The 3'-Terminal Nucleotide Sequences of Adenovirus Types 2 and 5 DNA

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Short nucleotide sequences at the 3'-termini of adenovirus types 2 and 5 DNA have been determined using T4 DNA polymerase as described by P. T. Englund (1972). The terminal sequences of both serotypes appear to be completely identical. Both molecular ends of type 2 as well as of type 5 DNA terminate with the sequence ...pCpC...pGpApTpG9, consistent with the presence of an inverted terminal repetition in adenovirus DNA.

The human adenoviruses type 2 (Ad2) and type 5 (Ad5) contain linear duplex DNA molecules with a molecular weight of about 24 x 10^6 (6, 13), which are homologous to a large extent (3). Denaturation of native adenovirus DNA followed by renaturation at low concentrations leads to the formation of single-stranded circles, indicating that adenovirus DNA contains termini with an inverted repetition, i.e., identical nucleotide sequences at both molecular ends of the type

\[
\begin{align*}
\text{3' terminal:} & \ A\ B\ C\ D \ldots \ D'\ C'\ B'\ A' \ a^9 \\
\text{5' terminal:} & \ A'\ B'\ C'\ D' \ldots \ D\ C\ B\ A \ a^9 
\end{align*}
\]

(4, 14). The actual nature of the termini and the function of this terminal repetition are still unknown.

The structure of the termini of adenovirus DNA is of particular interest from the point of view of DNA replication and transcription. Studies on the initiation of replication of Ad5 DNA have shown that the origin is located at the A-T rich molecular end, suggesting that a unique nucleotide sequence is situated at this side of the molecule (12). To obtain more information on the structure of the molecular ends of adenovirus DNA, we have analyzed the terminal nucleotide sequences. In this communication we report the determination of short nucleotide sequences at the 3'-termini of native Ad2 and Ad5 DNA using the method developed by Englund (1, 2). This method is based on the fact that T4 DNA polymerase contains a polymerizing as well as a 3'-5' exonucleolytic activity (5). Incubation of linear duplex DNA with this enzyme at 11 C in the presence of a single deoxyribonucleoside triphosphate (dNTP) leads to release of mononucleotides from the 3'-termini due to the exonucleolytic activity associated with the polymerase. Once a mono-

nucleotide is released that can be replaced by the enzyme's polymerizing activity by transfer from the added dNTP, a steady state of release and replacement is reached and the enzyme will not further penetrate into the DNA molecule. Using this exchange reaction, incubation of DNA in the presence of each of the four α-[\(^{32}\)P]dNTP's leads to a specific labeling of the first dAMP, dTMP, dGMP, or dCMP residue from the 3'-termini of a linear duplex DNA molecule. The relative order of these residues can be derived from the effect of unlabeled dNTP's on the incorporation, e.g., the 3'-terminal residue is that whose incorporation is unaffected by any one of the other dNTP's.

These data in combination with those of nearest neighbor analysis of the labeled DNAs establish the complete sequence of a short stretch of nucleotides from the 3'-termini of linear duplex DNA.

MATERIALS AND METHODS

Materials. Ad2 and Ad5 DNA were isolated from purified virions as described previously (11). Alkaline sucrose gradient centrifugations showed that more than 90% of the DNA was free of nicks. Unlabeled dNTP's were obtained from Sigma, St. Louis, Mo.; α-[\(^{32}\)P]dNTP's (specific activity > 100 Ci/mmol) were from New England Nuclear, Boston.

T4 DNA polymerase preparations were generous gifts from H. Heynker and B. M. Alberts. Micrococcal nuclease and spleen phosphodiesterase were purchased from Boehringer, Mannheim. Endonuclease R EcoRI was isolated as described by Mulder and Delius (8).

Incubation conditions. A typical reaction mixture (0.1 ml) contained 5 μg of adenovirus DNA, 7 μmol of Tris-hydrochloride, pH 8.0, 0.7 μmol of MgCl\(_2\), 1 μmol of 2-mercaptoethanol, 0.05 nmol of one α-[\(^{32}\)P]dNTP and 12 U of T4 DNA polymerase. Unlabeled dNTP was added when indicated in a concentration of 0.25 mM. The reaction mixture was incubated at 11 C for
150 min, after which 10 μl of 0.5 M EDTA were added to stop the reaction. The incorporation of radioactivity into acid-insoluble material was measured by adding samples of the reaction mixture to 0.3 ml of 0.3 M EDTA, 0.3 M inorganic pyrophosphate (PPi), pH 7.2, containing 0.3 mg of calf thymus DNA per ml. After addition of 2 ml of 5% trichloroacetic acid-0.01 M PPi, the acid-insoluble material was collected on Whatman GF C filter disks. The filters were washed six times with 10 ml of 2% trichloroacetic acid, 0.01 M PPi, rinsed once with ethanol, dried, and counted in a liquid scintillation counter.

Nearest neighbor analysis. Nearest neighbor analysis was performed essentially as described by Englund (2). After the T4 DNA polymerase reaction had been stopped, 40 μg of calf thymus DNA was added as carrier and the DNA was separated from α-[32P]dNTP's in the following way. DNA was precipitated by addition of 2 ml of 10% trichloroacetic acid, 0.01 M PPi, at 0 C and was centrifuged for 10 min at 15,000 × g. The supernatant was discarded and the precipitate was redissolved in 0.1 ml of 50 mM NaOH. This procedure was repeated four or five times; radioactivity in 0.1 ml of the supernatant had then reached background level (20 counts/min). The precipitate was washed with ice-cold ethanol, dried, and redissolved in 90 μl of 2.5 mM Tris-hydrochloride, pH 9.0, and 2.5 mM CaCl2. Then 15 U of micrococcal nuclease was added and the mixture was incubated at 37 C for 20 min. The pH was subsequently adjusted to 6.8 with 0.1 M HCl, and 0.2 U of spleen phosphorylase was added. Incubation was continued for 90 min at 37 C. The reaction mixture was chromatographed on Whatman no. 1 paper. The chromatogram was developed using saturated ammonium sulfate-1.0 M sodium acetate-isopropanol (80:18:2) as solvent (7, 15). After drying, the four nucleotide spots were visualized by UV light and cut from the chromatogram, followed by counting in a liquid scintillation counter.

Isolation of terminal fragments of adenovirus DNA. After stopping the T4 DNA polymerase reaction by addition of EDTA, the mixture was deproteinized by addition of 1.8 ml of 0.16 M KCl and 2.0 ml of chloroform-isomylalcohol (24:1), followed by shaking for 1 min. The aqueous layer was transferred to a centrifuge tube, two times its volume of ice-cold ethanol was added, and the mixture was allowed to stand at -70 C for 30 min. After centrifugation at -5 C for 30 min at 35,000 rpm, the supernatant was discarded. The precipitated DNA was redissolved in 90 mM Tris-hydrochloride, pH 7.9, 10 mM MgCl2, and endonuclease R- EcoRI was added in a quantity that digested the DNA completely in 45 min at 37 C. After digestion the viral DNA fragments were deproteinized as described above. Gel electrophoresis of the deproteinized DNA fragments was performed in 0.7% agarose containing ethidium bromide as described by Pettersson et al. (10). The positions of the fragments in the gels were visualized by UV light. The distribution of the radioactivity was determined after slicing of the gel. The slices were liquefied by heating, dissolved in counting solution, and counted in a liquid scintillation counter.

RESULTS

The effect of unlabeled dNTP's on the incorporation of various labeled nucleotides in adenovirus DNA. Figure 1 shows the time course of the incorporation of dCMP into Ad2 DNA in the presence of α-[32P]dCTP and T4 DNA polymerase. Similar results were obtained with each of the other α-[32P]dNTP's. The maximal incorporation levels with Ad2 as well as Ad5 DNA varied between 1.6 to 2.0 residues per DNA molecule. This is in agreement with an incorporation of one residue of each nucleotide per molecular end.

For sequence analysis the competitive effect of unlabeled dNTP's on the incorporation of each of the α-[32P]dNTP's into adenovirus DNA was studied. The results of these experiments with Ad2 as well as with Ad5 DNA are summarized in Fig. 2. For both types of DNA the incorporation of [32P]dGMP was not influenced by the presence of any one of the other dNTP's, whereas incorporation of [32P]dTMP was only inhibited by dGTP. Further, the incorporation of [32P]dAMP was inhibited by dGTP and dTTP, whereas the incorporation of [32P]dCMP was affected by all three remaining dNTP's.

These results indicate that for Ad2 as well as for Ad5 DNA the relative positions of the labeled residues from the 3'-termini are . . . C . . . A . . . T . . . G . . . . The relative positions at the left and right terminus must be identical since the inhibition of incorporation of one particular dNMP was always between 75 and 95%. If the relative positions at both termini were different, inhibition values around 50% may be expected, which were not observed.

The presence of identical relative positions at both 3'-termini was verified for Ad5 DNA by analyzing the incorporation of radioactivity at

![Fig. 1. Incorporation of [32P]dCMP into Ad2 DNA by T4 DNA polymerase. Ad2 DNA was incubated with T4 DNA polymerase and α-[32P]dCTP as described in Materials and Methods. Samples were taken at the times indicated and the incorporation was determined by trichloroacetic acid precipitation.](http://jvi.asm.org/DownloadedFrom/vol_15_1975/terminals_sequences_of_adenovirus_dna_269)
FIG. 2. Effect of unlabeled nucleotides on the incorporation of labeled nucleotides into Ad2 and Ad5 DNA. Ad2 or Ad5 DNA were incubated in the presence of T4 DNA polymerase and a single \( \alpha-[\text{32P}]dNTP \), with or without one of the other unlabeled dNTP's. The incubation conditions were as described in Materials and Methods. The bars indicate the relative incorporations in the presence and absence of the unlabeled dNTP's, respectively. The incorporation in the absence of unlabeled triphosphate was set at 100%.

TABLE 1. Incorporation of dAMP into the termini of Ad5 DNA

<table>
<thead>
<tr>
<th>Terminal fragment</th>
<th>Incorporation of ( \text{[3P]} \text{dAMP} ) (counts/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>- + dCTP + dGTP + dTTP</td>
</tr>
<tr>
<td>B</td>
<td>1638 1458 219 208</td>
</tr>
<tr>
<td></td>
<td>1345 1095 195 122</td>
</tr>
</tbody>
</table>

*Ad5 DNA was incubated with T4 DNA polymerase and \( \alpha-[\text{32P}]dATP \) in the presence or absence of one of the three unlabeled dNTP's as indicated. After the incubation the DNA was isolated, digested with endo R.EcoRI and fragments were separated by agarose-gel electrophoresis (see Materials and Methods). The Table shows the results of Cerenkov counting of the gel slices containing the terminal fragments A and B.
iting effect on the incorporation at both molecular ends. This is completely in agreement with the results shown in Fig. 2 and verifies that for Ad5 DNA the relative positions of the four dNMP’s are the same at the right and left 3’-terminus.

**Nearest neighbor analysis.** Further information on the nucleotide sequences at the 3’-termini was obtained from nearest neighbor analysis. Ad2 as well as Ad5 DNA were incubated with T4 DNA polymerase and each of the four α-[32P]dNTP’s, respectively, after which the viral DNA was precipitated with 10% trichloroacetic acid. After repeated washings the DNA was digested completely to 3’-mononucleotides with micrococcal nuclease and spleen phosphodiesterase. The mixture of mononucleotides was separated by paper chromatography, after which the radioactivity in each nucleotide was determined. Figure 3 shows the results of the nearest neighbor analysis of Ad2 and Ad5 DNA. In all cases the transfer of 32P to one of the four 3’-mononucleotides was 60 to 80%, whereas the remaining 20 to 40% of the radioactivity was found divided over the other three nucleotides. This indicates that the same dinucleotide sequences are present at both molecular ends. The results in Fig. 3 show that for Ad2 as well as Ad5 DNA both termini contain the dinucleotides pTpG, pApT, pGpA, and pCpC.

Combination of these data with the relative positions ... C ... A ... T ... G leads to the conclusion that the 3’-terminal sequences of Ad2 and Ad5 DNA are ... pCpC ... pGpAp-TpG. **DISCUSSION**

Using the T4 DNA polymerase technique
described by Englund (2) the following 3'-terminal sequence . . . pCpC . . . pGpApTpG is established at both ends of Ad2 and Ad5 DNA. During the specific labeling of the first residues we also observed some aspecific labeling varying between 10 to 20% in the inhibition reactions and about 30% in the transfer of label during the nearest neighbor analysis. Aspecific labeling might be caused by the presence of random single-stranded breaks at which the combined exonuclease-repair reaction might occur. A second factor might be the occasional "slipping" of T4 DNA polymerase past the first nucleotide in the sequence that can be exchanged. This process becomes more prominent at temperatures above 11°C (2).

The results presented in this paper are consistent with two possible structures for the adenovirus DNA termini. One possibility is that the ends are flush with the sequence

\[ pCpApTpC \ldots pGpG \ldots pCpC \ldots pGpApTpG \]

The other possibility is that there is a 5' single-stranded extension of one nucleotide with the sequence

\[ pCpApTpC \ldots pGpG \ldots pCpC \ldots pGpApTpG \]

Both structures would give identical results in the experiments described in this paper.

More complicated structures involving a 3' single-stranded extension are highly unlikely in view of the sensitivity of circular single-stranded adenovirus DNA to Escherichia coli exonuclease III as reported by Wolfson and Dressler (14).

The identical sequences at the 3'-ends of Ad2 and Ad5 DNA indicate a high degree of similarity between both DNA's. This is in agreement with earlier observations (3). The identical sequences at the 3'-ends of adenovirus DNA confirm the model of an inverted terminal redundancy proposed by Wolfson and Dressler (14) and Garon et al. (4).

Our data do not reveal a structural basis for the fact that the replication of Ad5 DNA always starts from the molecular right end of the molecule (12). Since the inverted repetition extends to the very end of the molecule, it seems unlikely that the origin of replication is located at the ultimate end of the righthand terminus. However, a location of the origin more internally has not been excluded and further studies on the structure of the molecular ends are in progress.

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LITERATURE CITED


