Site-specific nicking within the adenovirus inverted terminal repetition

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ABSTRACT

Site-specific nicking occurs on the l-strand, but not on the r-strand, of the adenovirus left inverted terminal repeat. Nicks are presumably introduced into double- or single-stranded DNA by a cellular endonuclease in an ATP-independent reaction. The consensus nick site has the sequence:

GGRGYG[↓]GGRNRNGTG.

INTRODUCTION

DNA molecules from all adenovirus serotypes have an inverted terminal repetition (ITR) (1,2). Many adenovirus ITRs have recently been sequenced: human adenovirus serotypes 2, 3, 4, 5, 7, 9, 10, 12, and 31 (3-10); simian adenovirus SA7 (4); mouse adenovirus FL (11); avian adenovirus CEL0 (12, 13); equine adenovirus (13); and infectious canine hepatitis virus ICHV (13). ITRs differ between serotypes in both sequence and length, but all ITRs share conserved or highly homologous sequences. The longest conserved sequence, ATAATATAC (nucleotides 9 through 17 from the ends), has been shown to control adenovirus replication (14) in a cell-free replication assay (15). Another conserved sequence, TGACGT, is located at or near the boundaries of the ITRs, but the function, if any, remains as yet undefined. In this paper we show that highly homologous G-rich sequences within the ITR are nicked at specific sites. The results suggest that a cellular endonuclease nicks double- or single-stranded DNA in an ATP-independent reaction. The consensus nick site has the sequence GGRGYG[↓]GGRNRNGTG. Although the functional significance of site-specific nicking within the ITR is not yet known, G-rich sequences similar to the adenovirus nick sites are also found at or near papovavirus replication origins (16).

MATERIALS AND METHODS

Materials. Restriction endonucleases, E. coli DNA polymerase I (Klenow fragment), E. coli exonuclease III, dNTPs, and ddNTPs were from Bethesda

Figure 1. Restriction endonuclease maps of XD-7 (left) and mKM4 (right). Double lines indicate pBR322 sequences, the thick line represents adenovirus sequences, and the thin line shows M13mp8 sequences. XD-7 contains the entire 1.35-kb XbaI-E fragment from the left end of type 2 adenovirus DNA inserted into the EcoRI site of pBR322. mKM4 contains the 1-kb HindIII-SmaI fragment of XD-7 inserted between the HindIII and SmaI sites of M13mp8. Single-stranded mKM4 has the adenovirus r-strand. The primer site lies just to the left of the HindIII site on mKM4.

Research Laboratories. The enzymes were used as recommended by the supplier. The synthetic 15-base primer was from P-L Biochemicals. [α - 32 P]dATP (800 Ci/mmol) was from New England Nuclear. Purified calf thymus topoisomerase I was generously provided by Dr. LeRoy F. Liu.

Construction of mKM4. XD-7 (15) and M13mp8 (17) have already been described. The 1-kb HindIII-SmaI fragment of XD-7 (Fig. 1) was cloned into M13mp8 cut with HindIII and SmaI. The 8.3-kb recombinant phage, called mKM4 (Fig. 1), was grown in *E. coli* JM103. Single-stranded, circular mKM4 DNA was purified by electrophoresis on a horizontal 0.8% agarose gel.

Labeling the adenovirus 1-strand. The annealing reaction (2.4 μ l) contained 300 ng mKM4 DNA, 0.5 ng primer, 100 mM NaCl, 17 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, and 0.8 mM EDTA. The solution was heated to 100°C for 3 min and then cooled to 22°C over a period of 45 min. Primer extension was carried out in a reaction mixture (5 μ l) containing 300 ng mKM4 DNA annealed to 0.5 ng primer; 120 mM NaCl; 18 mM Tris-HCl (pH 7.5); 14 mM MgCl₂; 4 mM dithiothreitol; 0.4 mM EDTA; 25 μ M each of dCTP, dGTP, and dTTP; 0.5 μ M [α - 32 P]dATP (2 μ Ci); and 0.2 unit *E. coli* DNA polymerase I (Klenow fragment). After incubating the solution for 10 min at 37°C, the dATP concentration was adjusted to 80 μ M and incubation continued for 10 min. Approximately 600 bases were polymerized under these conditions. Dideoxy sequence ladders were synthesized on mKM4 as described by Sanger *et al.* (18) and used as length standards.

Labeling the adenovirus r-strand. HindIII-cut XD-7 or pBR322 DNA (1 μ g) was digested for 30 min at 22°C with 5 units of *E. coli* exonuclease III in a 15 μ l reaction mixture containing 90 mM NaCl, 13 mM Tris-HCl (pH 7.4), and 5 mM MgCl₂. The reaction was stopped by phenol extraction. Approximately 400 bases were removed from each end of the linear DNA under these conditions (19). Exonuclease III-digested DNA was repaired in a 5 μ l reaction mixture containing 1 μ g DNA; 25 mM Tris-HCl (pH 7.5); 5 mM MgCl₂; 2 mM dithiothreitol; 50 μ M each dGTP, dCTP, and dTTP; 1 μ M [α -³²P]dATP (4 μ Ci); and 0.15 unit *E. coli* DNA polymerase I (Klenow fragment). After incubating the solution for 10 min at 37°C, the dATP concentration was adjusted to 80 μ M and incubation continued for 10 min.

Nicking reaction. Nuclear and cytoplasmic extracts were prepared from uninfected and adenovirus-infected HeLa cells as previously described (15). The protein concentration in the nuclear extracts ranged from 4 to 6 mg/ml, and the concentration in the cytoplasmic extract was 15 mg/ml. The standard reaction mixture contained 90 mM NaCl; 60 mM Hepes (pH 7.5); 9 mM Tris-HCl (pH 7.5); 12 mM MgCl₂; 6 mM dithiothreitol; 100 μ M each ddCTP, ddGTP, ddATP, and ddTTP; 2 μ l nuclear extract (8 to 12 μ g protein) from adenovirus-infected or uninfected HeLa cells; and 300 ng mKM4 DNA (or 1 μ g XD-7 or pBR322 DNA) in total volume of 10 μ l. Some reactions also contained 1 μ l cytosol (15 μ g protein). After incubation for 30 min at 37°C, the reaction was stopped by phenol extraction.

Topoisomerase I reaction. The reaction mixture contained 40 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 300 ng mKM4 DNA, and 150 ng calf thymus topoisomerase I in a total volume of 10 μ l. Incubation was for 30 min at 37°C.

Gel electrophoresis. Gel electrophoresis was carried out at 1000 V on 8% polyacrylamide gels containing 8 M urea (25 cm wide x 35 cm long x 0.3 mm thick). The running buffer contained 8 M urea, 50 mM Tris-borate (pH 8.3), and 1 mM EDTA. The gel was dried and autoradiographed after electrophoresis.

RESULTS

Site-specific nicking on the l-strand.

The strategy to map site-specific nicking on the l-strand of the cloned adenovirus left inverted terminal repetition is detailed in Fig. 2. ³²P-Labeled l-strand has the sequence shown below where the synthetic primer occupies the first fifteen nucleotides, the EcoRI site is underlined, and the

Figure 2. Strategy to map site-specific nicking on the adenovirus l-strand. A 15-base primer was annealed to single-stranded mKM4 and elongated with the Klenow fragment of DNA polymerase using [α - ^{32}P]dATP and unlabeled dCTP, dGTP, and dTTP. ^{32}P -Labeled DNA corresponds to the adenovirus l-strand. After incubation in a reaction mixture containing nuclear extract, nicks were located by the size of specific fragments on sequencing gels. The locations of nicks were further verified by subsequent cleavage with EcoRI as diagrammed: $x = x' + r$ where $r = 67$ bases. The thin line represents single-stranded, circular mKM4 DNA; double lines indicate the 15-base primer; and the thick, wavy line shows the ^{32}P -labeled adenovirus l-strand.

arrow indicates cleavage by EcoRI:

Numbers below the sequence indicate nucleotides from the 5' end of the primer whereas numbers above the sequence indicate nucleotides from the 5' end of adenovirus DNA (i.e., nucleotide 72 corresponds to adenovirus nucleotide 1). To test the strategy, mKM4 substrate containing ^{32}P -labeled adenovirus l-strand was cut with EcoRI. As expected, a 67-base fragment, designated r , was produced (Fig. 3, lane 2) which migrated with the ddG-terminated fragment within the EcoRI sequence (Fig. 3, lane G). When EcoRI-cut mKM4 substrate was subsequently incubated in a reaction mixture containing nuclear extract from uninfected HeLa cells, the EcoRI cohesive site was partially repaired as shown in Fig. 3 (lane 1) by fragments ranging in size from 67 bases (fragment r) to 71 bases (fragment r'). A faint ladder extending down at nucleotide intervals from fragment r to the size of the synthetic primer indicated limited exonucleolytic damage to the 3' end of fragment r , presumably due to exonuclease III-like activity. Addition of ddNTPs to the reaction mixture prevented repair of the EcoRI cohesive site, but did not eliminate 3'-exonucleolytic activity (not shown).

Figure 3. Analysis of site-specific nicking on the adenovirus 1-strand. Gel electrophoresis was for 3 hr. Lanes labeled G, C, A, and T contain dideoxy sequence ladders respectively terminating in ddG, ddC, ddA, and ddT. Lane 1: mKM4 cut with *EcoRI* and incubated with uninfected nuclear extract. Lane 2: mKM4 cut with *EcoRI* only. Lane 3: mKM4 incubated with uninfected nuclear extract containing ddNTPs and 4 mM ATP. Lane 4: mKM4 incubated with uninfected nuclear extract containing ddNTPs but no added ATP. Lane 5: Heat-denatured mKM4 incubated as described in Lane 3. Lane 6: Heat-denatured mKM4 incubated as described in Lane 4. $r = 67$ bases, $r' = 71$ bases, $x = 123$ bases, $y = 127$ bases, $z = 146$ bases, $u = 153$ bases, $v = 159$ bases, and $w = 164$ bases.

Fig. 3 also shows that a set of specific fragments was generated when ^{32}P -labeled mKM4 substrate was incubated in a reaction mixture containing nuclear extract from uninfected HeLa cells and ddNTPs. Six of the specific fragments, designated x, y, z, u, v, and w, arise from cleavages within the adenovirus ITR. Identical fragments were produced regardless of whether the mKM4 substrate was native (Fig. 3, lane 4) or denatured (Fig. 3, lane 6). Curiously, adding 4 mM ATP to the reaction mixture eliminated evidence of site-specific nicking (Fig. 3, lanes 3 and 5). Based on the concentration of ATP in nuclei isolated from uninfected or adenovirus-infected HeLa cells (20), we estimate that the endogenous ATP concentration in the nicking reaction is less than 0.2 μM . Even the addition of 0.5 mM ATP to the reaction completely abolished evidence of nicking (not shown). In contrast, the optimal ATP

Figure 4. High-resolution mapping of adenovirus 1-strand nicking. (A) Gel electrophoresis was for 8 hr. Lane G: Dideoxy sequence ladder terminating in ddG. Lane 1: mKM4 incubated with nuclear extract from adenovirus-infected cells. Lane 2: mKM4 incubated with nuclear and cytoplasmic extracts from adenovirus-infected cells. Lane 3: mKM4 incubated with nuclear extract from uninfected cells. (B) Gel electrophoresis was for 3 hr. Lane 1: mKM4 incubated with uninfected nuclear extract. Lane 2: mKM4 incubated with uninfected nuclear extract and then cut with *EcoRI*. *r* = 67 bases, *x'* = 56 bases, *y'* = 60 bases, *z'* = 79 bases, *u'* = 86 bases, *v'* = 92 bases, and *w'* = 97 bases.

concentration for *in vitro* adenovirus DNA replication is 2 mM (20). It is not clear whether ATP directly inhibits the nicking enzyme or an ATP-dependent process repairs the nicks or degrades the fragments.

High-resolution mapping (Fig. 4A) located the 3' ends of fragments *x*, *y*, *z*, *u*, *v*, and *w* at nucleotides 52, 56, 75, 82, 88, and 93 respectively within the ITR. Moreover, Fig. 4A shows that identical fragments were produced regardless of whether the nuclear extract was made from uninfected HeLa cells (lane 3) or adenovirus-infected HeLa cells (lane 1) or the nuclear extract was

supplemented with a cytoplasmic extract (lane 2). Neither the concentration of protein in the reaction (range: 0.8 to 2.7 mg/ml) nor the length of incubation (up to 60 min) affected the production of specific fragments. However, reactions with higher protein concentrations (compare lanes 1 and 2 in Fig. 4A with lane 3) or longer incubation times (not shown) showed increased exonucleolytic damage. We therefore conclude that specific fragments are created by endonucleolytic cleavages rather than by exonucleolytic damage. The locations of the nicks could be further verified by subsequent cleavage with EcoRI as shown in Fig. 4B (lane 2). A new set of fragments, identified as x', y', z', u', v', and w', was produced where each new fragment was exactly 67 bases shorter than the corresponding parental fragment. Only fragments cleaved by EcoRI could be located unambiguously. Whether nicking in the nuclear extract or cleavage by EcoRI occurred first is not important since a longer exposure of Fig. 3 (lane 1), where the order was reversed, also revealed fragments x', y', z', u', v', and w' (not shown). The sequences surrounding the nick sites are listed below where the arrows indicate the nicks and the numbers in parentheses identify the adenovirus nucleotides on either side of the nick:

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x: TGAGGG†GGTGGAGTT (52-53)
y: GGGGTG†GAGTTTGTG (56-57)
z: GGCGCG†GGGCGTGGG (75-76)
u: GGCGTG†GGAACGGGG (82-83)
v: GGAACG†GGGCGGGTG (88-89)
w: GGGGCG†GGTGACGTA (93-94)

GGRGYG†GGRNRNGTG consensus

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All cleavages occur between adjacent G residues. In each case at least nine of the surrounding fifteen nucleotides are G residues, and these neighboring G-rich sequences appear to be related as indicated by the derived consensus sequence. This is all the more surprising since these sequences overlap extensively as shown in Fig. 7 below. The role, if any, that neighboring sequences play in determining the specificity or frequency of cleavage is not yet known. If the intensity of fragment r, produced by EcoRI, is taken as a measure of complete cleavage, it is clear that the yield of fragment x is much lower. In fact, fragments y through w, which span the x cleavage site, could not be detected at all if x were completely cleaved. Consequently, cleavages at sites distal to fragment x from the primer are underestimated using the strategy outlined in Fig. 2.

Figure 5. Strategy to map site-specific nicking on the adenovirus *r*-strand and on pBR322. (A) XD-7 or pBR322 was linearized with *Hind*III, digested with exonuclease III, and repaired with the Klenow fragment of DNA polymerase using [α - 32 P]dATP and unlabeled dCTP, dGTP, and dTTP. 32 P-Labeled DNA (thick, wavy line) corresponds to the adenovirus *r*-strand in XD-7. (B) After incubation in a reaction mixture containing nuclear extract, nicks were located by the size of specific fragments on sequencing gels. The locations of nicks were further verified by subsequent cleavage with *Eco*RI as diagrammed: $p = p' + r''$ where $r'' = 35$ bases.

The *r*-strand is not nicked.

The strategy to map site-specific nicking on the *r*-strand of the cloned adenovirus left inverted terminal repetition is detailed in Fig. 5. When XD-7 substrate with 32 P-labeled adenovirus *r*-strand was incubated in a reaction mixture containing nuclear extract from uninfected HeLa cells and ddNTPs, a set of specific fragments was generated (Fig. 6A, lane 2). However, none of the specific fragments was altered by subsequent cleavage with *Eco*RI although the expected 35-base fragment r'' appeared (Fig. 6A, lane 1). We interpret this to mean that the fragments in Fig. 6A arose from cleavages on the 32 P-labeled strand at the other end of the linearized XD-7 molecule and that the *r*-strand of the adenovirus ITR was not nicked. Lack of cutting of the *r*-strand establishes that the *l*-strand cleavages are indeed nicks.

Failure to detect nicking on the *r*-strand of the ITR was not due to faulty experimental design. To test the strategy, 32 P-labeled pBR322 substrate was incubated in the nicking reaction. A set of specific fragments was produced (Fig. 6B, lane 2) which included all of the fragments displayed in Fig. 6A (lane 2) as well as at least one additional fragment designated *p*. Fig. 6B (lane 1) shows that a new fragment, called *p'*, which was 35 bases shorter than

Figure 6. Analysis of site-specific nicking on the adenovirus r -strand and on pBR322. Gel electrophoresis was for 3 hr. (A) Lane 1: XD-7 incubated with uninfected nuclear extract and then cut with *EcoRI*. Lane 2: XD-7 incubated with uninfected nuclear extract. (B) Lane 1: pBR322 incubated with uninfected nuclear extract and then cut with *EcoRI*. Lane 2: pBR322 incubated with uninfected nuclear extract. $r'' = 35$ bases, $p = 144$ bases, and $p' = 109$ bases.

p , appeared along with fragment r'' after subsequent cleavage with *EcoRI*. Fragments p and p' locate a site-specific nick on pBR322 within the sequence $AGGG^+GTT$ where the cleavage is between adjacent G residues at nucleotides 4251 and 4252 (21). Six of the seven nucleotides surrounding the pBR322 nick are identical to nucleotides around the nick defining fragment x .

Site-specific nicking is not due to topoisomerase I or topoisomerase II.

Site-specific nicking clearly cannot be caused by HeLa topoisomerase II since it requires ATP and makes double-stranded cleavages (22). To test for the involvement of topoisomerase I, purified calf thymus topoisomerase I was incubated with ^{32}P -labeled mKM4 substrate. Although a set of specific fragments was generated by topoisomerase I, none of the fragments coincided with fragments produced in the nicking reaction (not shown). Since calf thymus topoisomerase I and HeLa topoisomerase I cleave SV40 DNA at identical and specific sites (23), we conclude that site-specific nicking of the l -strand of the ITR is also not caused by HeLa topoisomerase I.

Figure 7. Sequence of the 1-strand of the type 2 adenovirus left inverted terminal repetition. Dots indicate every tenth nucleotide. Site-specific nicks are shown by the arrows. Brackets below the line enclose homologous G-rich sequences.

DISCUSSION

Site-specific nicking occurred on the l-strand, but not the r-strand, of the adenovirus left inverted terminal repetition in reaction mixtures containing extracts from uninfected as well as adenovirus-infected HeLa cells. The substrate for nicking could be either double-stranded or single-stranded DNA. The reaction did not require added ATP, and no nicks were observed in the presence of 4 mM ATP. Nicking clearly cannot be caused by HeLa topoisomerase II which requires ATP and makes double-stranded cleavages (22). Nicking is also probably not caused by HeLa topoisomerase I since a direct test eliminated calf thymus topoisomerase I, which cleaves SV40 at the same sites as HeLa topoisomerase I (23), as the nicking enzyme. The nature of the termini created at the nick site is not yet known. However, faint bands one nucleotide larger than the main cleavage fragments could often be detected. For example, a faint band is visible at position x+1 in Fig. 4A (lanes 1 and 3) and fragment y appears as a doublet differing by a single nucleotide in Fig. 4A (lanes 1 and 2). We interpret this to mean that one dideoxynucleotide residue can be added at the cleavage site, an indication perhaps that nicking yields a 3'-hydroxyl end. Nevertheless, other alternatives have not yet been excluded. For example, a phosphomonoesterase could convert 3'-phosphates into 3'-hydroxyls. We have detected a phosphomonoesterase activity which removes 5'-phosphates in the nicking reaction (not shown).

High-resolution mapping located the cleavage sites within the sequence of the adenovirus ITR. Fig. 7 shows that all cleavages occurred between adjacent G residues in highly homologous, G-rich sequences which, surprisingly, overlap extensively. The consensus nick site has the sequence GGRGYG GGRNRNGTG. All adenovirus serotypes, except the avian adenovirus CELO, have related G-rich sequences in the ITR (3-13). Moreover, G-rich sequences similar to the adenovirus nick sites are also found at or near papovavirus replication

origins (16). The functional significance, if any, of site-specific nicking within the ITR is not yet known. Deletions removing all of the nick sites in the ITR do not prevent adenovirus replication in a cell-free replication assay (14). However, all of the deletions are connected through a BamHI linker to a G-rich adenovirus sequence at the SstII site. Deletion mutant d130 was subcloned into M13mp8 and tested for l-strand nicking (not shown). A site-specific nick was mapped on d130 within the sequence CCGGATCCGG⁺GGGGA where the cleavage is between adjacent G residues, one G within the BamHI linker (underlined sequence) and the other G at adenovirus nucleotide 358. This G-rich sequence, present in all deletion mutants, may therefore be able to compensate for the loss of nick sites in the ITR.

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REFERENCES

1. Garon, C.F., Berry, K.W. and Rose, J.A. (1972) *Proc. Natl. Acad. Sci. USA* 69, 2391-2395.
2. Wolfson, J. and Dressler, D. (1972) *Proc. Natl. Acad. Sci. USA* 69, 3054-3057.
3. Steenbergh, P.H., Maat, J., van Ormondt, H. and Sussenbach, J.S. (1977) *Nucleic Acids Res.* 4, 4371-4389.
4. Tolun, A., Alestrom, P. and Pettersson, U. (1979) *Cell* 17, 705-713.
5. Dijkema, R. and Dekker, B.M.M. (1979) *Gene* 8, 7-15.
6. Arrand, J.R. and Roberts, R.J. (1979) *J. Mol. Biol.* 128, 577-594.
7. Shinagawa, M. and Padmanabhan, R. (1979) *Biochem. Biophys. Res. Commun.* 87, 671-678.
8. Shinagawa, M. and Padmanabhan, R. (1980) *Proc. Natl. Acad. Sci. USA* 77, 3831-3835.
9. Sugisaki, H., Sugimoto, K., Takanami, M., Shirolu, K., Saito, I., Shimojo, Y., Sawada, Y., Uemizu, Y., Uesugi, S.-I. and Fujinaga, K. (1980) *Cell* 20, 777-786.
10. Stillman, B.W., Topp, W.C. and Engler, J.A. (1982) *J. Virol.* 44, 530-537.
11. Temple, S., Antoine, G., Delius, H., Stahl, S. and Winnacker, E.-L. (1981) *Virology* 109, 1-12.
12. Alestrom, P., Stenlund, A., Li, P. and Pettersson, U. (1982) *Gene* 18, 193-197.
13. Shinagawa, M., Ishiyama, T., Padmanabhan, R., Fujinaga, K., Kamada, M., and Sato, G. (1983) *Virology* 125, 491-495.

14. Enns, R.E., Challberg, M.D., Ahern, K.G., Chow, K.-C., Mathews, C.Z., Astell, C.R. and Pearson, G.D. (1983) *Gene* 23, 307-313.
15. Pearson, G.D., Chow, K.-C., Enns, R.E., Ahern, K.G., Corden, J.L. and Harpst, J.A. (1983) *Gene* 23, 293-305.
16. Seif, I., Khoury, G. and Dhar, R. (1979) *Cell* 18, 963-977.
17. Messing, J. (1981). in *Third Cleveland Symposium on Macromolecules: Recombinant DNA*, Walton, A., Ed., pp. 143-153, Elsevier, Amsterdam.
18. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.
19. Guo, L.-H. and Wu, R. (1982) *Nucl. Acids Res.* 10, 2065-2084.
20. De Jong, P.J., Kwant, M.M., van Driel, W., Jansz, H.S. and van der Vliet, P.C. (1983) *Virology* 124, 45-58.
21. Sutcliffe, J.G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* 43, 77-90.
22. Miller, K.G., Liu, L.F. and Englund, P.T. (1981) *J. Biol. Chem.* 256, 9334-9339.
23. Edwards, K.A., Halligan, B.D., Davis, J.L., Nivera, N.L. and Liu, L.F. (1982) *Nucl. Acids Res.* 10, 2565-2576.