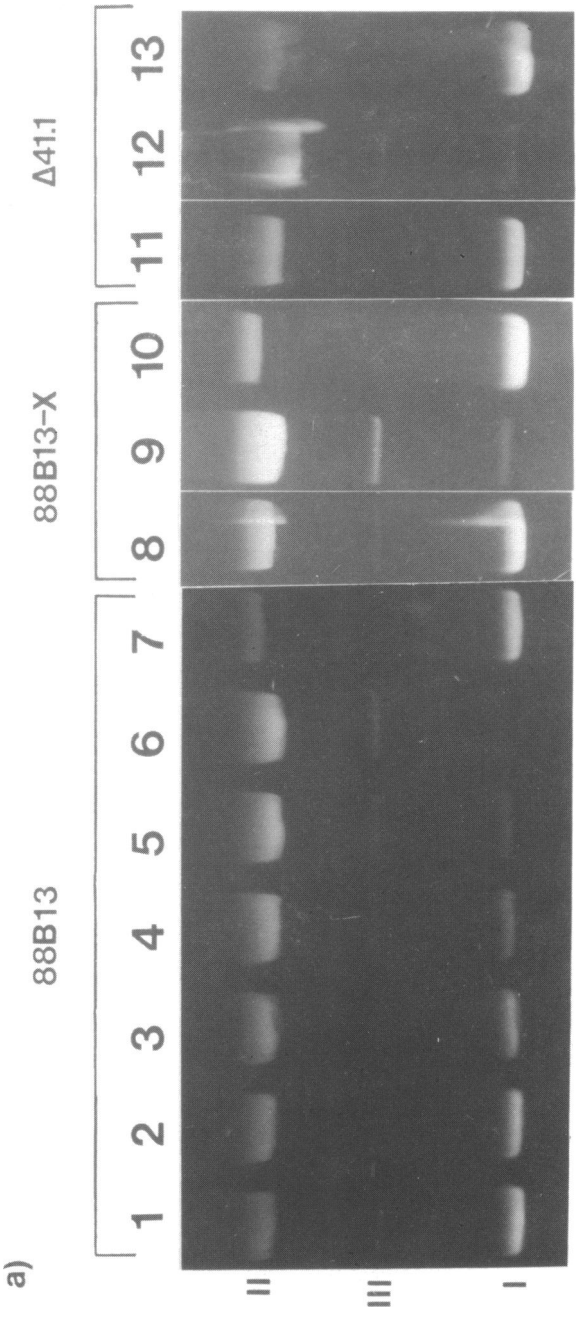
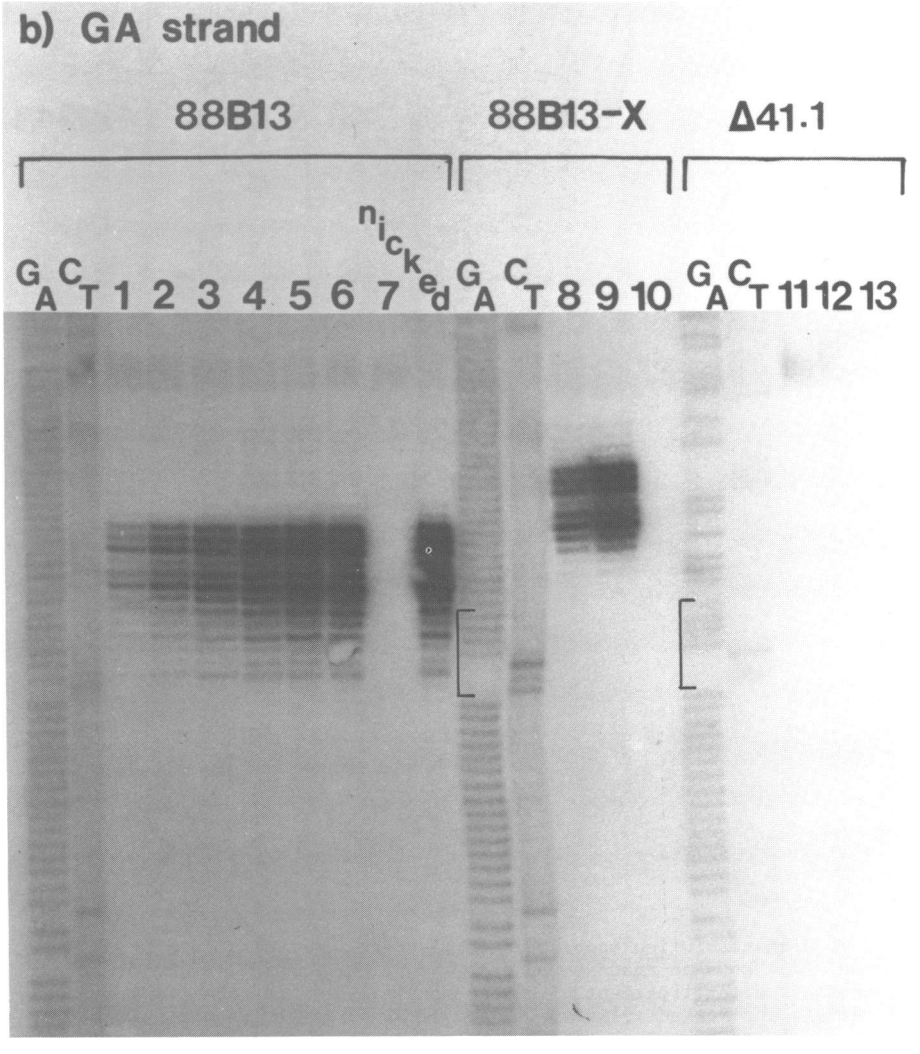


Figure 2: Mapping S1 cleavage sites in 88B13, 88B13-X and Δ41.1. The indirect end-labelling procedure was used to map S1 cleavage relative to the Bam HI site as described in Materials and Methods. The Eco RI-Bam HI fragment indicated in Figure 1 was used as a probe. Lanes 1,3 and 5; supercoiled plasmids 88B13, 88B13-X and Δ41.1 respectively incubated with S1 nuclease followed by Bam HI digestion as described in Materials and Methods. Lanes 2, 4 and 6: The linear controls for S1 digestion of 88B13, 88B13-X, and Δ41.1 respectively; these plasmids were first cut with Bam HI and then treated with S1 nuclease as described in Materials and Methods. M: molecular weight markers.


a)
88B13
88B13-X
Δ41.1
1
2
3
4
5
6
7
8
9
10
11
12
13
II
III
I


b) GA strand
88B13
88B13-X
Δ41.1
nicked
G A C T 1 2 3 4 5 6 7
G A C T 8 9 10
G A C T 11 12 13

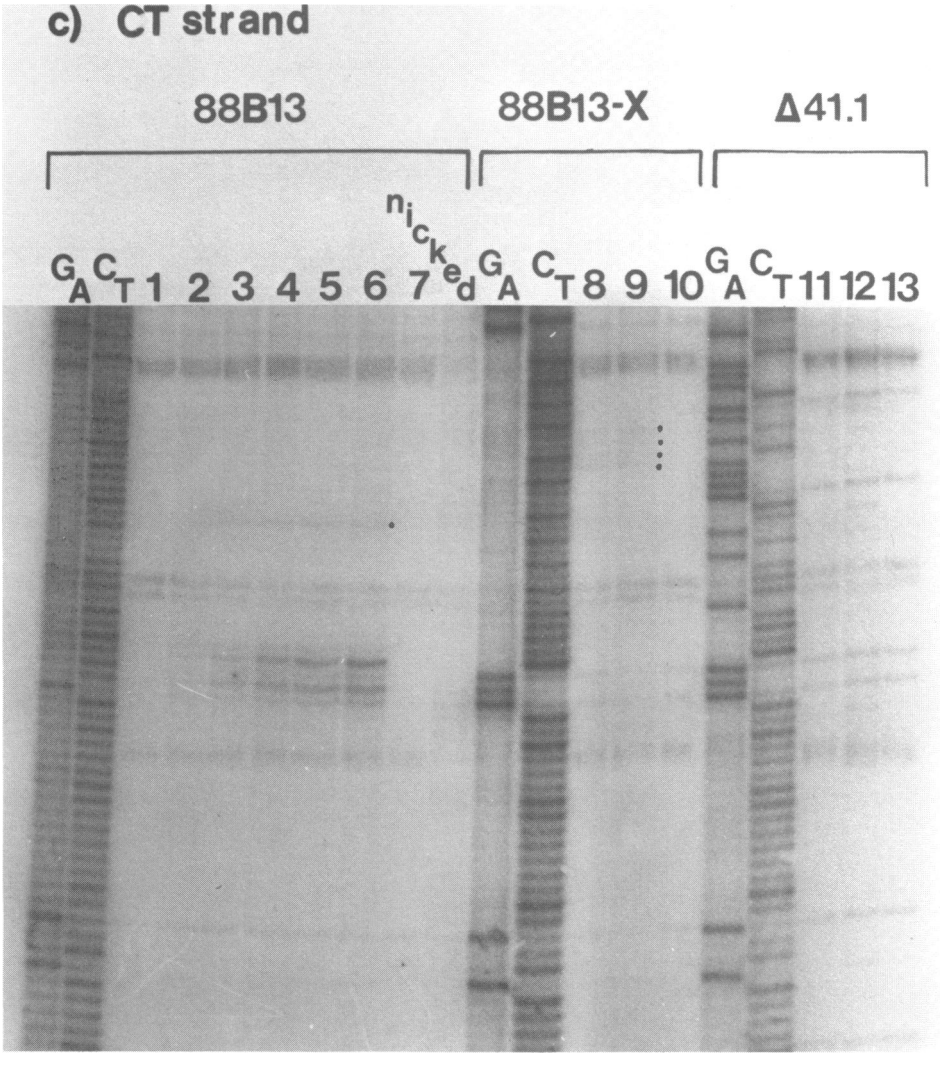


Figure 3: High resolution mapping of S1 cleavage sites 5′ to hsp 26.
Panel (a) shows an agarose gel analysis of 88B13, 88B13-X and Δ41.1 plasmid DNA's after treatment with S1 nuclease under the conditions described in Materials and Methods; lanes 3, 8 and 11 are 2 minute incubations, lanes 1, 2, 4 and 5 are 0.5, 1, 5 and 10 minute incubations respectively, and lanes 6, 9 and 12 are 20 minute incubations. As a control lanes 7, 10 and 13 show DNA that was incubated in S1 buffer in the absence of S1 for 20 minutes. I,II and III indicate supercoiled, open circle and linearized plasmids respectively.
Panel (b) shows aliquots of the same samples seen in (a) analysed by indirect end-labelling after Hha I digestion and electrophoresis on a 6% polyacrylamide sequencing gel (27). Samples labelled GA and CT are sequencing

ladders used as markers. The sample designated "nicked" was open circle DNA electrophoretically purified from an S1-treated preparation of 88B13 subsequently digested with Hha I. The probe used in this experiment was the phage T7 RNA polymerase transcript specific for the GA strand as described in Materials and Methods. The bracket indicates the Xho I linker in 88B13-X which causes all upstream sequences (including the site of S1 cleavage) to shift upwards several nucleotides on the sequencing gel.

Panel (c) shows aliquots of the same samples as in (b) but hybridized this time to a phage T3 RNA polymerase transcript specific for the CT strand. Dots indicate faint S1 cleavage sites.

Autoradiographic exposures have been standardized relative to the intensity of the markers for each experiment.

Note that since chemical sequencing eliminates a nucleoside (20) the equivalent oligonucleotide in the sequencing ladder runs one base lower than its counterpart in the S1 samples and further, that the equivalent oligonucleotide in the sequencing ladder in (b) has a 3'-phosphate group which causes these fragments to run approximately an additional half a nucleotide ahead, thus displacing the sequencing markers 1.5 nucleotides from the S1 samples (46).

between positions -69 and -110. 'CT' strand refers to the complement of this strand.) The cleavage by S1 nuclease on the transcribed strand lies in a stretch of purines mapping between positions -86 and -96. There is a cluster of approximately 4-6 strong cuts (lane 1) and a series of weaker ones that are seen on longer incubation with S1 (lanes 2-6). When the S1 sites are mapped on the CT strand (Figure 3c) cleavage is not detected until longer incubation times (compare 3b lane 1 to 3c lanes 1 and 6) and there appear to be just two strong cleavage sites at positions -86 and -84.

To determine whether any strand bias exists in the initial S1 cleavage, open circle DNA was electrophoretically purified from an S1 treated sample and subjected to the same analysis as above. Interestingly the nicks map on the GA strand only; when probed specifically for the CT strand there is no cleavage (compare lane 'nicked' in Figure 3b and 3c). The cleavage patterns for 88B13 5' to hsp 26 are summarized in Figure 7.

An alternate means of studying this unusual DNA structure is to define the sequences required to create it. We generated a series of deletions that remove the S1 sensitivity 5' to hsp 26 in 88B13, the smallest of which was 46 bp long. A schematic diagram illustrating the protocol used to generate these deletions is shown in Figure 4 and further described in Materials and Methods. 88B13-X is a useful intermediate for generating deletions in and around the S1 site because it introduces a unique restriction site at which the plasmid can be linearized. The site of insertion of the Xho I linker in 88B13 is consistent with the site of linearization by S1 nuclease as determined in Figure 3. We believe that the loss of two nucleotides in 88B13-X may be due to nibbling by S1 nuclease.

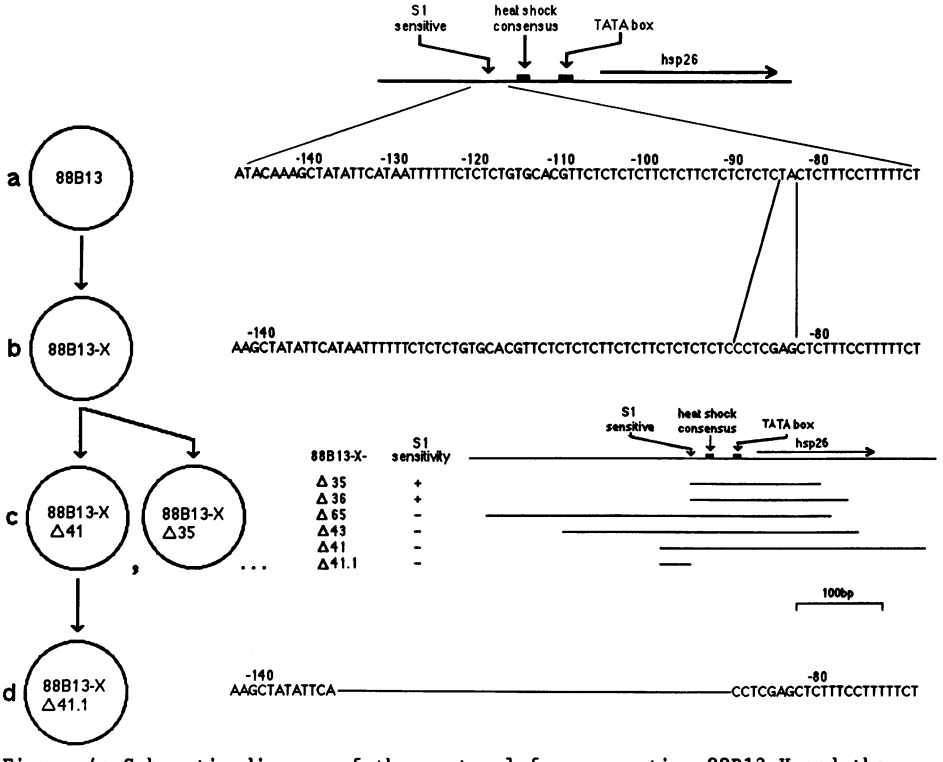


Figure 4: Schematic diagram of the protocol for generating 88B13-X and the various deletions of this plasmid.

a: 88B13 and the sequence in and around the S1 sensitive site 5′ to hsp 26.

b: 88B13-X was generated by linearizing 88B13 with S1 and inserting an Xho I linker. The resulting sequence is as indicated. In generating the plasmid two nucleotides, TA, at positions -84 and -85 were deleted from 88B13 and one G was lost from the Xho I linker.

c: The five deletions Δ35, Δ36, Δ65, Δ43 and Δ41 were generated by linearizing 88B13-X with Xho I and deleting sequences with exo III followed by S1 digestion; then the Xho I linker was reinserted. These plasmids were mapped and assayed for S1 sensitivity as described in Materials and Methods.

d: Δ41.1 was generated by recombining in vitro Δ41 with 88B13-X. A Xho I-Bgl II fragment of 88B13-X was used to replace the Xho I-Bgl II fragment which was partly deleted in Δ41. The resulting sequence is as indicated.

88B13-X was tested for the presence of an S1 site 5′ to hsp 26 at the level of resolution of agarose gels, the site appeared identical to the one in 88B13 (Figure 2, lanes 3 and 4), being in the same location and supercoil-dependent. Mapping at the sequence level (Figure 3b and 3c, lanes 8-10) demonstrates that cleavage occurs on the GA strand at the same dinucleotides as for 88B13. Note that the cleavage pattern appears to be shifted up relative to that of 88B13, due to the insertion of the Xho I

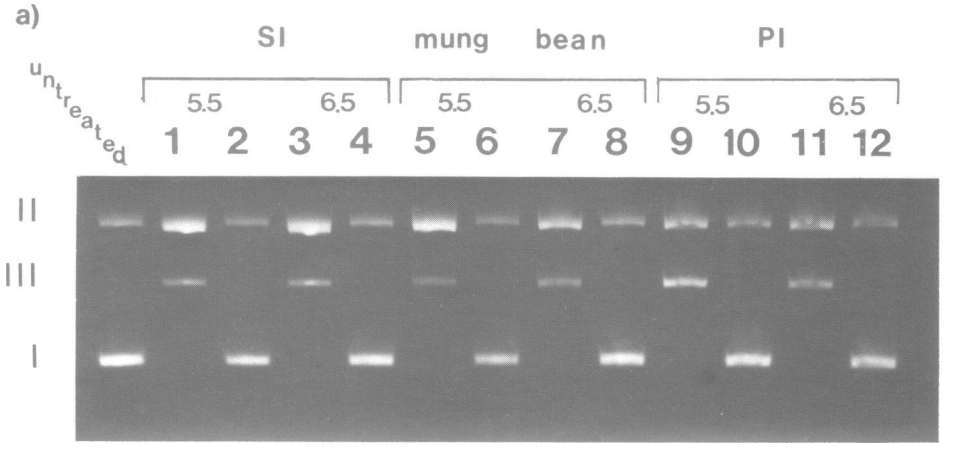
linker which is indicated in Figure 3. 88B13-X was then used to generate deletions in the region of the S1 sensitivity 5′ to hsp 26 by linearizing the plasmid with Xho I and deleting nucleotides by exonucleaseIII followed by S1 digestion. The Xho I linker was reinserted in all cases. Attempts to produce deletions using Bal 31 were unsuccessful, suggesting that this sequence is resistant to digestion by that enzyme. We generated a variety of deletions, five of which (Δ35, Δ36, Δ65, Δ43 and Δ41) were mapped (±10 bp) and assayed for the S1 sensitive site 5′ to hsp 26 (data not shown). A summary of the results is given in Figure 4c. Given the results with these relatively large deletions we deduced that the sequences responsible for generating the S1 site lay in the common sequences deleted in Δ41, Δ43 and Δ65. To test this directly we generated a small deletion of 46 bp, Δ41.1, by in vitro recombination of Δ41 and 88B13-X (see methods section); the sequence is shown in Figure 4d. Δ41.1 was assayed for S1 sensitivity at both the level of resolution of agarose gels (Figure 2, lanes 5 and 6) and acrylamide gels (Figure 3b and 3c, lanes 11-13); in both cases there is no evidence of prominent S1 cutting in the region 5′ to hsp 26, indicating that we have deleted the sequences responsible for S1 sensitivity at this location. A few weak nicks are distributed across the fragment we have examined (Figure 3b and 3c, lanes 12-13); however, they are not clustered in a single region and do not constitute the appearance of a new prominent S1 cleavage site.

We have also examined the cleavage pattern of a variety of single strand-specific nucleases 5′ to hsp 26 in supercoiled plasmid 88B13. All samples treated with nuclease were equalized for the extent of nicking as shown in Figure 5a. Figure 5b shows that mung bean and P1 nucleases, at pH 5.5, have similiar cleavage patterns to S1 nuclease. Similar strand specificity to that seen with S1 nuclease is inferred by the much stronger nicking on the 'GA' strand than on the 'CT' strand by these enzymes (data not shown). The pattern of site specific cleavage for all three enzymes is substantially weakened at pH 6.0 (data not shown) and essentially disappears at pH 6.5 (Figure 5). That no prominent S1 cleavage site appears at pH 6.5 elsewhere in the plasmid is shown in the following experiment. Figure 6 shows the S1 cleavage at pH 5.5 (lanes 1 and 2) and pH 6.5 (lanes 3 and 4) mapped across the length of 88B13 using a unique Sac I site within the plasmid and probing bidirectionally; panel A was hybridized with an Eco RI-Sac I fragment (Figure 1 probe c) and panel B with a Sac I-Bam HI fragment (Figure 1 probe b). The cleavage site 5′ to hsp 26 shows the expected pH dependency and is absent at pH 6.5, as is a site near hsp 28, but no novel cleavages are seen at the higher pH. Interestingly, there are many sites, particularly within pBR322,

that do not appear to change with the increased pH illustrating the heterogeneity amongst S1 nuclease sensitive DNA structures. All of these nucleases have a low pH optima so in theory the absence of cleavage at the higher pH may be due either to a loss of the DNA structure or to a loss of enzyme activity. However, since the degree of digestion in the S1, mung bean and P1 samples under the different pH conditions has been equalized (Figure 5a), simple loss of enzyme activity is eliminated as a possible reason for failure to nick this site at pH 6.5. If there is an effect on the enzyme, then a more complex hypothesis involving loss of enzyme specificity for this unusual DNA structure would be needed. N.crassa nuclease, which has a neutral pH optimum, does not cleave the DNA at this site at a level significantly above the surrounding sequences at pH 5.5 to pH 7.0 (data not shown). A recent report (28) also describes the use of venom phosphodiesterase at pH 9.0 to cleave an S1 sensitive region. The S1 cleavage site we have examined in this work is not sensitive to cleavage by this enzyme (data not shown). The results of the single strand nuclease cleavage studies at the site 5′ to hsp 26 in plasmid 88B13 are summarized in Figure 7. These findings suggest that the DNA structure may be pH dependent, being present in solution only at pH 6 or lower.

DISCUSSION

A summary of the supercoil-dependent single strand specific nuclease cleavage sites 5′ to hsp 26 in 88B13 is presented in Figure 6. The results indicate that the double stranded cut that was mapped by Selleck et al (9) appears to arise from linearization of the plasmid opposite a cluster of



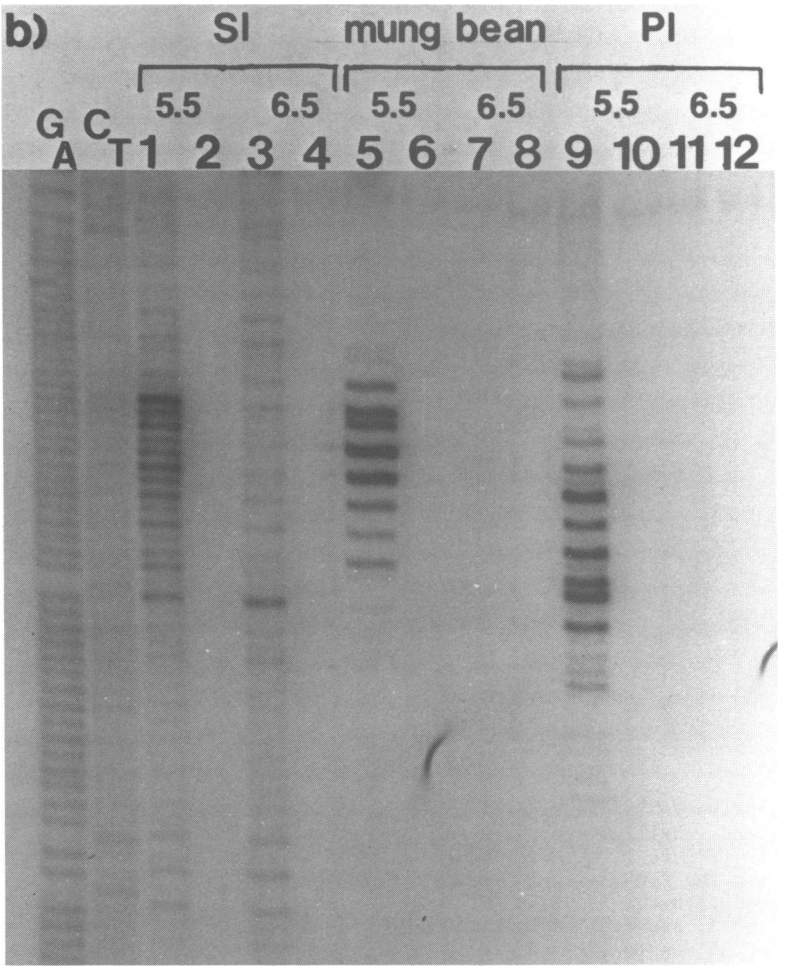


Figure 5: High resolution mapping of single strand specific nuclease cleavage sites 5' to hsp 26 in plasmid 88B13. Plasmid DNA was incubated with S1, mung bean and P1 nucleases at pH 5.5 and 6.5 as described in Materials and Methods. Panel (a) shows an agarose gel analysis of 88B13 DNA after treatment with S1, mung bean and P1 nuclease at pH 5.5 (lanes 1,5 and 9, respectively) and pH 6.5 (lanes 3,7 and 11, respectively). All samples were approximately equalized for the extent of nicking. As a control, DNA was incubated in single strand nuclease buffer at the appropriate pH without nuclease. These samples are shown to the right of each nuclease treated sample in the even numbered lanes. Panel (b) shows aliquots of the same samples as seen in (a) analyzed by indirect end-labelling, specific for the GA strand, after Hha I digestion and electrophoresis on a sequencing gel as described in Figure 3. Lanes labelled GA and CT are sequencing ladders used as markers as described in Figure 3. Note that the S1 samples shown here represent a more extensive digestion of 88B13 than those shown in Figure 3 and that much weaker non-pH dependent sites are apparent.

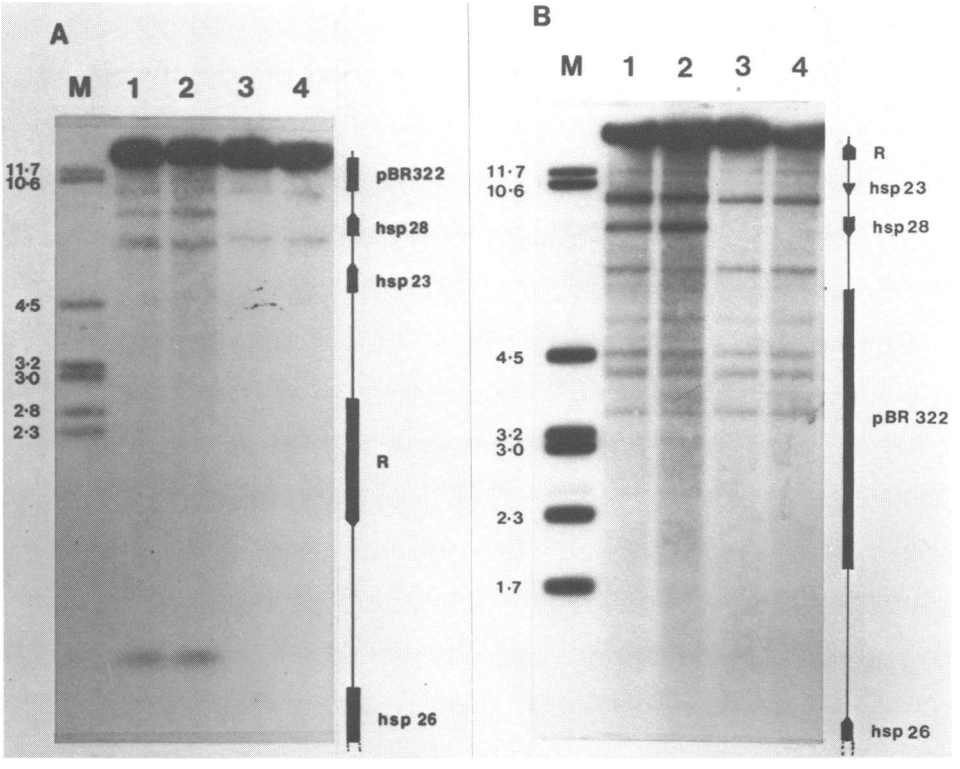


Figure 6: Mapping S1 cleavage sites in 88B13 at pH 5.5 and pH 6.5. The indirect end-labelling procedure was used to map S1 cleavage relative to the Sac I site (Figure 1) as described in Materials and Methods. Panel A is probed with the Eco RI-Sac I fragment indicated in Figure 1 as probe c. Panel B is probed with the Sac I-Bam HI fragment indicated in Figure 1 probe b. Lanes 1 and 2; supercoiled plasmid 88B13 incubated with S1 nuclease at pH 5.5 for 15 and 30 minutes, respectively. Lanes 3 and 4; supercoiled plasmid 88B13 incubated with S1 nuclease at pH 6.5 for 15 and 30 minutes, respectively. M: molecular weight markers.

nicks on the GA strand. Initial nicking occurs exclusively on the GA strand as a cluster of six strong sites between positions -96 and -90, and a series of weaker sites, seen with longer incubation in the presence of S1, extending to position -84 (Figure 3b). The linearization of the plasmid is due to cleavage on the CT strand; the most prominent sites in this case are at positions -86 and -84 (Figure 3c). One of these latter sites was also reported in an earlier study of S1 cleavage using a smaller plasmid which included only the 5′ flanking region of hsp 26. In this study, a sequence level analysis was carried out only for the CT strand. Several other sites

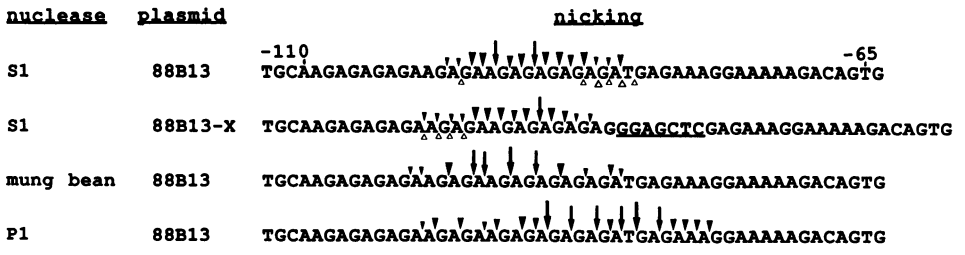


Figure 7: The location of S1, mung bean and P1 nuclease cleavage sites 5′ to hsp 26 in 88B13. Filled arrows indicate the sites of nicking on the GA strand and the height of the arrow approximates the relative intensity of cutting. Open arrows indicate nicking on the CT strand where determined. S1 cleavage of the GA and CT strands of 88B13 and 88B13-X were mapped from data shown in Figure 3b and 3c. Mung bean and P1 cleavage patterns on 88B13 at pH 5.5 were mapped from data shown in Figure 5b. The Xho I linker in 88B13-X is underlined.

were also reported in the region from positions -80 to -120 and a model structure based on slippage loops postulated (29). Examination of both strands clearly demonstrates that the major S1 cleavage in the region occurs on the GA strand. The cleavage pattern is not symmetrical; the results do not support the earlier model. We have also examined a subclone of 88B13, containing approximately 700 bp of 5′ flanking sequence of hsp 26 in a Pst I-Eco RI fragment (Figure 1) and observe the same pattern of cleavage as we detect for 88B13 (data not shown).

The S1 cleavage pattern for 88B13-X is very similiar to the pattern for 88B13 indicating that the insertion of the Xho I linker does not significantly affect the location or extent of cleavage by S1 nuclease. That the flanking region downstream of position -84 appears to play little or no role in the formation of the supercoil-dependent DNA structure is demonstrated by plasmids Δ35 and Δ36 (Figure 4c), which are missing sequence between the S1 site and the gene and yet remain S1 sensitive. On the other hand, when we delete 46 bp (-86 to -132) in plasmid Δ41.1, the sensitivity to S1 disappears. This stretch of homopurine/homopyrimidine sequence is thus responsible for the S1 sensitivity.

We have also examined this sequence with other single strand-specific nucleases and find that it is sensitive to both mung bean and P1 nucleases. Although the actual cleavage sites and extent of cleavage are different for each nuclease, it is clear that the same stretch of homopurine/homopyrimidine sequence 5′ to hsp 26 in 88B13 is recognized in each case. This indicates that the presence of an alternate DNA structure is the basis

for the sensitivity to these nucleases. This sensitivity is strand specific, certainly for S1 nuclease and probably for the other nucleases as well.

The sensitivity to S1, mung bean and P1 nucleases appears to be pH dependent cleavage occuring at pH 5.5 but not pH 6.5. This may reflect the inability of the nucleases to recognize an alternate DNA structure at higher pH, since these nucleases have low pH optima (30). However, given that the enzymes continued to nick the plasmid DNA at higher pH, it appears likely that the lack of specificity is due to the inability of the DNA to form the requisite structure at higher pH. Figure 6 illustrates that S1 sensitive sites associated with hsp 26 and hsp 28 disappear at pH 6.5 but that no prominent S1 cleavage sites appear elsewhere in 88B13 at the higher pH. It is clear that there are sites within 88B13 that remain suceptible to cleavage by S1 nuclease at pH 6.5; these presumably represent other classes of S1 sensitive DNA structures. Thus, since we have equalized our samples for nicking, cleavage events which were occuring over a stretch of ~30 bp must now be distributed over some 16 kb. S1 cleavage sites, both on the GA and CT strands, were generated at pH 5.0 in the presence of 200mM NaCl. We have investigated the cleavage in a similiar fashion at pH 5.0 in the presence of a variety of salt concentrations ranging between 0 and 300mM. At the highest salt concentration there is only slight inhibition of S1 cleavage at this site with no detectable change in specificity (data not shown).

Our results are consistent with those of other investigators (31-37) who have detected S1 sites in eukaryotic promoter regions. In general these sites reside in predominantly homopurine/homopyrimidine stretches, and most are supercoil dependent (31-36). The nature of these DNA structures is as yet unknown, although there has been much speculation and a number of hypotheses put forth (38-41). One model suggested by Pulleyblank and coworkers (39) is of an altered helix with non-Watson Crick basepairing in the homopurine/homopyrimidine region due to protonation of the CT strand. This might explain the non-equivalent cleavage by S1 nuclease on the different strands and also the requirement for a lower pH. It is interesting to note that in their studies of a plasmid containing a 45 bp insertion of d(TC)n.d(GA)n, the cleavage preference is on the CT strand. However, the authors report that in some circumstances cleavage occurs preferentially on the GA strand and that an S1 sensitive conformation can occur on either strand as a conseqence of the protonation of the CT strand (39). Sensitivity to cleavage by single strand nucleases is a common feature to a variety of possible alternate DNA structures such as cruciforms and B-Z DNA junctions (42-45). It is likely that the particular DNA structure studied here does not fall into these

categories, since cleavage is highly asymmetric, rather than the symmetric pattern anticipated in the above cases.

The possible biological role of these DNA structures, sensitive to single strand nucleases,is unknown. There have been a number of examples of S1 sites mapped in isolated nuclei in the 5′ flanking regions of genes (12,13,35). In some instances the nuclear sites correspond to sites that are supercoil-dependent S1 sensitive sites in plasmid DNA. This taken with the observation that S1 sites map near or within DNase I hypersensitive sites suggests that these structures may indeed play some role in formation of active chromatin and the regulation of gene expression. We are in the process of testing these possibilities by using germline transformation to introduce sequences containing hsp 26 and the 5′ flanking region, with or without the small deletion carried in plasmid Δ41.1, into Drosophila in an attempt to determine if these sequences , and the structure they form in vitro, are important in the formation of chromatin structures and gene expression.

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