ACTION OF S_1 NUCLEASE ON NICKED CIRCULAR SIMIAN VIRUS 40 DNA.

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SUMMARY SV40 DNA form II (FO II) containing on average more than one single strand nick per molecule was treated with S_1 nuclease. Linear duplex molecules of unit length (FO III) were generated at enzyme concentrations sufficient to achieve 95% hydrolysis of at least 100 times the amount of single-stranded DNA. Therefore, S_1 nuclease introduces under the described conditions only one double strand break per molecule despite the presence of several single strand nicks.

INTRODUCTION Single strand specific S_1 nuclease (1,2) is capable of cleaving both strands of superhelical and of relaxed Simian virus 40 (SV40) and of polyoma DNA to form linear duplex molecules (3,4,5). It was suggested that cleavage of superhelical DNA occurs at partially single stranded regions (3,4,5) while cleavage of relaxed DNA FO II, which is devoid of such regions, takes place at or near the single strand nick (4,5). Thus, S_1 nuclease might be a reagent to extend single strand nicks into double strand breaks (4).

To test this hypothesis we have treated SV40 DNA FO II, generated either by radiation damage or by DNase with S_1 nuclease. We have analysed the reaction products both by alkaline sucrose velocity sedimentation and by agarose gel electrophoresis. We shall describe conditions under which S_1 nuclease introduces only one double strand break per molecule of FO II regardless of the number of single strand nicks present.

MATERIALS AND METHODS

Purification of SV40 DNA. Infection of CV-1 cells with SV40, radioactive labeling, and extraction of SV40 DNA FO I were as described (6).

Preparation of radiation damaged $[^3H]$-SV40 DNA FO II. $[^3H]$-SV40 DNA FO II originated spontaneously from superhelical DNA after storage for 6 months, when 64% of the FO I DNA had been relaxed

probably owing to radiation damage (7). The specific activity of the [^{3}H]-SV40 DNA FO II was 1.64 x 10^5 cpm/μg.

Preparation of DNase-nicked SV40 DNA FO II. The reaction mixture (1.1ml) contained 5μg of SV40 DNA FO I, 0.015μg DNase (electrophoretically pure bovine pancreatic deoxyribonuclease Schwarz/Mann, Orangeburg, N.Y.) and 10mM $MgCl_2$ in 10mM Tris-HCl, pH 7.2, 1mM EDTA and 5% glycerol. After 30 minutes of incubation at 0°C 100% of FO I are converted under these conditions to FO II as determined by agarose slab gel electrophoresis. To generate [^{3}H] SV40 DNA FO II with on average only one to two nicks per molecule, the same procedure was followed, except that incubation at 0°C with DNase was stopped after 5 minutes. FO II (23%) was separated from FO I (77%) by electrophoresis and recovered by homogenization of the agarose as described (8).

Alkaline velocity centrifugation. The samples were layered on 5-20% sucrose gradients, pH 12.7, containing 1M NaCl, 0.1M Tris, 0.01M EDTA and 15μg unlabeled calf thymus DNA. Centrifugation took place in a Spinco SW41 rotor at 20°C, 30.000 rpm, for 18 hours.

S_1 nuclease preparation and assay. The S_1 nuclease was prepared as described by Vogt using 20g of Takadiastase (2), up to and including the sulfo-Sephadex chromatography. The assay mixtures (0.10ml) were as described (2) except that carrier calf thymus DNA was omitted. The reactions were carried out at 37°C for 30 minutes. Under these conditions 2μl of S_1 nuclease hydrolysed denatured [^{3}H]-SV40 DNA FO II (2μg) to the extent that 95% of the radioactivity was no longer in acid precipitable form, while native SV40 DNA FO II was not rendered acid soluble.

Agarose slab gel electrophoresis. Electrophoresis in 1.4% agarose gels was performed as described (9). The radioactivity of the sliced gels was determined as described (10).

RESULTS

Analysis of the S_1 nuclease reaction products. Radiation damaged SV40 DNA FO II was treated with S_1 nuclease and analysed in alkaline velocity gradients. The sedimentation profile of the untreated [^{3}H] FO II sample together with a sedimentation marker is shown in Fig.1A. Only a minority of the molecules (approximately 15%) sedimented at 18S as unnicked single-stranded circles. The rest of the molecules sedimented either as single strands of unit length (16S) or, to a large extent, as fragments that were shorter than

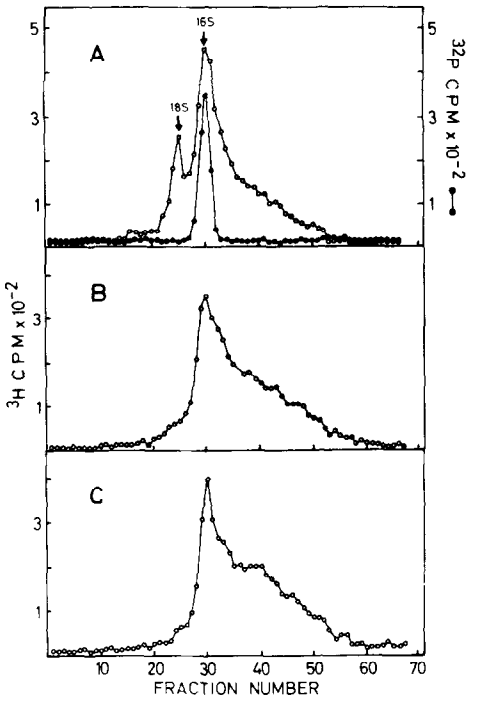

Fig. 1: Alkaline velocity sedimentation analysis of radiation damaged S_1-nuclease-treated, [^{3}H] SV40 DNA FO II. In each case 0.027 µg [^{3}H]-DNA (4.5 x 10^3 cpm) were assayed. (A) -o-o- without enzyme; -●-●- 16S marker generated by treating [^{32}P] SV40 DNA FO I (0.1µg) with Eco R_I (0.5µl, 30 minutes, 37°C); (B) + 3µl S_1; (C) + 10µl S_1.

unit length. Treatment of the DNA with either 2 or 10µl of S_1 nuclease caused disappearance of the structures sedimenting at 18S (Figs.1B,C).

For further investigation agarose slab gel electrophoresis was employed. The untreated control (Fig.2A) consisted exclusively of circular structures (FO II). No FO III, which migrates faster in the gel, could be detected. Since the alkaline velocity sedimentation analysis (Fig.1A) revealed that not more than 15% of these molecules contained an intact circular strand, it is clear that the remainder of the molecules bear at least one nick in each one of their strands. One would expect, therefore, that S_1 nuclease should convert at least 85% of the FO II molecules to duplex structures shorter than unit length, if the enzyme would continue single-strand nicks to double strand breaks. The results depicted

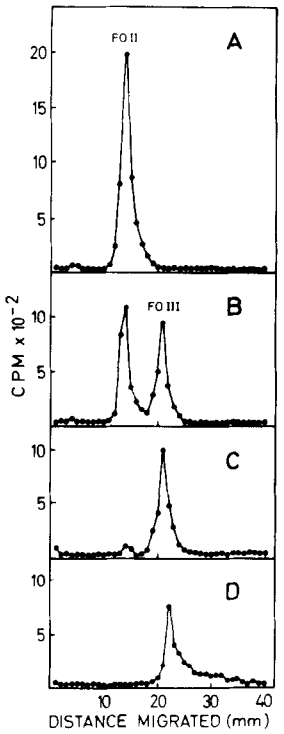

Fig. 2: Slab gel electrophoresis of S_1 treated radiation damaged [^{3}H] SV40 DNA FO II. The DNA (0.027µg) described in legend to Fig.1 was used (A) without enzyme; (B) + 0.5µl S_1; (C) + 5µl S_1; (D) + 10µl S_1. After electrophoresis the gel was sliced and the radioactivity contained in each slice was determined.

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in Fig.1 are not consistent with this prediction. Incubation of the substrate for 30 minutes with 0.5µl of S_1 nuclease generated 40% FO III, and no smaller fragments could be discerned in the gel (Fig.2B). A ten-fold increase in enzyme concentration converted 95% of the FO II molecules to FO III. Only when 10µl of the enzyme was applied some smaller products appeared that migrated faster than FO III, although it may be estimated that still 50% of the molecules consisted of structures of unit length (Fig.2C). Even after digestion with 30µl of S_1 nuclease approximately 45% of the molecules had retained a size corresponding to unit length.

As another nicked circular substrate FO II obtained by brief DNase-treatment of [^{3}H] FO I was used (insert in Fig.3A). Most of these molecules contained only one or two nicks as confirmed by the

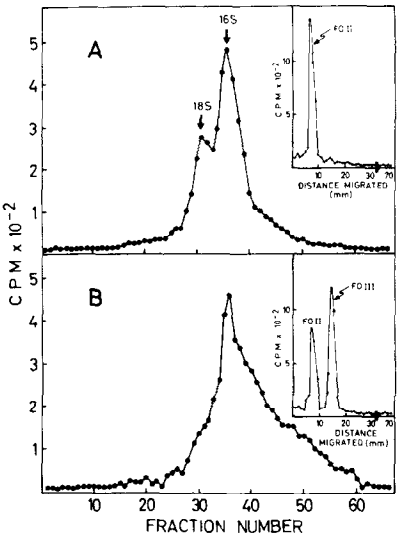

Fig. 3: Alkaline velocity sedimentation analysis of DNase-generated [^{3}H] SV40 FO II. 0.046µg DNA (7.7 x 10^3 cpm) were assayed. (A) without enzyme; (B) + 3µl S_1; the respective inserts show analysis of the DNA samples in agarose slab gels.

result of the alkaline velocity sedimentation analysis (Fig.3A) which revealed a relatively sharp profile of unnicked rings and of prevailing linear strands of unit length. After treatment of the substrate with S_1 nuclease most rings disappeared, the relative amount of 16S-components remained constant, and smaller fragments were generated (Fig.3B). Continuation of one of the pre-existing nicks to a double strand break would not produce much material sedimenting in alkali slower than strands of unit length. Product analysis in agarose gels (insert in Fig.3B) showed that most of the circular FO II molecules were converted to FO III without generating smaller linear products. Taken together, both results argue for de novo introduction of a double strand break by S_1 nuclease.

Also, when FO II (4.7µg) bearing on average four or more nicks was treated with S_1 (10µl) FO III was the only reaction product (results not shown here).

Kinetics of the reaction. Increasing concentrations of S_1 nuclease were added to constant amounts of radiation damaged [^{3}H] FO II, and the reaction products were electrophoresed in agarose

gels. The rate of conversion of FO II to FO III was determined from the quantitative evalution of the distribution of radioactivity in the sliced gels (see also Fig.2). The result of these experiments is summarized in Fig.4. The conversion of FO II to FO III is clearly dependent on the concentration of the S_1 nuclease. With increasing concentration of the enzyme an increasing formation of FO III could be noticed. Close to 100% conversion to FO III was observed when 5µl of S_1 nuclease were used. Within the range covered by application of 0.5 to 5µl of enzyme, FO III was the sole reaction product, as emphasized already in the preceding section (Fig.2). Only when the enzyme concentrations exceeded 10µl was it possible to generate products smaller than FO III. The single-strand-specificity of the S_1 nuclease and its ability to generate FO III from nicked FO II were investigated simultaneously by adding increasing amounts of single-stranded substrate to constant amounts of both radiation damaged $[^3H]$ FO II and of S_1 nuclease (Fig.5). The main result to be pointed out is, that both activities occured concurrently, even in the presence of an excess of single-stranded substrate. The ability to convert FO II to FO III was reduced faster than the single-strand-hydrolysing activity.

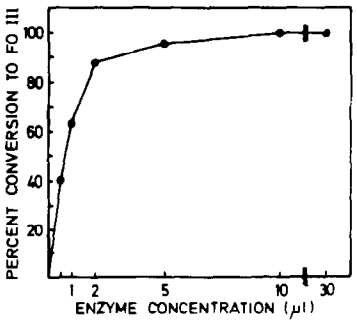

Fig. 4: Kinetics of $[^3H]$ SV40 DNA FO III formation. Radiation damaged $[^3H]$ SV40 DNA FO II was treated with increasing amounts of S_1 enzyme and analysed as described in Fig.2. The percentage of conversion to FO III was calculated by determining the amount of radioactivity of each peak (see Fig.2).

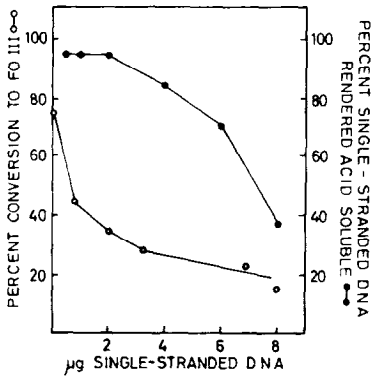

Fig. 5: S_1 nuclease treatment of radiation damaged [^{3}H] SV40 DNA FO II in the presence of single stranded DNA. The reaction mixture (o-o) contained [^{3}H] FO II (0.027μg, 4.5 x 10^3 cpm), 2μl S_1 nuclease, and the indicated amounts of CV-1 cell DNA (6), which had been denatured (7 min, 100°C) and was then quenched in ice. Product analysis was performed as described in the legend to Fig.4. The single-strand activity (●-●) of the S_1 nuclease was assayed by treating the indicated amounts of denatured [^{3}H] CV-1 DNA (7800 cpm/μg DNA) with 2μl of S_1 nuclease. The undigested DNA was precipitated after addition of 100μg bovine serum albumin with an equal volume of 10% trichloroacetic acid and was collected on Sartorius filters. Both the radioactivity on the filters and the radioactivity contained in the filtrates were determined.

DISCUSSION

The results reported in this paper have shown that S_1 nuclease contains an activity which opens nicked circular SV40 DNA FO II to linear duplex FO III of unit length. This reaction is dependent on the concentration of the S_1 nuclease as shown by the kinetics in Fig.4. Over a wide range, comprising a ten-fold increase of enzyme concentration, FO III is generated exclusively and no smaller DNA duplex structures were produced. If, under these conditions pre-existing single-strand nicks were continued by the enzyme to double strand breaks, then a steady decrease of FO III with concomitantly increasing amounts of smaller products should be observed. The enzyme concentration that was required to convert almost all nicked FO II to FO III (5μl) is sufficient to achieve 95% hydrolysis of at least a 100-fold larger amount of single-stranded DNA.

Our results extend the reports of other groups who have suggested that S_1 nuclease continues all single-strand nicks to

double-strand breaks in nicked circular substrates. This suggestion was made first by Beard et al. (4) who have studied [^{32}P] radiation damaged SV40 DNA. Similar conclusions were drawn recently by Germond et al. (5) who used polyoma DNA. Both groups have based their interpretation on data obtained by sucrose gradient analysis. The resolution provided by agarose gels is, however, superior to velocity sedimentation. This may account for the different conclusions that we have reached.Our experiments indicate that primarily FO III is formed after S_1 treatment of FO II, probably because of de novo introduction of a double strand break,rather than owing to extension of one out of several single-strand nicks to a double-strand break.

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