S1 sensitive sites in adenovirus DNA

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ABSTRACT

S1 nuclease has been used as a probe for regions of DNA secondary structure in supercoiled recombinant plasmids containing adenovirus (Ad) DNA sequences. In the sequences examined two S1 sensitive sites were identified in the left-terminal 16.5% of Ad 12 DNA, one of which aligned approximately with an inverted repeat region in the DNA sequence. In addition an S1 sensitive site was dictated by a potential cruciform structure in the region of the Ad 2 major late promoter. In contrast to the expected cleavage site at the loop of the cruciform, cleavage occurred at the base of the stem in the region of the TATA box. All three S1 sensitive sites identified were more sensitive to S1 than the endogenous sites in the parent plasmids.

INTRODUCTION

The adenovirus genome comprises a linear double-stranded DNA molecule typically of about 35,000 base pairs (bp) with a protein of 55,000 molecular weight covalently linked to the S1 termini and an inverted terminal repetition of about 100 bp. Transcription from the viral DNA occurs in the nucleus and is conventionally divided into two phases: early transcription from several promoters which are differentially regulated and expressed, and late transcription, after DNA synthesis has begun, in which transcripts from the major late promoter at about 16.4 map units are processed to form the mRNAs coding for the majority of the late viral polypeptides (for review see 1). It is not yet known whether the viral DNA, which in the virion is found in a chromatin form together with viral histone-like core proteins V and VII (2, 3), remains associated with the viral core proteins during early transcription and DNA replication or whether it assumes an alternative conformation, possibly in association with cellular histones.

Changes in chromatin structure have previously been correlated to differential gene expression by identification of regions of DNAse 1...
sensitivity and hypersensitivity (4, 5, 6). The presence of such sites may be related to alterations in the protein composition of the chromatin or to variations in the secondary structure of the DNA itself. The ability of DNA sequences to adopt a particular conformation in response to their environment is most easily studied by using small covalently closed circular DNA molecules. Recent work has shown that the single-strand specific nuclease S1 from Aspergillus oryzae can introduce site-specific double-strand cuts close to the centre of symmetry of inverted repetitions separated by between 2 and 6 nucleotides in such circular DNAs (7, 8, 9). This S1 cleavage only occurs in highly negatively supercoiled substrates (10) and it is postulated that in underwound DNA inverted repeats form hairpin loops, or cruciform structures, stabilized by the negative superhelicility of the DNA.

In a preliminary study to examine the possibility that DNA secondary structure may play a role in regulation of adenovirus replication we have used nuclease S1 to probe for regions of secondary structure in three recombinant plasmids containing adenovirus DNA. The results indicate that two S1 sensitive sites, more stable than the endogenous plasmid sites, are present within the left-terminal 16.5% of Ad 12 DNA. Furthermore, S1 nuclease also cleaves at a site dictated by the Ad 2 major late promoter. This latter cleavage site is not, as expected, at the loop of the potential cruciform formed from sequences around the major late promoter, but at the base of the stem, close to or within the TATA box.

MATERIALS AND METHODS

Preparation of plasmid DNA

Plasmid DNA was grown in E.coli strain HB 101 and prepared by a standard procedure involving amplification with chloramphenicol, alkaline/SDS lysis of the cells and precipitation of E.coli DNA (11) before purification of plasmid on CsCl equilibrium gradients.

S1 cleavage of plasmid DNA

S1 nuclease reactions were performed in buffer containing 30 mM K-acetate pH 4.8, 100 mM NaCl and 3 mM ZnSO₄. The concentration of S1 used was adjusted to convert all form I plasmid into 50% form II, 50% linear during 5 minutes incubation at 37°C. Reactions in 100 µl therefore routinely contained: 5-10 µg plasmid DNA, 10 µl 10 x reaction buffer and 5 µl of a 1 in 200 dilution of S1 nuclease (400 units/µl) and
H$_2$O to a final volume of 100 µl. The final concentration of enzyme was therefore approximately 1 unit/µg of plasmid DNA and the reaction was carried out at 37°C for 5 minutes.

**Hybridisation and blotting procedures**

Hybridisation was carried out following a modified procedure of Southern (15). The agarose gel was blotted onto a nitrocellulose filter and after baking at 80°C for 3 hr was dipped into 3 x SSC for 15 minutes prior to soaking in a buffer containing 50 mM Tris pH 7.5, 0.5% SDS, 1 mM EDTA, 3 x SSC, 0.1% PVP, 0.1% Ficoll, 0.1% BSA, 50% formamide, 100 µg/ml denatured calf thymus DNA, overnight at 37°C. The filter was then transferred to the same buffer containing denatured $^{32}$p labelled nick-translated adeno 12 DNA (10$^7$ cpm/ml) and incubated at 37°C for 24 hr before washing 3 times for 5 minutes in 2 x SSC, 0.1% SDS at room temperature. After further washings at 60°C for 2½ hr in 2 x SSC, 0.1% SDS and at room temperature in 2 x SSC, 0.1% SDS, the filter was finally rinsed 3 times for 5 minutes with 2 x SSC before being dried at 80°C and mounted for autoradiography.

**Enzymes**

Restriction enzymes were obtained from Bethesda Research Laboratories (BRL) or New England Biolabs and S1 nuclease from BRL.

T4 polynucleotide kinase and bacterial alkaline phosphatase were obtained from BRL. DNA 5'-termini were labelled according to Maxam and Gilbert (12).

DNAse EP (Sigma) and Pol 1 (Biolabs) were used in nick-translation of Ad 12 DNA isolated from purified virions (13) following the procedure outlined by Rigby et al., (14).

**Electrophoresis**

DNA samples were analysed by electrophoresis in 1% agarose gels stained with ethidium bromide (16) or in 5% polyacrylamide gels (17).

**RESULTS**

Identification of S1 sensitive sites in parent plasmid pAT 153

The recombinant plasmids used in this study are presented in Fig. 1. Plasmid RIC1 contains the left-terminal 16.5% (Eco R1 C fragment) of Ad 12 inserted via Eco R1 linkers into the Eco R1 site of pAT 153, a derivative of pBR 322 (18). B17 comprises the 816 bp Taq I fragment of Ad 2 containing the major late promoter at 16.4 map units inserted into the...
Figure 1. Representation of plasmids used in this study. Tick marks inside circles indicate distances of 1 Kb from the Eco RI site in pBR 322 or pAT 153. Thick lines denote Ad inserts with the orientation of the insert for RIC1 and XD7 indicated by the distance in map units (m.u.) from the left end of the Ad genome and that of the 816 bp insert in B17 by the position of the major late promoter (MLP). Locations of the unique restriction enzyme sites (Eco RI, Hind III, Bam HI and Sal I) in pBR 322 and pAT 153 are shown and the distance of restriction sites (in Kb) from the Eco RI site in RIC1 are given in brackets. Note that these representations are not to scale.

Repaired Bam HI site of pBR 322 and XD7 the left-terminal Xba I E fragment of Ad 5 inserted via Eco RI linkers into the Eco RI site of pBR 322 (19).

Previous reports have located three major S1 sensitive sites, corresponding to inverted repeats, within pBR 322 (7, 8). These sites, designated the major, minor and subminor, are located at 3.06 kb, 3.23 kb and 3.12 kb respectively from the Eco RI site. Initial experiments were therefore performed to determine whether these sites were conserved in pAT 153. Plasmid DNA was cut with S1 until the supercoiled form I DNA was converted into 50% linear, 50% relaxed circular DNA and, after removal of S1 nuclease by phenol extraction and ethanol precipitation, cleaved with restriction enzymes Hind III or Sal I before analysis by agarose gel electrophoresis. The stained gel pattern showed, for Hind III, four major S1 dependant DNA bands and a range of minor fragments (Fig. 2) indicating...
site specific cleavage by S1 nuclease at at least two major sites. Sizing of these fragments and those produced by S1/Sal I digestion, against Hind III and Xba I cut Ad 5 marker DNA, identified two S1 cleavage sites (±100 bp): one of these, at about 2.5 Kb from the Eco RI site is in good agreement with the reported cleavage sites for pBR 322 which in pAT 153 would be located at 2.44 kb, 2.61 kb and 2.50 kb. The other site, at about 1.26 Kb, might correspond to a region of dyad symmetry comprising 8 bases separating a 9 base inverted repeat identified by Lilley (8) and located at 1.33 Kb from the Eco RI site in both pBR 322 and pAT 153. The experimental results are shown in Fig. 2 and the sizes of the various DNA fragments together with the likely locations of the S1 cleavage sites are schematized in Fig. 3. The probable S1 cleavage sites indicated in Fig. 3 are deduced from all the possible alternative S1 sensitive sites shown in Fig. 4. Only those DNA fragments with sizes known from published
sequence data were used as size markers since discrepancies were evident between restriction fragment sizes determined from sequence data and the published restriction maps.

**S1 sensitive sites in RIC1**

Having identified the major endogenous S1 cleavage sites in pAT 153, the recombinant Ad 12-pAT 153 plasmid RIC1 was examined. Similar experiments were performed and showed that S1 site-specific cuts were introduced into the RIC1 DNA (Figs. 2, 3, and 4) but that these no longer corresponded to the endogenous sites in pAT 153. Further analyses using restriction enzymes Bam HI and Eco RI as well as Sal I and Hind III confirmed these results and two major S1 cleavage sites were identified.
"Maps of plasmids pAT 153, (a), and RIC1, (b) showing all possible alternatives for the locations of SI cleavage sites derived from the data presented in Figs. 2 and 5. The distances of the alternative SI sensitive sites and restriction sites from the Eco RI site in pAT 153 or the Eco RI site in RIC 1 are given in Kb. The figures underlined denote the positions of sites considered to be most consistent with the available data (shown in Figs. 2, 3 and 5). (It is not possible to distinguish between the possible sites located around 2.5 Kb from the Eco RI site for the SI/Sal digest of pAT 153).

The results from these experiments are summarised as part of Fig. 3. Restriction with Eco RI, which excises the Ad 12 insert, demonstrated unambiguously that one SI dependent fragment ('a1 in Fig. 5) was larger than linear pAT 153 DNA and therefore was a product of SI cleavage within the Ad 12 insert. Sizing of the SI dependent fragments identified band 'd' in Fig. 4 as the complement to 'a'. The predicted size of the complement to 'c' would place it at the same position as the linear pAT 153 DNA and it therefore would not be seen in the stained gel. However, a
Figure 5: (A) Ethidium bromide stained agarose gel showing products of SI nuclease and restriction enzyme digestion of plasmid RIC1. The results are summarised in Fig. 3. (B) Southern blot of gel shown in (A) after hybridization to nick-translated Ad 12 DNA and autoradiography.

Southern blot of this gel, hybridized to nick-translated Ad 12 DNA confirmed that bands 'a', 'c' and 'd' were of Ad 12 origin and that the predicted band 'b' was present. The conditions of hybridization were such that no hybridization to pAT 153 or Ad 5 DNA was evident.

The locations of these SI cleavage sites were at 1.2 kb (+0.1 kb) and 2.0 kb (+0.1 kb) from the left hand end of Ad 12. A computer search of the known Ad 12 DNA sequence using SEQ3 (25) identified several potentially stable cruciform structures including one located at 1.1 kb from the left end (A in Fig. 6), corresponding well with one of the SI cleavage sites. The criteria used for the prediction of stability of a potential cruciform structure were those outlined by Lilley (9) whereby stability is proportional to the length of the stem but inversely proportional to the loop size and limited G-T or C-A base pairing do not significantly reduce stability but C-T or G-A pairing or loop-outs are highly destabilizing. The potential cruciform at 1.1 kb abides by these rules and should therefore be stable. This cruciform is located at a functionally significant site - at the splice acceptor for the Ela genes of Ad 12. This suggests that a functional role for this inverted repeat might lie in conferring a secondary structure to the primary Ela transcripts, facilitating splicing. No cruciform of similar stability
could be identified in the region of the 2.0 kb S1 cleavage site and although it was possible to construct a cruciform (C in Fig. 6) this structure, according to the rules outlined above, should be highly unstable. (It cannot be ruled out, however, that the SEQ3 program failed to detect a more stable structure in this region.)

A third inverted repeat potentially capable of forming a cruciform (B in Fig. 6), although not as stable as A (since it contains 7 bp in the loop) is also predicted but could not be aligned to any S1 sensitive site. Similar experiments were performed using a plasmid containing the left-terminal Xba I E fragment of Ad 5 (1344 bp) inserted into pBR 322. In this case no S1 cleavage sites within the Ad DNA were observed (data not shown). Although computer analysis of this Ad 5 sequence identifies several potentially stable cruciform structures these were all of lower predicted stability than the endogenous pBR 322 cruciforms.

S1 cleavage dictated by the Ad 2 major late promoter

The adenovirus genome contains a large inverted repeat located at the major late promoter (26) which should, in a supercoiled molecule, be capable of forming a highly stable cruciform structure (D in Fig. 6), more stable than those in pBR 322 DNA. A plasmid (B17) containing the (Ad 2) major late promoter was therefore used in experiments similar to those described above. As expected, S1 treatment of B17 introduced a single site-specific cut in the region of the major late promoter. This site was mapped by S1 cleavage and subsequent restriction with Bam HI or Hind
Figure 7: Autoradiograms of 5% polyacrylamide gel analyses of S1 cut, restricted, 5' end-labelled plasmid B17 DNA. Markers of Hae III restricted pAT 153 or Taq I cut pX 174 DNA are shown with sizes in base pairs indicated. Linear pAT 153 (Z) is marked and the Bam HI excised 816 bp Ad 2 DNA fragment (Y) and the smaller fragment generated by Hind III cleavage of B17 (X) correspond to X and Y in Fig. 8. The arrows indicate the fragments used to locate the S1 cleavage site and correspond to the smaller S1/restriction cleavage products.

Ill, followed by S1 end-labelling with [γ-32P]ATP and polynucleotide kinase and analysis on 5% polyacrylamide gels with appropriate size markers. The result is shown in Fig. 7 with a map of the restriction sites shown in Fig. 8. It is clear that S1 cleaves B17 once at a specific site in the Ad 2 DNA. Although the site of the larger S1/Bam
Figure 8. Linear restriction map of B17 between the unique Eco Rl and Sal I sites. Thick line denotes pBR 322 DNA and thin line the Ad 2 Insert (816 bp). The major late promoter (MLP) is indicated and 202 and 251 indicate distances (in bp) from the centre of the CGTT sequence at the loop of the cruciform (see Fig. 5) to the end of the double-stranded DNA after restriction with Hind III or Bam HI. X and Y correspond to bands in Fig. 7. (Restriction sites were determined from the known Ad 2 sequence (26)).

cleavage product was estimated to be about 590 bp based on its mobility, more accurate determination of the S1 cleavage site was obtained by sizing the smaller S1/restriction cleavage product for which there were more suitable size markers. The expected length of the smaller cleavage products of S1/Hind III and S1/Bam HI digestion would be 202 and 251 bases respectively if cleavage occurred at the centre of symmetry of the inverted repeat (26). However the apparent sizes of the corresponding S1/restriction fragments appeared to be about 226 ± 3 bp using Taq I P174 restriction fragment markers and about 234 ± 3 bp using Hae III P153 restriction fragment markers after digestion of B17 with Bam HI. When B17 was restricted with Hind III and analysed using the Hae III P153 markers the S1 cleavage product had a size of 224 ± 3 bp (see Fig. 7). This apparent variation in the size of the fragment generated by S1 using these procedures is probably related to sequence dependent differences in electrophoretic mobilities of the marker fragments since the size of these fragments was assessed using the published sequence data (27, 20). Notwithstanding these variations this clearly places the S1 cleavage site near the base of the stem in the region of the TATA box, indicating a region of single-stranded DNA located asymmetrically at the base of the stem.
DISCUSSION

In this study we have shown that S1 nuclease can cut pAT 153 at specific sites and that such sites can also be detected in adenovirus DNA.

Two S1 sensitive sites in pAT 153, at about 2.5 Kb and 1.26 Kb from the Eco RI site, were identified. That located at 2.5 Kb corresponds to the region of S1 sensitive sites reported for pBR 322 (7, 8), demonstrating the conservation of such sites in related plasmids. The site at 1.26 Kb does not align with a potentially highly stable cruciform and cleavage at this site has not previously been reported.

Three S1 sensitive sites have been identified in the adenovirus DNA examined, two within the left-terminal 16.5% of Ad 12 and one dictated by the Ad 2 major late promoter. It is important to stress that the methods used will only detect S1 sensitive sites that are more sensitive than the endogenous plasmid sites. The potential for formation of S1 sensitive sites within the Ad DNA effectively deletes the S1 sensitivity of sites in the parent plasmid. Similar results describing the transmissibility of cleavage sites have been demonstrated previously (9, 28).

Of the S1 cleavage sites identified in RIC1 one approximately aligns with a predicted potentially stable cruciform at about 1.12 kb, another at 2.0 kb does not align with a predicted stable secondary structure, and a predicted secondary structure at 1.61 kb does not correspond to an S1 cleavage site. The inability to correlate an S1 sensitive site in pAT 153 to any potentially stable cruciform together with the results of S1 cleavage of RIC 1 indicates that factors other than the dimensions of inverted repeats must be involved in determining the hierarchy of S1 sensitivities. Such factors might include the presence of other alternative DNA conformations as well as the degree of negative super-helicity and possibly the size of the DNA molecule. The conditions of S1 cleavage may also be important, particularly in terms of salt concentration and temperature. Larsen and Weintraub (28) reported that under some conditions S1 sensitive sites observed in pBR 322 DNA could not be aligned to either an AT rich sequence, as found for S1 cleavage of SV40 DNA at high temperatures (7), or to regions of potentially stable secondary structure. Lilley (8) also showed some variation with temperature in S1 cleavage of plasmids. It is also possible that the DNA slippage mechanism of Hentschel (29), whereby a region of DNA slips to pair with a repeated sequence to one side resulting in unpaired loops of DNA, might explain the cleavage site at 2.0 kb in RIC 1 and at 1.26 Kb in pAT 153.
While this manuscript was in preparation results on the S1 sensitivity of a plasmid containing an adenovirus major late promoter were reported by Larsen and Weintraub (28). However, these workers only mapped the cleavage site close to or at the major late promoter. Although the precise location of the cleavage site in B17 can only be determined by direct sequence analysis, the data presented here strongly suggest that cleavage occurs at or very close to the base of the cruciform stem in the region of the TATA box. This contrasts with the results of others who demonstrated S1 cleavage at the loops of cruciforms in Col E1 and pBR 322 (7, 8, 9). Although the S1 cleavage site in B17 is clearly governed by the major late promoter the question why only one side of the base of the stem is cut must be addressed. Examination of the DNA sequence at the base of the cruciform stem shows there is considerable C-A and G-T base pairing in the region of the TATA box (Fig. 6). In a supercoiled molecule or in a situation where the cruciform is stabilized by interaction with protein, the constraints on the DNA resulting from the region of transition from the flanking DNA into the stem of the cruciform might result in a relatively loose structure. In most cruciforms this region would be small compared to the loop but with the major late promoter, and possibly other similar promoters, the AT-rich TATA box together with the high degree of C-A pairing could result in a more open structure in which the TATA box would continuously form part of the cruciform and revert to the usual B form of DNA. Since the DNA pairing with the TATA box in the cruciform is GC-rich this region might be more stable as part of the surrounding DNA. As such there would be a bias towards the TATA box side of the cruciform in susceptibility to S1 nuclease. These ideas are summarized in Fig. 9. It is assumed that the DNA sequence in this region in the plasmid corresponds with the published data (26). Thus it is conceivable that an alteration in or near the TATA box could result in a mismatch leading to S1 sensitivity. However, this is unlikely since other studies have shown that at least 3 base mismatches are required for S1 cleavage (30, 31).

It is not known whether chromatin is under torsional constraints but any mechanism whereby conformational features of DNA are formed in response to superhelicity is of obvious interest. Also, many of the potential secondary structures that, by Lilley's (9) guidelines, are unstable in naked DNA may be stabilized by interaction with protein in chromatin. Evidence has recently been provided for some degree of
Figure 9. Possible secondary structures in the region of the major late promoter. See text for further details.

correlation between S1 sensitive sites in chromatin and regions of DNase I hypersensitivity, which are retained in the same DNA regions when subcloned into pBR 322 (28). In the adenovirus genome itself potentially stable cruciform structures are present at many sites, some of which are located at regions of obvious functional significance such as promoters, splice junctions or transcription termination signals (unpublished observations). The observation of similar secondary structures in DNA from a variety of sources (32, 33, 34) together with their apparently nonrandom distribution (35) suggests they may play an important role in such processes as DNA replication, transcription and splicing, providing recognition signals for interaction with proteins and, in some cases such as the major late promoter, facilitating destabilization of the DNA helix.

This study concentrates exclusively on the presence of S1 sensitive sites in recombinant Ad DNA molecules as a necessary prelude to the analysis of the secondary structure of Ad chromatin in the virion, in productively infected cells and in an integrated state, and its relationship to transcription and replication. Adenovirus DNA may therefore
prove a convenient model for the structure of eukaryotic chromatin in general.

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