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Adenovirus terminal protein protects single stranded DNA from digestion by a cellular exonuclease

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ABSTRACT

Adenovirus 5 DNA-protein complex is isolated from virions as a duplex DNA molecule covalently attached by the 5' termini of each strand to virion protein of unknown function. The DNA-protein complex can be digested with E. coli exonuclease III to generate molecules analogous to DNA replication intermediates in that they contain long single stranded regions ending in 5' termini bound to terminal protein. The infectivity of pronase digested Adenovirus 5 DNA is greatly diminished by exonuclease III digestion. However, the infectivity of the DNA-protein complex is not significantly altered when up to at least 2400 nucleotides are removed from the 3' ends of each strand. This indicates that the terminal protein protects 5' terminated single stranded regions from digestion by a cellular exonuclease. DNA-protein complex prepared from a host range mutant with a mutation mapping in the left 4% of the genome was digested with exonuclease III, hybridized to a wild type restriction fragment comprising the left 8% of the genome, and transfected into HeLa cells. Virus with wild type phenotype was recovered at high frequency.

INTRODUCTION

The Adenovirus genome exists within the virion (1,2) and in infected cell nuclei (3) as a DNA-protein complex: a 35 kb duplex DNA molecule covalently attached via the 5' termini of each strand to terminal proteins. In the virion, these terminal proteins have a molecular weight of 55 K daltons (2). The genomes of Ø29, a duplex DNA bacteriophage of B. subtilis (4,5,6) and of picornaviruses (7,8), also have terminal proteins covalently bound to the 5' ends of polynucleotide chains. The function of these terminal proteins is not well understood. It is established that in Ø29 the terminal protein is required for DNA replication (9,10) and in an in vitro Adenovirus replication system, the terminal protein is required for template activity of the DNA-protein complex (11).

Adenovirus DNA replication starts at either DNA end and proceeds by a strand displacement mechanism (12,13). Daughter strands are initiated at or near the termini and synthesized in a 5' to 3' direction with concomitant dis-

placement of the parental strand of the same polarity. The second daughter molecule is generated by complementary strand synthesis from the 3' end of the displaced parental strand. Replicative intermediates are generated which have long single stranded regions having 5' termini covalently bound to terminal protein (E.L. Winnacker, personal communication).

In this work we show that Adenovirus 5 DNA-protein complex isolated from virions can be digested with E. coli exonuclease III (Exo III) to generate molecules analogous to replicative intermediates. Exo III digested DNA-protein complex contains long single stranded regions with 5' termini bound to terminal protein. We find that the infectivity of the DNA-protein complex is not diminished by Exo III digestion. This is in marked contrast to the infectivity of Adenovirus DNA in which terminal protein has been removed by pronase digestion. The infectivity of pronase digested DNA is greatly diminished by Exo III digestion. These results indicate that terminal protein protects 5' terminated single stranded regions of viral DNA from digestion by a cellular 5' exonuclease.

Exo III digested DNA-protein complex should be useful for methods of mutagenesis which require single strand regions of DNA (14,15,16) and for marker rescue of mutations mapping at the end of the genome by hybridization of wild type DNA restriction fragments to the single stranded regions prior to transfection (17).

MATERIALS AND METHODS

Enzymes. E. coli exonuclease III was purified by the method of Rogers and Weiss (18) from E. coli harboring the plasmid pSGR. This plasmid carries the structural gene for Exo III as well as bacteriophage λ replication functions, and after thermal induction the cellular concentration of Exo III increases 125 fold compared to wild type E. coli (19). The preparation was >95% pure as judged by SDS-polyacrylamide gel electrophoresis. Restriction endonuclease Sma I was a gift from Constance Cepko. Nuclease S1 was purchased from Boehringer Mannheim Biochemicals. E. coli DNA polymerase I Klenow fragment was purchased from Biolabs, Beverly, Mass.

Preparation of Ad5 DNA-protein complex and Ad5 DNA. DNA-protein complex was prepared from CsCl gradient purified Ad5 virions (20) by lysis with an equal volume of 8 M guanidine-HCl (Schwartz-Mann ultra pure) followed by equilibrium density centrifugation in 4 M guanidine-HCl, 3.03 M CsCl, 10 mM Tris, pH 8.0, 1 mM EDTA in polyalomar tubes in the type 65 rotor at 40,000 rpm for 40 hr at 4° (1). Peak fractions were pooled and stored at 4° in the

density gradient solution. Aliquots were removed and dialyzed exhaustively against 10 mM Tris, pH 8.0, 1 mM EDTA at 4° prior to enzyme digestion or transfection. The transfecting activity of Adenovirus DNA-protein complex is somewhat variable from experiment to experiment (21). However, we estimate that on average the infectivity of preparations stored in this way fell with a half-life of approximately one month. Ad5 DNA was prepared by phenol extraction of SDS-lysed pronase digested virions as described (20).

Transfections. Transfection of Ad5 DNA and DNA-protein complex was by the method of Graham and van der Eb (22) using HeLa DNA as carrier. Four hours following transfection of HeLa cells in 60 mm plates, the media was removed and 1 ml of 25% glycerol in phosphate buffered saline (PBS) was added to the plate. After 1 min it was removed, the plates were washed two times with 5 ml PBS and overlayed with agar overlay medium (23). Four hours after transfection into 293 cells, plates were simply overlayed with agar medium.

Enzyme digestion of DNA. For Exo III digestion, DNA or DNA-protein complex in 50 mM Tris, pH 8.0, 3 mM $MgCl_2$, 1 mM EDTA was warmed to 37° prior to addition of enzyme to 10 units or 50 units per µg DNA. At 50 units per µg digestion proceeded at a rate of approximately 500 nucleotides per min, and at 10 units per µg at approximately 300 nucleotides per min. In experiments where Exo III digested DNA or DNA-protein complex was used in transfections, digestion was terminated by addition of EDTA to 5 mM and incubation on ice. In the experiment in which Exo III digested DNA and DNA-protein complex were further digested with Sma I endonuclease, Exo III digestion was terminated by incubation at 68° for 10 min prior to digestion with Sma I.

Hybridization of Hind III G of wild type Ad5 to Exo III digested DNA-protein complex prepared from Ad5 hr1. Ad5 hr1 (24) was grown on 293 cells (25), virions were purified, and complex prepared as described above. The Hind III G fragment was purified by electrophoresis in Sea Plaque agarose (FMC Corporation, Rockland, Maine) followed by phenol extraction of the melted slice containing the Hind III G fragment, butanol extraction, and ethanol precipitation of the DNA (Russell Higuchi, manuscript in preparation). The molar equivalent of 5 µg of Ad5 DNA was ethanol precipitated with 20 µg E. coli tRNA carrier. The pellet was dissolved in 10 µl 0.1 N NaOH and incubated at room temperature for 10 min. Following chilling in an ice bath, 10 µl 1 M Tris, pH 7.4, was added, and subsequently 2.5 µg of Ad5 hr1 DNA-protein complex digested with 10 units/µg Exo III for 5 min at 37° was added. NaCl was added to 0.5 M and the solution was incubated at 68° for 10 min (>20 $C_o t_{\frac{1}{2}}$).

RESULTS

DNA-protein complex is a substrate for Exo III. The terminal protein of the Adenovirus DNA-protein complex is covalently bound to the 5' phosphate of each strand. It has a molecular weight of 55,000 daltons (2), and its hydrophobicity results in the formation of multimeric complexes and circular structures due to protein-protein interactions when the complex is removed. from protein denaturing solvents (21). The size of the terminal protein and its tendency to aggregate in non-denaturing solutions suggested that the complex might be resistant to Exo III digestion from the ends of the genome because of steric hindrance of Exo III attack at the termini. To test this possibility, ^{32}P labeled Ad2 DNA-protein complex and pronase digested DNA were treated with Exo III in parallel reactions and the rate of digestion was determined (Figure 1).

At 50 units Exo III per μg DNA, digestion followed essentially similar kinetics for DNA-protein complex and DNA, with an initial rate of ∿500 nucleotides digested (1.5% of the genome) per minute at each end. After 120 min of digestion, an additional 50 units Exo III/μg was added to the reaction, and following an additional 30 min of digestion ∿45% of the DNA of both

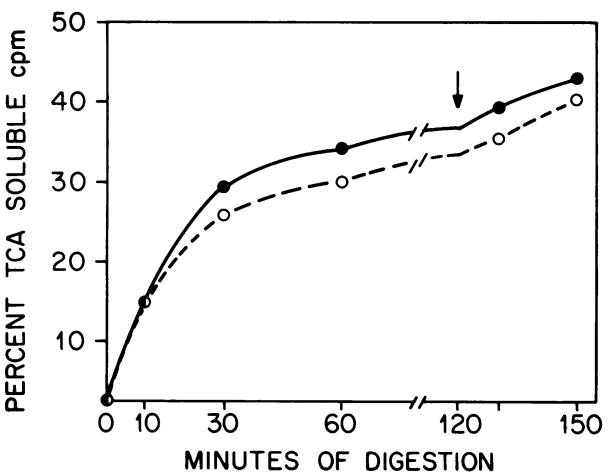

Figure 1. Kinetics of digestion of Ad2 DNA-protein complex and Ad2 DNA by Exo III. Uniformly ^{32}P labeled Ad2 DNA-protein complex and Ad2 DNA were treated with 50 units/μg Exo III at 37°. Aliquots of the reaction were withdrawn at the times shown and the fraction of cpm soluble in cold 5% trichloroacetic acid were determined. An additional 50 units/μg DNA were added to the reaction after 120 min (arrow). ●——●, Ad2 DNA-protein complex; O——O, Ad2 DNA.

pronase digested DNA and DNA-protein complex was digested to acid solubility, close to the theoretical limit of 50% (26).

To determine whether or not digestion had occurred from the ends of the genome in the DNA-protein complex, aliquots of the two reactions were removed during the course of digestion, Exo III was thermally inactivated, and the DNA was further digested by the restriction enzyme Sma I. Sma I does not digest single stranded DNA. Thus, if Exo III digestion proceeds past the terminal Sma I sites at each end of the genome, digestion will yield the internal Sma I fragments, but the terminal and penultimate fragments at each end of the genome will not be produced. Rather, a partially duplex fragment will be produced composed of one strand (the strand with its 5' end at the end of the genome) equal in length to the sum of the terminal plus penultimate fragments and the other, Exo III digested strand, shorter than the length of the penultimate restriction fragment and terminating at the internal restriction site.

The Exo III, Sma I digested DNA and DNA-protein complex were analyzed by electrophoresis on an alkaline agarose gel followed by autoradiography of the gel (Figure 2). Denaturation of the strands in the alkaline gel allows the unique length undigested strands of the fused terminal fragments to migrate independently of the Exo III digested strands which are heterogeneous in length. After 10 min of Exo III digestion, the left terminal fragments J and E are not observed. Instead, new fragments equal in length to J + E and J + E + L are observed in both the DNA and DNA-protein complex samples. Similarly, the right terminal fragments C, G and K are absent from the 10 min Exo III digested samples and a new fragment equal in length to C + G + K is observed. Additional high molecular weight bands are also observed at lower intensity and result from molecules in which Exo III digestion has proceeded into Sma I F at the left and Sma I A at the right. Quantitation of counts in the J + E and J + E + L band and the C + G + K band by densitometry of the autoradiogram indicate that the 5' terminal strands at each end of the genome remain intact in >80% of the Exo III digested molecules.

After longer periods of Exo III digestion, still higher molecular weight bands are observed on the alkaline gel (Figure 2). This indicates that even after extensive Exo III digestion with this enzyme preparation the long single stranded regions that are generated from the ends of the genome remain intact in a substantial fraction of molecules. However, even after long periods of digestion, some counts remain in Sma I fragments near the termini, indicating that in both the DNA and DNA-protein complex preparations, a small

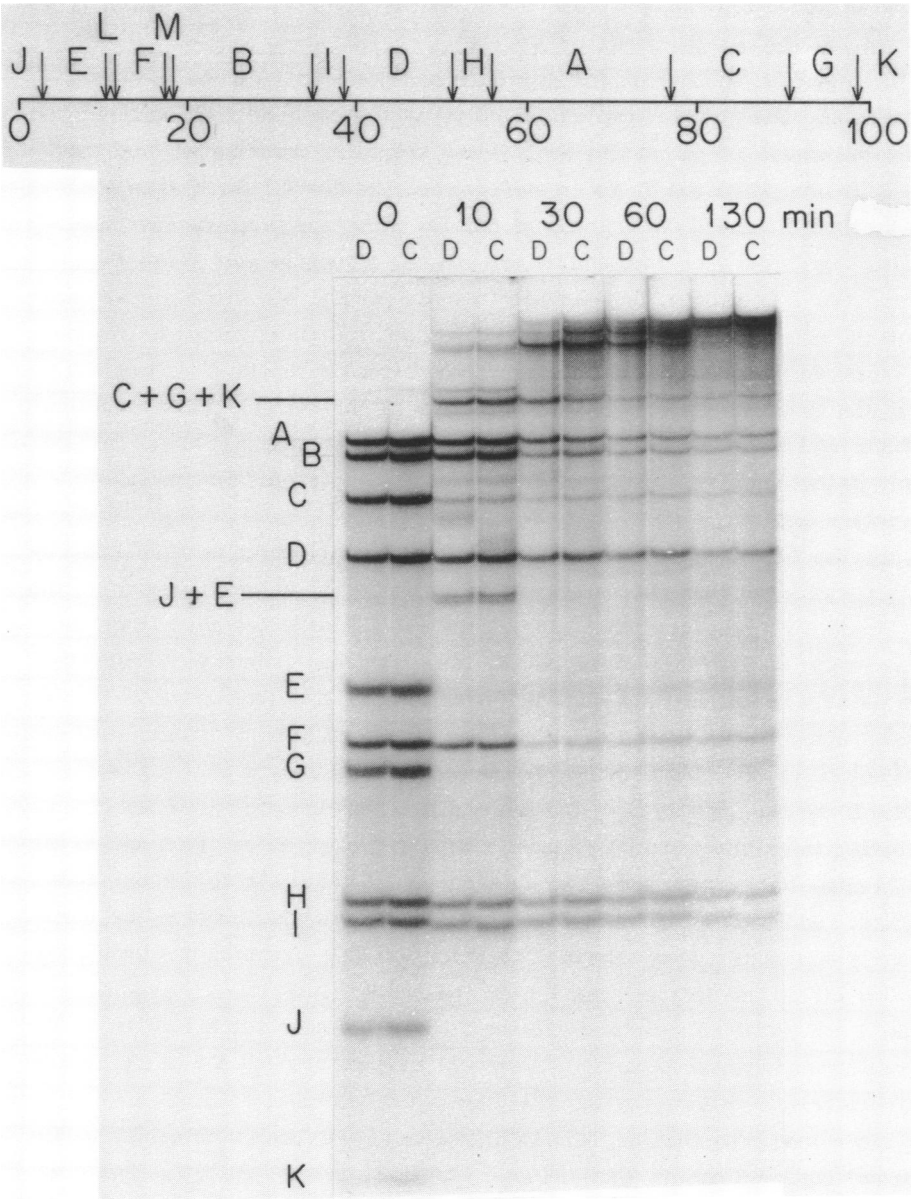

Figure 2. Autoradiogram of an alkaline agarose gel of Exo III and Sma I digested Ad2 DNA-protein complex and Ad2 DNA. DNA is shown in D lanes, DNA-protein complex in C lanes. Minutes of digestion are shown. The Sma I map of Ad2 DNA is shown at the top.

fraction of ends are resistant to Exo III digestion.

These results demonstrate that pronase digested DNA and the DNA-protein complex are equally good substrates for Exo III digestion. Two other experiments were performed to further substantiate that the DNA-protein complex is a substrate for Exo III. DNA-protein complex was digested for 5 min with 10 units Exo III/μg and, following thermal inactivation of Exo III, to completion with Sma I. The products were electrophoresed in 1.4% agarose in neutral buffer containing 0.1% sarkosyl along with a sample of the same preparation of DNA-protein complex digested with Sma I and with marker Sma I digested Ad5 DNA. Covalent linkage of the end protein to the terminal fragments of the Sma I digested DNA-protein complex causes these fragments to electrophorese with decreased mobility compared to the terminal fragments of Sma I digested DNA (E. Winnacker, personal communication). This was observed with our DNA-protein complex preparation, and, following Exo III digestion, the terminal and penultimate Sma I fragments were lost (data not shown). Thus we demonstrated that terminal protein was present on the DNA immediately before Exo III digestion, and that Exo III attacked the termini of the DNA-protein complex.

In the second experiment, DNA and DNA-protein complex were digested in parallel for 5 min with 10 units Exo III/μg DNA. The two samples were then pronase digested, phenol extracted and used as substrates for repair synthesis using α-^{32}P TTP and the Klenow fragment of E. coli polymerase I. After complete repair, the labeled DNAs were digested with Sma I and electrophoresed through a neutral agarose gel. The two Exo III digested samples incorporated the same mass of TTP (equivalent to $\sim$1500 nucleotides at each end of the genome), and autoradiography of the gel revealed that >90% of the label was incorporated into the two terminal and two penultimate Sma I fragments (data not shown). These results again demonstrate that the rate of Exo III digestion of DNA and of DNA-protein complex is equivalent and that the 5' ends of each strand which are rendered single stranded by Exo III digestion are not nicked during digestion with this preparation of Exo III.

Exo III digestion does not diminish the infectivity of DNA-protein complex. DNA-protein complex was digested with Exo III at a concentration of 10 units/μg for time periods from 30 sec to 8 min, digesting 150 to 2400 nucleotides from the 3' ends of each strand. The treated complex was then used to infect HeLa cells by the $Ca_2(PO_4)_3$ transfection method of Graham and Van der Eb (22). The specific infectivity of Exo III digested DNA-protein complex did not vary significantly from that of untreated complex, even after

digestion of 2400 nucleotides from the 3' ends of each strand (Figure 3).

These results for DNA-protein complex contrasted with findings for pronase digested DNA. The specific infectivity of untreated pronase digested DNA is 10^2 - 10^3 fold lower than that of the DNA-protein complex (21). Exo III digestion of 600 to 2400 nucleotides from the 3' ends of each strand of pronase digested Ad5 DNA decreased its infectivity an additional factor of 10^2 - 10^3 (Fig. 2).

To be certain that the infectious activity of Exo III treated complex was indeed due to partially single stranded molecules, we tested the sensitivity of this infectivity to S1 nuclease. Simply subjecting fully duplex DNA-protein complex to the low pH and high salt conditions required for single strand specific S1 digestion, without addition of S1, decreased the infectivity of the complex to 25% that of untreated complex (Table 1, experiment 1).

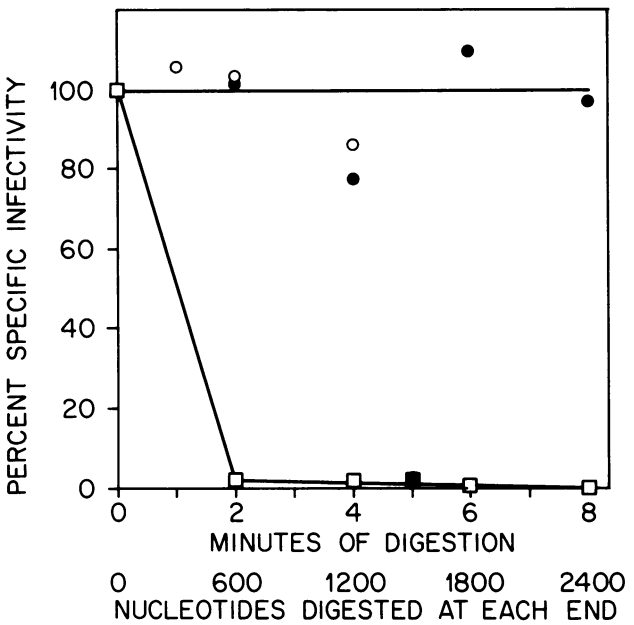

Figure 3. Specific infectivity of Ad5 DNA-protein complex and Ad5 DNA following Exo III digestion. The 0 time sample was withdrawn immediately before addition of enzyme. Circles and squares show the specific infectivity as percentage of the 0 time sample for Ad5 DNA-protein complex and for Ad5 DNA, respectively. Open and closed symbols refer to results for experiments 1 and 2, respectively. The specific infectivities of 0 time samples in experiments 1 and 2 were 5600 plaques/μg and 4800 plaques/μg for DNA-protein complex, and 51 plaques/μg and 24 plaques/μg for DNA, respectively.

Table 1. Sensitivity of infectivity to S1 digestion

Experiment	Percent infectivity of untreated DNA-protein complex		
	Untreated	S1 digestion conditions without S1[a]	S1 digestion conditions with S1[b]
1 Duplex	100	25	22
Exo III, 5 min	100	25	1.4
2 Duplex	100	not done	30
Exo III, 5 min	100	not done	1.1

a: 5 µg DNA-protein complex were incubated at 20° in 1 ml 0.25 M NaCl, 0.03 M $NaCH_3COO$, pH 4.5, 1 mM $ZnCl_2$, 5% glycerol. After 30 min EDTA was added to 5 mM.

b: 5 µg DNA-protein complex were incubated as in (a) except that 1000 units S1 were added to the 20° incubation.

Addition of S1 to the incubation mixture did not lead to a significant further decrease in the infectivity of duplex DNA-protein complex. The infectivity of Exo III digested complex was also decreased about four fold simply by incubation in S1 digestion buffer. However, unlike duplex DNA-protein complex, addition of S1 to the incubation mixture resulted in a substantial further decrease in the infectivity of Exo III-digested complex (Table 1, experiments 1 and 2). The S1-resistant infectivity of this preparation may be due to the small fraction of molecules which are resistant to Exo III digestion (Fig. 1).

Hybridization of wild type Ad5 Hind III G to Exo III-digested Ad5 hr1 DNA-protein complex results in recovery of wild type virus at high frequency. As a test of the utility of Exo III digested complex, we examined the frequency of marker rescue of a mutation mapping at the left end of the Ad5 genome following hybridization to a left end restriction fragment prepared from wild type virus.

The host range mutant Ad5 hr1 plaques efficiently on 293 cells, a line of Ad5 transformed human embryonic kidney cells, but not on HeLa cells (24). The mutation responsible for this phenotype has been mapped to within the left 4.5% of the genome (23). We prepared DNA-protein complex from Ad5 hr1 virions and digested the mutant complex with 10 units/µg Exo III for 5 min, digesting approximately 1500 nucleotides (4.3% map units) from the 3' end of each strand. Hind III G fragment (mapping from 0 - 8.0%) equimolar to 5 µg of Ad5

DNA was denatured, mixed with 2.5 µg of Exo III digested Ad5 hr1 DNA-protein complex and hybridized to ∿20 x $C_0t_{\frac{1}{2}}$. The products of the hybridization were then transfected into HeLa and 293 cells.

Sharp et al. (21) found that the specific infectivity of Ad5 DNA-protein complex varied over a range of ∿100 fold from experiment to experiment. We have found this also, particularly following hybridization of restriction fragments to Exo III digested complex (see Table 2, plaques/µg of Ad5 hr1 complex on 293 cells). For this reason, in order to best measure the relative infectivity of a DNA-protein complex preparation on HeLa and 293 cells, the same $Ca_2(PO_4)_3$ precipitate was transfected into 293 and HeLa cells at approximately the same time, and the ratio of infectivity in HeLa and 293 cells was determined.

In two successive experiments, the ratio of infectivity of HeLa to 293 cells of Ad5 hr1 complex hybridized to Hind III G increased at least 100 fold compared to the HeLa/293 specific infectivity of Exo III digested complex taken through the same procedure but without addition of Hind III G to the hybridization reaction (Table 2). Furthermore, in experiment 1, 3 out of 3 plaques picked from HeLa cells following transfection of the unhybridized Ad5 hr1 DNA-protein complex were still host range in phenotype, whereas 3 out of 3 plaques picked from HeLa cells transfected with the hybridized complex had wild type phenotype. Thus, hybridization of wild type sequence to mutant sequence rendered single stranded by Exo III digestion resulted in rescue of the wild type phenotype at high frequency.

DISCUSSION

In this work we show that Adenovirus DNA-protein complex prepared by guanidine HCl lysis of virions (1) is a substrate for exonuclease III of E. coli. That is, the 55 K dalton hydrophobic protein covalently bound to the 5' end of each strand in the DNA-protein complex (2) does not inhibit the attack

Table 2. Marker rescue of hr1 with Hind III G

Experiment	Hybridize to Hind III G	Plaques/µg HeLa	293	HeLa/293
1	-	87	35,000	0.0025
	+	460	1,680	0.27
2	-	<1	2,000	<0.0005
	+	510	6,200	0.082

of Exo III on the 3' ends of each strand. Furthermore, we demonstrate that Exo III digestion of the DNA-protein complex does not significantly diminish its specific infectivity even after digestion of 2400 nucleotides from the 3' end of each strand. In contrast to this finding, Exo III digestion of pronase digested DNA markedly diminishes its infectivity.

The DNA-protein complex is 10^2 - 10^3 fold more infectious than Adenovirus DNA isolated from pronase digested virions (21). Sharp et al. (21) demonstrated that in vitro the DNA-protein complex is resistant to the ATP dependent exonuclease of H. influenzae (equivalent to rec BC exonuclease V of E. coli) whereas pronase digested Adenovirus DNA, like other linear DNAs, is sensitive to this nuclease. These authors suggested that the resistance of the DNA-protein complex to an equivalent enzyme in animal cells might account in part for the increased infectivity of the DNA-protein complex compared to DNA.

The failure of Exo III digestion to diminish the infectivity of DNA-protein complex contrasts with the marked effect of Exo III digestion on the infectivity of pronase digested DNA. This result suggests that the terminal protein protects the single stranded termini of the genome from digestion by a cellular exonuclease. Exo III digested DNA-protein complex is analogous to Adenovirus DNA replicative intermediates in that it contains single stranded regions which end in 5' termini attached to terminal protein. In particular it is very similar to type II replicative intermediates (12). Thus, the findings here suggest that one function of the terminal protein is to protect the displaced parental single strands generated during DNA replication from digestion by cellular exonuclease.

Exo III digestion resects the r-strand at the left end of the Adenovirus genome and the l-strand at the right end of the genome. Transcription of the r-strand between approximately 500 and 1500 nucleotides from the left end of the genome (early region IA) is necessary for production of a pre-early function required for expression of early regions IB, II, III, and IV in HeLa cells (27,28,29). Thus it is very likely that repair synthesis performed by cellular enzymes follows transfection of Exo III digested complex regenerating the left end of the r-strand and allowing transcription of early region IA.

Exo III digested complex should be useful for the generation of mutations in the termini of the genome using single strand specific mutagens such as $NaHSO_3$ (14,30), and for marker rescue of mutations mapping near the termini following hybridization to terminal restriction fragments. We have demonstrated the feasibility of the latter procedure by showing efficient marker rescue

of the Ad5 hr1 mutation which maps between 0 and 4.5 map units (23) with the Ad5 Hind III G restriction fragment (0 - 8.0 map units).

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