Biochemical Method for Mapping Mutational Alterations in DNA with S1 Nuclease: The Location of Deletions and Temperature-Sensitive Mutations in Simian Virus 40*

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ABSTRACT. S1 nuclease (EC 3.1.4.X), a single-strand-specific nuclease, can be used to accurately map the location of mutational alterations in simian virus 40 (SV40) DNA. Deletions of between 32 and 190 base pairs, which are at or below the limit of detectability by conventional electron microscopic analysis of heteroduplex DNAs, have been located in this way. To map a deletion, a mixture of unit length, linear DNA, prepared from the SV40 deletion mutant and its wild-type parent, are denatured and reassmeled to form heteroduplexes. S1 nuclease can cleave heteroduplexes at the nonbase-paired region to produce fragments whose lengths correspond to the position of the deletion. Similarly, specific fragments are produced when S1 nuclease cleaves a heteroduplex formed from the DNAs of SV40 temperature-sensitive mutants and either their revertants or wild-type parents. Thus, the positions of the nonhomology between these DNAs can be determined.

S1 nuclease (EC 3.1.4.X) from Aspergillus oryzae degrades single- but not double-stranded DNA (1); nevertheless, superhelical simian virus 40 (SV 40) (2) and polyoma (3) DNAs are converted to unit length linear molecules by this enzyme. Presumably, this occurs because unpaired, or weakly hydrogen-bonded regions, susceptible to S1 nuclease, occur or can be induced in the strained superhelical molecule. Since nicked circular molecules appeared to be intermediates in the double-strand cleavage, we surmised that the enzyme was capable of cleaving the intact strand opposite or near the nick (2).

This property suggested that S1 nuclease could be used to map the location of small deletions, insertions, or, in fact, any difference in base sequence between otherwise homologous DNAs. For example, heteroduplex DNA molecules formed from the complementary strands of a deletion mutant and wild-type SV40 DNA contain a single-strand loop at a position corresponding to the deletion (Fig. 1). If S1 nuclease can digest this single-strand loop and subsequently cleave the intact strand at the nick (or gap), fragments whose length corresponds to the position of the deletion loop should be generated. In principle, insertion mutations could also be located this way.

Here we report that S1 nuclease can indeed be used to locate a deletion of as few as 32 base pairs. Moreover, S1 nuclease also cleaves heteroduplex molecules formed from the DNAs of SV40 temperature-sensitive mutants and either their revertants or wild-type parents. If, as is likely, the base sequence of the temperature-sensitive mutant DNA differs from the wild-type or revertant DNA by a single base change, this method could be useful for accurately mapping point mutations, thereby making reliance on genetic recombination unnecessary.

MATERIALS AND METHODS

Cells and Viruses. The origin and the procedures for the growth of primary African green monkey kidney cells (AGMK) and the established monkey kidney cell lines (CV-1P and MA-134) have been described (4). All virus stocks and virus DNA were prepared in MA-134 cells. Plaque assays were performed on CV-1P cells. Mutants dl-808 and dl-861 are viable deletion mutants of SV40 (5, 6). SV40 mutants ta430 and ta602, as well as their wild-type parents, were obtained from Peter Tegtmeier (7) and Robert Martin (8), respectively. Temperature-sensitive mutants were grown at 32°. Spontaneous revertants of these mutants were from plaques that arose on cell monolayers incubated at 41°.

Enzymes. S1 nuclease was prepared from "Enzopharm" powder (Enzyme Development Corp., New York) by the procedure of Vogt (9) with minor modifications. One unit of S1 nuclease releases 1.0 nmol of nucleotides per min at 37° when acting on sonicated, denatured salmon sperm DNA at pH 4.4 in the presence of 0.5 mM Zn²⁺ and 280 mM Na⁺. S1 nuclease obtained from a commercial source (Miles Laboratories, Inc.) was also found to give good results in the types of experiments reported here, although the "nibbling" effect (see Results) was greater. EcoRI restriction endonuclease was prepared by the procedure of Greene et al. (10). HpaI and HpaII restriction endonucleases were prepared essentially by the procedure of Sharp et al. (11).

DNA Substrates. SV40 DNA was extracted according to Hirt (12) from MA-134 cells infected at a multiplicity of <0.05 PFU per cell when >90% of the cells showed cytopathic effect. Covalently closed viral DNA [SV40 (I)] was purified directly from the supernatant by adding CaCl₂ to 1.56 g/cm³ and ethidium bromide to 200 μg/ml and centrifuging to equilibrium. The band of SV40 (I) DNA was collected, and the ethidium bromide was removed by passing the DNA through Dowex-50 (13). Full length linear DNA or fragments derived from it were prepared with restriction endonucleases using published protocols (11, 14), and then the DNA was repurified by velocity sedimentation in a neutral CaCl₂ gradient.

Cleavage of Heteroduplex DNA with S1 Nuclease. Heteroduplexes of EcoRI or HpaII endonuclease-generated linear...
SV40 DNAs were prepared by denaturing a mixture containing equal parts of two DNA species (1.5–6 μg/ml of each) in NaOH (0.1 N). After 10 min at room temperature, the solution was titrated to pH 7–8 with HCl, the Na+ concentration was raised to 300 mM, and the DNA was reannealed at 68° for 3 min. The reannealed DNA was treated with S1 nuclease (135 units/μg of DNA) in the presence of Zn++ (4.5 mM), Na+ (280 mM), and CH3COO- (30 mM) at pH 4.4. After 30 min at 25°, the reaction was terminated by raising the pH to 0.05 volume of Tris base (2 M), and by increasing the Na+ concentration to 500 mM. To decrease the volume and Na+ concentration of the sample prior to gel electrophoresis, the DNA was precipitated at −20° after the addition of yeast RNA (20 μg/ml) and 2 volumes of ethanol.

In our earlier experiments there was some cleavage by S1 nuclease of homoduplex DNA within A+T-rich regions. To minimize the technical difficulties caused by this background, we sought digestion conditions that would reduce or randomize the location of such cleavages. Under the conditions we adopted (4.5 mM Zn++, 280 mM Na+), the difference in Tm between two DNAs of substantially different base composition is eliminated (Table 1). In 4.5 mM Zn++, Micrococcus luteus (72% G+C) and salmon sperm (43% G+C) DNAs melt at the same temperature (Tm), and over a narrower temperature range (ΔT) than was the case with our earlier conditions (0.5 mM Zn++). With these reaction conditions inadvertent cleavages are nearly random and, therefore, any fragments generated are more evenly distributed throughout the analytical electrophoresis gels.

Gel Electrophoresis. Agarose gels (1.2%, 6 mm in diameter, 200 mm long) were prepared in Tris-borate buffer (89 mM Tris-Oh, 88 mM borate acid, 2.5 mM EDTA, pH 8.2) (10). Samples were applied in 60 μl of Tris-borate buffer containing sucrose (20% w/v). Electrophoresis was at 40 V for 17 hr. The DNA bands were stained with ethidium bromide and visualized using a short wavelength ultraviolet light. The fluorescent bands were photographed using a Vivitar orange (62) filter and Kodak TX-135 film. The negatives were scanned with a Joyce, Loebl and Co. microdensitometer.

RESULTS

S1 Nuclease Cleaves Duplex DNA at the Site of a Single-Stranded Nick. Essential to our approach of mapping deletions is the predicted ability of S1 nuclease to digest the single-stranded loop of a deletion heteroduplex and to cleave the resulting nicked or gapped duplex structure at the interruption (see Fig. 1). The first expectation seemed plausible from the known activity of the enzyme (1), but, although earlier experiments (2) had indicated that S1 nuclease could carry out the second step, we sought more convincing evidence of this ability. Accordingly, SV40 DNA nicked once in either of its two strands at the HpaII endonuclease restriction site (located 0.735 SV40 fractional length clockwise from the EcoRI endonuclease-cleavage site), was converted to linear DNA by cleavage with EcoRI endonuclease. These molecules migrate in agarose gels as unit length linear SV40 DNA (Fig. 2A). This uniquely nicked linear DNA is cleaved by S1 nuclease to form two fragments, 0.26 and 0.73 SV40 fractional length (Fig. 2B), measured from the mobility of fragments of known size (Fig. 2C). Because S1 nuclease appears to "nibble" the ends of duplex DNA molecules during the course of the reaction, the length of the fragments is slightly underestimated. The extent of "nibbling" is difficult to determine accurately but is about 30 base pairs or 0.006 SV40 fractional length. Applying this correction, it is clear that the nicked DNA molecules have been cleaved by S1 nuclease at or very near 0.735, the map coordinate of the HpaII endonuclease restriction site.

S1 Nuclease Cleaves Heteroduplex DNAs at the Site of Deletion Loops. Either naturally arising (5) or biochemically generated (6) mutants of SV40 with deletions of the HpaII endonuclease cleavage site are suitable model substrates for testing the validity of the mapping procedure. DNAs from mutants dl-808 and dl-861, which contain deletions of about 190 and 32 base pairs, respectively (0.035 and 0.007 SV40 fractional length), as well as wild-type DNA were converted to unit length linear DNAs by digestion with EcoRI endonuclease. Electron microscopic examination of heteroduplexes formed from dl-808 and wild-type DNA showed a barely detectable denaturation loop 0.245 SV40 fractional length from the nearest EcoRI endonuclease generated end (5). Cleavage of these same heteroduplex structures with S1 nuclease produced two fragments of about 0.73 and 0.24 SV40 fractional length (15). Thus, S1 nuclease can digest the single-stranded denaturation loop (0.035 SV40 fractional length) and cleave the heteroduplex at that point. The deletion loop in heteroduplexes formed from dl-861 and wild-type DNA is too small to be seen in the electron microscope; nevertheless, these molecules are readily cleaved into two fragments whose lengths are 0.74 and 0.28 SV40 fractional length (corrected for the "nibbling" effect mentioned above).

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**Table 1. Effect of Zn++ on melting transitions of DNA**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>M. luteus DNA</th>
<th>Salmon sperm DNA</th>
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<tbody>
<tr>
<td>S1 nuclease reaction buffer containing 0.5 mM Zn++</td>
<td>85.8</td>
<td>77.5</td>
</tr>
<tr>
<td>S1 nuclease reaction buffer containing 4.5 mM Zn++</td>
<td>74.5</td>
<td>74.2</td>
</tr>
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Micrococcus luteus and salmon sperm DNAs were heated at approximately 1°/min in the indicated buffers. Temperature was measured with a thermocouple in a cuvette in the sample changer, and melting transitions were monitored at 250 nm with a Gilford model 2000 spectrophotometer. Tm is the melting temperature and ΔT is the breadth of the transition from 1/4 to 3/4 maximum hyperchromicity.

**Fig. 1.** Putative cleavages of heteroduplexes which contain mismatched regions susceptible to S1 nuclease. A duplex DNA with a single nick (middle) is proposed as an intermediate in the S1 nuclease cutting reaction. There is no yet direct evidence for such an intermediate.
S1 Nuclease Cleaves Heteroduplexes Formed Between Wild-Type, Temperature-Sensitive, and Revertant DNAs. Since S1 nuclease could cleave heteroduplexes at the position of small single-stranded loops, we wished to test whether the enzyme could cleave heteroduplex DNA at mismatches due to single base differences. Lacking DNAs with defined single base changes, we examined the susceptibility of heteroduplexes formed from EcoRI endonuclease-cleaved temperature-sensitive (ts), revertant (−R) and wild-type SV40 DNAs. Both tsA30 and tsD202 were induced by hydroxylamine and though the nature and number of changes in the base sequence are not known, they are probably single base changes.

Because the DNA of tsA30 and its revertant, tsA30-R, were expected to have the lowest number of base pair differences, we examined the S1 nuclease susceptibility of their heteroduplex first. As expected, the mixed homoduplexes of EcoRI endonuclease-cut mutant and revertant DNAs did not yield specific fragments during S1 nuclease digestion (Fig. 4A). But the heteroduplex of the temperature-sensitive and revertant DNA was cut to produce two easily discernible fragments of 0.68 and 0.32 SV40 fractional length (Fig. 4B). This places the mismatch between tsA30 and its revertant at either 0.32 or 0.68 on the SV40 map. These two alternatives could be distinguished by examining the S1 nuclease cleavage products of the corresponding heteroduplex formed from HpaII endonuclease-cleaved mutant and revertant DNA. In this instance the mismatch would be expected to be in a different location relative to the ends of the heteroduplex. Since S1 nuclease cleaved these heteroduplex DNAs, generating fragments of 0.59 and 0.42 SV40 fractional length (data not shown), the mismatch between tsA30 and its revertant must be located at 0.32 on the SV40 map.

The analysis of the S1 nuclease cleavage products of the heteroduplex between tsA30 and wild-type DNA revealed at least one mismatched sequence (Fig. 4C). Cleavage of the heteroduplex formed from EcoRI endonuclease-generated linear tsA30 and wild-type DNA with S1 nuclease yielded...
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different digestion conditions, S1 nuclease produced another,
apparently specific, cleavage which yielded small quantities
duplexes formed from
tatively, there is more than one mismatch in the heteroduplex of
of fragments of
and wild-type DNA and only the site that yields the
fractio
S1 and wild-type DNA; the other
cleavage yielded the two fragments previously obtained with
the heteroduplex prepared from taASO and taASO-R DNA.
Thus, taASO contains at least one alteration relative to its
wild-type parent; this occurs at 0.38 on the SV40 map (Fig.
The alteration causing reversion of the taASO phenotype
is, therefore, a second-site mutation which occurred at 0.32 on
the SV40 map. This indicates that the map coordinates 0.32 to
0.38, which is the region within which the mutant and second
site revertant differ from wild-type, lie within the A comple-
mlementation group of SV40. This conclusion is reinforced by the
findings of Lai and Nathans (16), who have determined by
marker rescue experiments that the taASO mutation is located
within Hind II + III fragment H (0.375-0.43 map position)
and the taASO mutation (another ta allele) occurs within
Hind II + III fragment I (0.325-0.375 map position) (see
Fig. 5). Additional experiments with other ta mutants or A
mutants resulting from deletions and insertions should better
define the physical limits of the A cistron.
The S1 nuclease mapping procedure has also been used to
locate a second SV40 cistron. Heteroduplex molecules formed
from taD80 and a spontaneous revertant, taD80-R, were
digested with S1 nuclease as indicated above (data not shown).
A fragment 0.91 SV40 fractional length was produced by S1
nuclease cleavage of the heteroduplex formed from EcoRI
endonuclease-cut DNAs and a fragment 0.82 SV40 fractional
length from the heteroduplex of HpaII endonuclease-cut
DNAs. These results are consistent with the existence of a
nonhomology between taD80 and its revertant at 0.91 on the
SV40 map.
In this instance also, the reversion does not restore the
wild-type sequence. This conclusion follows from the finding
that though S1 nuclease can cleave the heteroduplex formed
from taD80-R and wild-type DNA, it fails to cleave the hetero-
duplex generated from taD80 and wild-type DNA. Perhaps
the particular mismatch generated in the heteroduplex mode
from taD80 and wild-type DNA cannot be readily "recog-
nized" or cleaved by S1 nuclease.
Here, too, the data of Lai and Nathans (16) substantiate
our assignment of the D cistron to a region of the physical
map containing the coordinate 0.91. They have found that
Hind II + III fragment E (map position 0.86-0.945) contains
the sequence modified by the taD80 mutation.

Fig. 5. The locations of point mutations on the SV40 chromo-
some as determined by the S1 nuclease procedure. The solid
circles on the map represent points at which Hind II + III endo-
nuclease-cut, and the letters designate the fragments produced
by this cleavage (17).

with S1 nuclease (Fig. 4D). One specific cleavage generated
the same two fragments as those obtained from the hetero-
duplex prepared from taASO and wild-type DNA; the other
cleavage yielded the two fragments previously obtained with
the heteroduplex prepared from taASO and taASO-R DNA.

predominantly fragments of 0.62 and 0.38 SV40 fractional
length. In several earlier experiments, carried out under
different digestion conditions, S1 nuclease produced another,
apparently specific, cleavage which yielded small quantities
of fragments of 0.58 and 0.42 SV40 fractional length (15).
Only a hint of such a cleavage is seen with the present reaction
conditions (Fig. 4C). Possibly the earlier result was spurious
and there is indeed only one S1 nuclease-sensitive mismatch
in the heteroduplex of taASO and wild-type DNA. Alterna-
tively, there is more than one mismatch in the heteroduplex
of taASO and wild-type DNA and only the site that yields the
fragments of 0.62 and 0.38 SV40 fractional length is appreci-
ably cleaved by S1 nuclease.

A decision as to whether the mismatch occurs at 0.38 or
0.62 on the SV40 map was made, as mentioned earlier, using
analogous heteroduplex molecules generated from HpaII
endonuclease-cut taASO and wild-type DNA; the mismatch
can be assigned to 0.38 map position (data not shown).

Heteroduplexes produced from taASO-R and wild-type
DNAs yield two specific classes of fragments after cleavage

Fig. 4. Cleavage by S1 nuclease of heteroduplexes prepared
from EcoRI endonuclease-generated linear DNAs containing
putative single mismatches. The S1 nuclease reactions con-
tained 10 μg/ml of DNA. Samples of 0.7 μg of DNA were applied
to each gel. The vertical lines drawn through the DNA bands
serve to match pairs of fragments whose sizes add up to unit
length SV40 DNA. (A) S1 nuclease-treated homoduplexes. (B)
S1 nuclease-treated heteroduplexes formed from taASO and taASO-
R DNAs. (C) S1 nuclease-treated heteroduplexes formed from
taASO and wild-type DNAs. (D) S1 nuclease-treated hetero-
duplexes formed from taASO-R and wild-type DNAs.

<table>
<thead>
<tr>
<th>Fractional Length</th>
<th>Hind II</th>
<th>Hind III</th>
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<tbody>
<tr>
<td>0.32</td>
<td>E</td>
<td>K</td>
</tr>
<tr>
<td>0.36</td>
<td>F</td>
<td>J</td>
</tr>
<tr>
<td>0.42</td>
<td>G</td>
<td>I</td>
</tr>
<tr>
<td>0.58</td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td>0.62</td>
<td>D</td>
<td>M</td>
</tr>
<tr>
<td>0.735</td>
<td>C</td>
<td>N</td>
</tr>
</tbody>
</table>
DISCUSSIONS

There seems little doubt that S1 nuclease can be used to map deletions and insertions (or, in fact, any change that produces single-strand segments in heteroduplex structures) in DNA. As with electron microscopic examination of heteroduplexes, the method permits accurate analysis of the location and size of these and other gross perturbations of DNA structure. Of particular importance, however, is the ability of the S1 nuclease-mapping procedure to detect and locate deletions and insertions that are too small to be seen by electron microscopy.

A most promising but still provisional conclusion is that S1 nuclease can detect and map single base changes as well. We are presently cautious because, though reasonable, it is still unproven that the temperature-sensitive DNAs differ from their revertant or wild-type DNAs by only single bases at the sites where S1 nuclease acts. Hopefully, experiments with DNAs having known base changes at specific locations can resolve this uncertainty.

Our limited experience with the method has already indicated several shortcomings. Under the conditions used, cleavage of heteroduplexes with putative single base mismatches is slower and more limited in extent than that observed with deletion and insertion heteroduplexes. The rate-limiting step in this cleavage is the introduction of the first nick at the site of the mismatch, since molecules containing a single-stranded scission are quite efficiently cleaved by the nuclease (Fig. 2). As a consequence of the limited cleavage at the site of possible single base mismatches, the signal (specific cutting at the mismatch) to noise (random cutting of the heteroduplex DNA) ratio is low. Several parameters may be tested to deal with this problem: the conditions required to ensure perfect renaturation in the formation of the heteroduplex substrates for S1 nuclease; the conditions that minimize the existence of regions of even transient single-strandedness (e.g., reaction conditions that eliminate or randomize transient single-strandedness due to differences in base composition and sequence, Table 1); the efficiency of S1 nuclease cleavage of mismatches involving different base pairs occurring within different sequences (e.g., A/G versus T/C and the influence of neighboring stretches of high or low G + C content). A collateral problem related to the noise or nonspecific cleavages is the possibility that sequence heterogeneity can result during propagation of a supposedly homogeneous DNA. With increasing experience we should also be better able to assign error limits to the lengths of the fragments and, therefore, to obtain more precise assignments of the map position of mutations.

Even at its present stage of development, the S1 nuclease procedure has permitted us to map several changes in the A and D cistrons of SV40 (Fig. 5). For example, at least one site (0.38 on the SV40 map) distinguishes the base sequence of tsA30 and its wild-type parent. Only one difference was detected between the sequence in tsA30 and its revertant tsA50-R, and that occurs at 0.32 on the map. Consistent with this is the fact that tsA50-R differs from the wild-type at a minimum of two map locations: 0.38 and 0.32. Thus, the revertant mutation has occurred at a second site. Although we could infer the existence of a region of nonhomology at map position 0.91 between tsD802 and tsD808-R and between tsD820-R and wild-type DNAs, we have been unable to detect a mismatch in the heteroduplex formed between tsD802 and wild-type DNA. The reason for this is unknown and further experiments are needed.

Some mention should be made of several ramifications of the method. First, it provides an approach to genetic mapping in systems where genetic recombination is lacking or difficult to measure. Second, the procedure enables one to locate genotypic alterations that cause no detectable change in phenotype. Third, the ability of S1 nuclease to cleave a duplex DNA at a mismatch may make it possible to isolate discrete segments of a genome if that segment can be bounded by small deletions. For example, a heteroduplex between two different deletion mutants should yield three fragments after S1 nuclease cleavage, one of which contains the segment between the two mutations.

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