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Temperature-Sensitive Initiation and Elongation of Adenovirus DNA Replication In Vitro with Nuclear Extracts from H5ts36-, H5ts149-, and H5ts125-Infected HeLa Cells

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Adenovirus DNA replication was studied in vitro in nuclear extracts prepared from HeLa cells infected at the permissive temperature with H5ts125, H5ts36, or H5ts149, three DNA-negative mutants belonging to two different complementation groups. At the restrictive temperature, H5ts125 extracts, containing a thermolabile 72-kilodalton DNA-binding protein, enable the formation of an initiation complex between the 82-kilodalton terminal protein precursor (pTP) and dCTP, but further elongation of this complex is inhibited. Wild-type DNA-binding protein or a 47-kilodalton chymotryptic DNA-binding fragment can complement the mutant protein in the elongation reaction. No difference in heat inactivation was observed between wild-type extracts and H5ts36 or H5ts149 extracts when the replication of terminal *Xba*I fragments of adenovirus type 5 DNA-terminal protein complex was studied. In contrast, the formation of a pTP-dCMP initiation complex, as well as the partial elongation reaction up to nucleotide 26, were consistently more temperature sensitive in mutant extracts. The results suggest that the H5ts36/H5ts149 gene product is required for initiation of adenovirus type 5 DNA replication and that the 72-kilodalton DNA-binding protein functions early in elongation.

Adenovirus type 5 (Ad5) contains a linear duplex DNA molecule of about 35,000 base pairs. An inverted repeat of 103 base pairs is present at the termini (31), and a 55-kilodalton (kd) protein (terminal protein [TP]) is covalently bound to both 5′ ends (27, 28). The DNA replicates in the nuclei of permissive cells by a strand displacement mechanism (34).

Initiation of Ad5 DNA replication can be studied in vitro by using nuclear extracts of infected HeLa cells (6). Recent studies indicate that initiation proceeds by a protein priming mechanism in which the viral 80- to 87-kd TP precursor (pTP) has an important function. In the presence of an appropriate template, pTP binds a dCMP residue which is the first nucleotide of the nascent strand. The free 3′-OH group of dCMP can serve as a primer for subsequent elongation (5, 8, 22, 26, 32). Late in infection the pTP is processed to the 55-kd mature TP (7).

At least two other viral proteins are required for DNA replication, represented by two DNA-negative complementation groups. One group, containing the H5ts125 mutant (10) as prototype, is defective for the 72-kd DNA-binding protein (DBP) (35), which is encoded by the E2A region.

This protein serves multiple functions in DNA replication. Based on temperature shift-up experiments in vivo, a role in initiation has been proposed (36). In vitro experiments with antibody to the 72-kd DBP (37) and complementation studies with H5ts125 (14, 16) have suggested that this protein is also involved in elongation.

A second group of DNA-negative mutants (group N) is represented by H5ts36, H5ts37, H5ts149, and H5ts69 (11, 13, 38). These mutations have been mapped between 18.5 and 22.0 map units in the E2B region of the viral genome (11), but neither the corresponding protein nor its role has been unequivocally established. Preliminary experiments in vivo have suggested a role for the H5ts36 product in initiation (36).

We investigated the function of the H5ts125 and H5ts36/H5ts149 products in more detail.

Addition of Ad5 DNA or DNA-TP, previously digested with *Xba*I, to nuclear extracts led to the labeling of both terminal and internal fragments (Fig. 1). Preferential labeling of the terminal fragments C and E was observed only when DNA-TP was used as the template. These fragments had a reduced mobility in agarose–0.1% sodium dodecyl sulfate (SDS) gels due to the presence of covalently bound protein. The reduction in electrophoretic mobility was 6%, compared to 4% for non-radioactive terminal

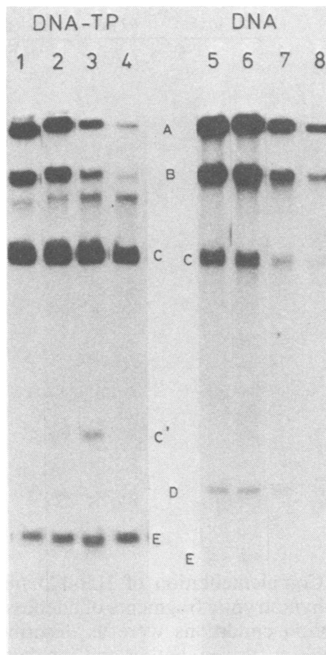

FIG. 1. In vitro DNA replication with *Xba*I-digested Ad5 DNA-TP and DNA at various aphidicolin concentrations. The reaction mixtures for in vitro DNA synthesis contained, in 30 μl, 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.5), 4 mM $MgCl_2$, 0.4 mM dithiothreitol, 1.7 mM ATP, 5 mM creatine phosphate, 5 μg of creatine phosphokinase per ml, 17 μM each of dATP, dGTP, and dTTP, 8 μM [α-^{32}P]dCTP (3 to 7 Ci/mmol, purchased from New England Nuclear Corp. at a specific activity of 3,185 Ci/mmol), 7 μl of Ad5 nuclear extract, 50 ng of *Xba*I-digested Ad5 DNA-TP or Ad5 DNA as template, and various concentrations of aphidicolin. Nuclear extracts were prepared essentially by the method of Challberg and Kelly (6). HeLa cells, maintained in suspension culture in Eagle minimum essential medium supplemented with 6% newborn calf serum, were infected at 37°C with purified Ad5 virus at a multiplicity of 10^4 physical particles per cell. To determine the optimum quantity for in vitro DNA replication, the nuclear extracts were first titrated. Ad5 DNA-TP was prepared by the method of Stillman (32) from Ad5 virions which were purified twice by CsCl centrifugation. Ad5 DNA was obtained from Ad5 DNA-TP by pronase digestion at 10 mg/ml for 1 h at 37°C and subsequent phenol extraction and ethanol precipitation, and was dissolved in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. After incubation for 1 h at 37°C the reaction was stopped by addition of EDTA and SDS to concentrations of 10 mM and 0.1%, respectively, and samples were directly electrophoresed on 1% agarose gels containing 40 mM Tris-hydrochloride (pH 7.8)–1 mM EDTA–5 mM sodium acetate–0.1% SDS. After electrophoresis the gels were partially dehydrated, and radioactivity was located by autoradiography. Aphidicolin concentrations: lanes 1 and 5, without aphidicolin; lanes 2 and 6, 1 μM; lanes 3 and 7, 10 μM; lanes 4 and 8, 100 μM.

fragments containing the 55-kd TP (not shown). This is in agreement with the presence of the larger pTP at the termini of newly synthesized strands (5).

An additional labeled band (C') was sometimes observed between C and D (Fig. 1, lanes 2 and 3). This band became more pronounced upon longer incubation periods and was sensi-

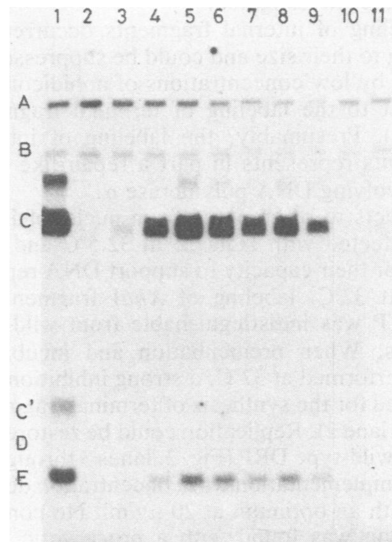

FIG. 2. Temperature-sensitive replication of DNA-TP with H5ts125 nuclear extract and complementation with adenovirus DBP. H5ts125, originally isolated by Ensinger and Ginsberg (10), was propagated as described (35). Reaction mixtures were as described in the legend to Fig. 1 with 7 μl of H5ts125 nuclear extract and 10 μM aphidicolin. The mutant nuclear extract was prepared as described (Fig. 1), but the infection of HeLa cells was performed at 32.5°C, 5 mM hydroxyurea was added at 4 h postinfection, and nuclei were prepared at 40 h postinfection. Ad5 DBP was isolated from washed nuclei as described by Schechter et al. (29) with slight modifications. DNA-cellulose chromatography was performed as described previously (37), including a sodium dextran sulfate wash. As judged by Coomassie blue staining, the protein was 98% pure. DBP was dialyzed against 0.05 M ammonium bicarbonate, freeze-dried, and stored at −80°C. Bacteriophage fd gene 5 DBP was obtained from Bethesda Research Laboratories. Before use in the complementation assay, the adenovirus and fd gene 5 DBPs were briefly dialyzed against 20 mM Tris-hydrochloride (pH 8.0)–100 mM NaCl–10% sucrose (DBP-buffer). The reaction mixtures, without template, were preincubated for 15 min at the incubation temperature. Incubations were started by the addition of 50 ng of *Xba*I-digested Ad5 DNA-TP and were performed for 30 min. Incubation temperatures: lane 1, 32°C; lanes 2 through 11, 37°C. Additions to the reaction mixtures: lanes 3 through 9, Ad5 DBP in increasing amounts of 0.1, 0.3, 0.6, 1.2, 1.8, 2.4, and 3.6 μg, respectively; lane 10, 1.4 μg of fd gene 5 DBP; lane 11, 6 μl of DBP-buffer.

tive to single-strand-specific nucleases. Its position in the gel coincided with that of denatured C fragments. Most likely this single-strand C originates from multiple rounds of replication (15). Upon longer exposure times a labeled band could also be seen at the single-strand E position. A third additional band with changing intensities often appeared just beneath the *Xba*I-B fragment (Fig. 1). The nature of this band was not further investigated.

Labeling of internal fragments occurred according to their size and could be suppressed up to 90% by low concentrations of aphidicolin, in contrast to the labeling of terminal fragments (Fig. 1). Presumably, the labeling of internal fragments represents in part a repair-like reaction involving DNA polymerase α.

Extracts were prepared from nuclei of HeLa cells infected with H5ts125 at 32.5°C and analyzed for their capacity to support DNA replication. At 32°C, labeling of *Xba*I fragments of DNA-TP was indistinguishable from wild-type extracts. When preincubation and incubation were performed at 37°C, a strong inhibition was observed for the synthesis of terminal fragments (Fig. 2, lane 2). Replication could be restored by adding wild-type DBP (Fig. 2, lanes 3 through 9). This complementation was concentration dependent with an optimum at 20 μg/ml. No complementation was found with a procaryotic DBP (bacteriophage fd gene 5 product) (Fig. 2, lane 10).

The native adenovirus DBP has an extended structure and can be cleaved easily by a mild chymotrypsin treatment (23). This cleavage site is probably located in a region around amino acid 130 (19). Digestion produces a 47-kd C-terminal fragment which binds to single-stranded DNA and a 24- to 26-kd N-terminal fragment which does not bind to DNA and contains the majority of the phosphate groups (17, 23).

Both fragments were prepared from purified 72-kd DBP, separated by DNA-cellulose chromatography, and assayed for complementing activity in vitro (Fig. 3). Only the C-terminal fragment could substitute for the intact DBP. In the particular experiment shown in Fig. 3 the optimum concentration for complementation was 33 μg/ml with 72-kd DBP and 40 μg/ml with the 47-kd fragment. Corrected for the difference in molecular weight, this indicates that the 47-kd fragment has 54% of the complementing activity of the native adenovirus DBP.

Incubation of nuclear extracts and DNA-TP in the presence of [α-^{32}P]dCTP leads to the formation of a 80- to 87-kd pTP-dCMP complex (8, 22, 26). The reaction requires $MgCl_2$ and ATP, which can be substituted by other purine triphosphates (8a). Pronase-treated DNA-TP could not support initiation.

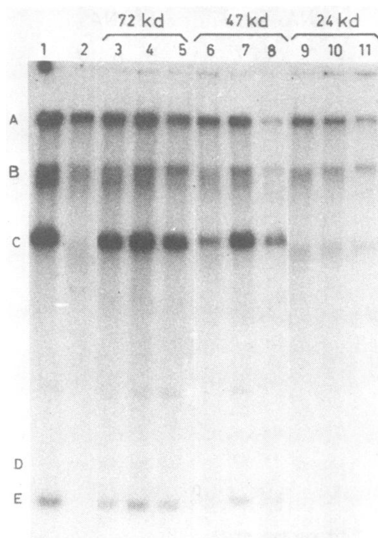

FIG. 3. Complementation of H5ts125 nuclear extract with chymotryptic fragments of adenovirus DBP. The incubation conditions were as described in the legend to Fig. 2. Preincubation and incubation times were 15 and 60 min, respectively. For the preparation of chymotryptic fragments, Ad5 DBP was dissolved in 10 mM Tris-hydrochloride (pH 8.0)–50 mM NaCl at a concentration of 1 mg/ml and digested with 0.3 μg of chymotrypsin (Miles) per ml for 30 min at 20°C. The reaction was stopped by addition of 20 μg of 2-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) per ml. NaCl was added to 0.15 M, and the fragments were separated on a single-stranded DNA-cellulose column (1 ml/mg of protein). The DNA-bound protein eluted with 2 M NaCl; 87% of it was a 47-kd fragment, and 13% was a 43-kd fragment. The flowthrough was a homogeneous 24-kd polypeptide. Both protein fractions were stored at −80°C. Before use, the chymotryptic fragments were briefly dialyzed against DBP-buffer. Incubation temperatures: lane 1, 32°C; lanes 2 through 11, 37°C. Additions to the reaction mixtures were as follows: lanes 3 through 5, 0.5, 1.0, and 2.0 μg of 72-kd DBP, respectively; lanes 6 through 8, 0.4, 1.2, and 2.4 μg of 47-kd fragment, respectively; lanes 9 through 11, 0.5, 1.0, and 2.0 μg of 24-kd fragment, respectively.

A partial elongation product could be formed by further addition of dATP, dTTP, and ddGTP. Under these conditions elongation proceeds only to nucleotide 26, the first G residue. Incorporation of ddGMP blocks further elongation, leading to the synthesis of a 88-kd product together with the initiation product (22).

Extracts from H5ts125-infected cells were assayed for initiation and partial elongation at 32 and 38°C. Figure 4A shows that the initiation

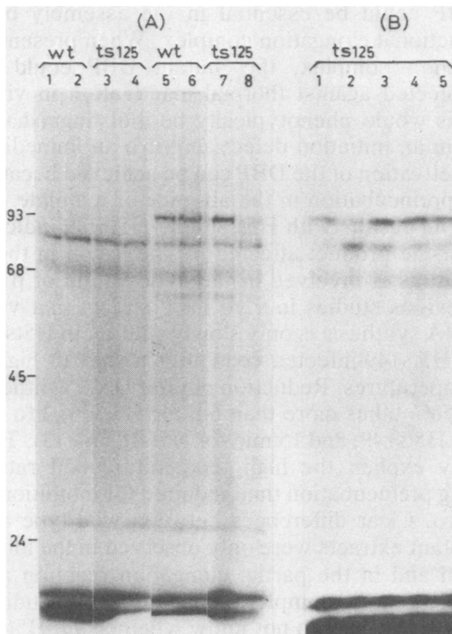

FIG. 4. Synthesis of pTP-dCMP and the partial elongation product with H5ts125 nuclear extract and complementation of the mutant defect. Reaction mixtures for the pTP-dCMP joining reaction contained, in 50 μl, 40 mM HEPES-KOH (pH 7.5), 4 mM $MgCl_2$, 0.2 mM dithiothreitol, 1.6 mM ATP, 4 mM creatine phosphate, 4 μg of creatine phosphokinase per ml, 1 μM [α-^{32}P]dCTP (50 to 200 Ci/mmol), 40 μM ddATP, 0.2 mM aphidicolin, 100 ng of Ad5 DNA-TP, and 12 μl of nuclear extract from cells infected either at 32.5°C with H5ts125 or at 37°C with Ad5. For the formation of an oligonucleotide of 26 bases joined to pTP, the partial elongation product, ddATP was omitted and 40 μM each of dATP, dTTP, and ddGTP was included. Preincubations were for 15 min at the incubation temperature in the absence of Ad5 DNA-TP; incubations were for 30 min in the presence of the template and were stopped by the addition of 7 μl of 200 mM EDTA. Macromolecules were precipitated with 20% trichloroacetic acid. Precipitates were directly dissolved in Laemmli buffer (20), the pH was adjusted to neutral with 1 M Tris base, and samples were analyzed by SDS-polyacrylamide electrophoresis according to Laemmli (20) with gels containing 10% acrylamide. Polyacrylamide gel electrophoresis was performed in the presence of the molecular weight markers phosphorylase *a* (93 kd), bovine serum albumin (68 kd), ovalbumin (45 kd), and chymotrypsinogen (24 kd). After electrophoresis the gels were stained, dried, and autoradiographed. (A) Inactivation of the H5ts125 extract. Odd-numbered lanes, 32°C; even-numbered lanes, 38°C. Lanes 1 through 4, initiation conditions; lanes 5 through 8, partial elongation conditions. Lanes 1, 2, 5, and 6, Ad5 wild-type extract; lanes 3, 4, 7, and 8, H5ts125 mutant extract. (B) Complementation of H5ts125 nuclear extract with adenovirus DBP under partial elongation conditions. Lane 1, 32°C; lanes 2 through 5, 38°C. Ad5 DBP was added as follows: lane 3, 0.5 μg; lane 4, 1.5 μg; lane 5, 3.0 μg.

was not diminished at 38°C compared to 32°C. In contrast, the synthesis of a partial elongation product was severely inhibited at 38°C. Quantification showed an inhibition of the labeling of the elongation product of 90 ± 5% at 38°C. As with the synthesis of *Xba*I terminal fragments, addition of wild-type DBP repaired the defect (Fig. 4B).

To analyze the function of the mutant group N product, nuclear extracts were prepared from HeLa cells infected at 32.5°C with H5ts36 or H5ts149 and preincubated at increasing temperatures to inactivate the mutant product. Inhibition of DNA replication occurred at higher temperatures (Fig. 5). A preincubation of 45 min at 39.5°C was at least required to obtain 95% inactivation. Under these conditions, which are more rigorous than with H5ts125, wild-type extracts were also considerably inactivated, presumably because of inhibition of the elongation process. Although a small difference in temperature sensitivity was observed in a number of experiments (not shown), definite conclusions could not be drawn from this experimental approach. In contrast, a pronounced difference was observed consistently between the temperature sensitivities of wild-type and mutant extracts when pTP-dCMP synthesis or the partial elongation reaction was studied (Fig. 6). In wild-type extracts these reactions were more resistant to higher temperatures than the complete replication with *Xba*I terminal fragments.

Quantification of the radioactivity in the 82-kd band at initiation conditions showed that at 39.5°C the H5ts36 product was 50% inhibited, H5ts149 was 95% inhibited, and the wild-type reaction was slightly increased (130%).

The H5ts125 mutation is a single nucleotide alteration leading to a Pro → Ser transition at amino acid 413 in the C-terminal part of DBP (19). This mutation leads to a thermolabile DBP which is not able to support replication at the nonpermissive temperature and is gradually degraded intracellularly. Our results indicate that H5ts125 nuclear extracts, when thermally inactivated, are blocked in elongation but still allow the synthesis of a pTP-dCMP initiation complex, suggesting that the 72-kd DBP is not required for this reaction. Based on complementation studies with purified components (9) or replication with extracts from H5ts125-infected cells grown at the nonpermissive temperature (8), other investigators reached the same conclusion. We cannot exclude the possibility that the DBP is required for initiation at much lower concentrations than for elongation, or that a host protein present in the nuclear extracts can substitute for viral DBP in initiation.

The defect in elongation that we observed could be complemented by 72-kd DBP or the

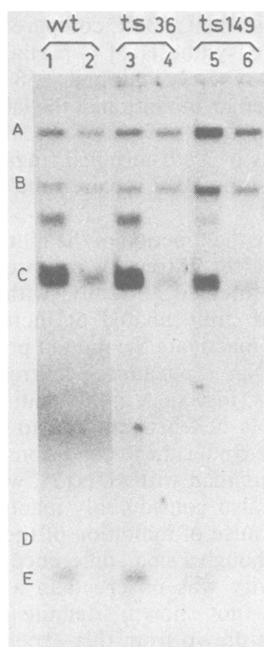

FIG. 5. Replication of *Xba*I fragments of Ad5 DNA-TP with Ad5, H5ts36, and H5ts149 nuclear extracts. H5ts36 (38) and H5ts149 (13) were propagated at 32.5°C. The mutant nuclear extracts were prepared as described in the legend to Fig. 2. Preincubations without and incubations with template were for 45 and 60 min, respectively. All reaction mixtures contained 10 μM aphidicolin. Lanes 1, 3, and 5, 33°C; lanes 2, 4, and 6, 39.5°C. Lanes 1 and 2, 11 μl of Ad5 extract; lanes 3 and 4, 9 μl of H5ts36 extract; lanes 5 and 6, 7 μl of H5ts149 extract.

chymotryptic C-terminal fragment, but not by the N-terminal fragment, in agreement with the results of Ariga et al. (2). The C-terminal part of the protein is underphosphorylated, which suggests that phosphorylation is not required for the elongation function of DBP, nor for DNA binding (17, 23), and that the 47-kd fragment can perform all functions required for adenovirus DNA replication. The N-terminal fragment is most likely involved in transcription regulation and not in DNA replication. This agrees well with the location of the H5hr404 mutation in this part of the protein (19), which influences late transcription but not DNA replication (18).

The results obtained with H5ts125 in vivo and in vitro seem contradictory. In H5ts125-infected cells, replicative intermediates can be completed after a shift to 39°C (36), whereas the elongation process is inhibited in vitro at this temperature. One explanation for this discrepancy could be that replication occurs in at least three steps: initiation, formation of an elongation complex, and finally the elongation reaction itself. The DBP could be essential in the assembly of a functional elongation complex. When present in such a complex, the mutant DBP could be protected against thermal inactivation in vivo. This would phenotypically be indistinguishable from an initiation defect. In vitro an immediate inactivation of the DBP can be achieved because of preincubation in the absence of template.

Our results with H5ts36 and H5ts149 indicate that the product affected by the lesion in these mutants is involved in the cytidylation of pTP. Previous studies in vivo have shown that viral DNA synthesis is only slowly shut off in H5ts36- or H5ts149-infected cells after a shift to higher temperatures. Reduction of viral DNA synthesis to 50% takes more than 6 h for H5ts36, 2 to 3 h for H5ts149, and 15 min for H5ts125 (4, 13). This may explain the high temperature and rather long preincubation time required for inhibition in vitro. Clear differences between wild-type and mutant extracts were only observed in the initiation and in the partial elongation reaction and not when the complete replication was studied. Therefore, we do not know whether the H5ts36 product is also functioning during elongation.

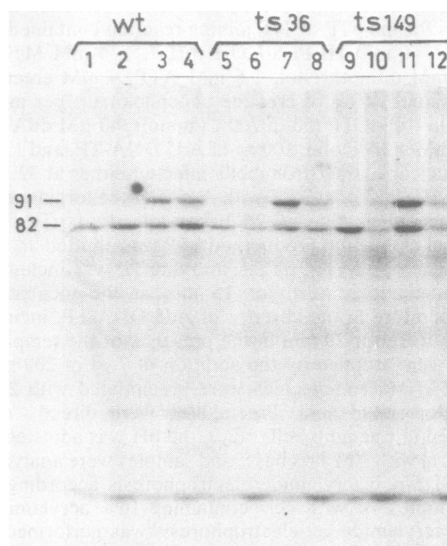

FIG. 6. Formation of initiation and partial elongation products with Ad5, H5ts36, and H5ts149 nuclear extracts under permissive and nonpermissive conditions. Reaction mixtures were as described in the legend to Fig. 4 with 18 μl of the wild-type or mutant extract. Preincubation and incubation times were 45 and 60 min, respectively. Odd-numbered lanes, 33°C; even-numbered lanes, 39.5°C. Lanes 1, 2, 5, 6, 9, and 10, Formation of the 82-kd initiation product; lanes 3, 4, 7, 8, 11, and 12, formation of the 91-kd partial elongation product. Lanes 1 through 4, Ad5 wild-type extract; lanes 5 through 8, H5ts36 extract; lanes 9 through 12, H5ts149 extract.

The H5ts36 mutation has been mapped between 18.5 and 22.0 map units on the adenovirus genome (11). By cell-free translation, Stillman et al. (33) have demonstrated that three proteins are encoded on the viral L-strand between coordinates 11 and 31.5 with sizes of 105, 87, and 75 kd. The 87-kd protein is identical to the pTP; the status of the 75-kd protein is not clear (3, 33). Recent sequence data (1, 12, 30) have shown that the N-group mutants lie within a region of the genome coding for a 120-kd protein, which does not overlap with the pTP-coding region. In agreement with this, we have observed that DNA-TP isolated from H5ts36 virions supported initiation at the nonpermissive temperature as well as wild-type-derived DNA-TP did, suggesting that the H5ts36 55-kd TP is not temperature sensitive (unpublished data).

The 120-kd protein may be identical to the 140-kd protein which is found in close association with pTP and which possesses DNA polymerase activity (9, 21). Mutations in this protein may influence pTP cytidylation directly, or they could change the interaction with pTP in such a way that synthesis of a pTP-dCMP complex becomes more difficult. Alternatively, the H5ts36 protein may function to unwind the origin of replication and thus expose a single-stranded nucleotide sequence that is able to bind pTP. Recently, Nagata et al. (25) reported that the single-strand-specific adenovirus DBP inhibits initiation with DNA-TP as template, unless a 47-kd cellular protein is present. This suggests that local unwinding of the adenovirus origin can occur during initiation.

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ADDENDUM IN PROOF

After this manuscript was submitted, Stillman et al. (B. W. Stillman, F. Tamanoi, and M. B. Mathews, Cell 31:613–623, 1982) showed that H5ts149 nuclear extracts, prepared from cells infected at the nonpermissive temperature, could be complemented for formation of the pTP-dCMP complex with a 140-kd E2B protein possessing DNA polymerase activity.

LITERATURE CITED

1. **Aleström, P., G. Akusjärvi, M. Pettersson, and U. Pettersson.** 1982. DNA sequence analysis of the region encoding the terminal protein and the hypothetical N-gene product of adenovirus type 2. J. Biol. Chem. **257**:13492–13498.
2. **Ariga, H., H. Klein, A. J. Levine, and M. S. Horwitz.** 1980. A cleavage product of the adenovirus DNA binding protein is active in DNA replication *in vitro*. Virology **101**:307–310.
3. **Binger, M. H., S. J. Flint, and D. M. Rekosh.** 1982. Expression of the gene encoding the adenovirus DNA terminal protein precursor in productively infected and transformed cells. J. Virol. **42**:488–501.
4. **Carter, T. H., and H. S. Ginsberg.** 1976. Viral transcription in KB cells infected by temperature-sensitive "early" mutants of adenovirus type 5. J. Virol. **18**:156–166.
5. **Challberg, M. D., S. V. Desiderio, and T. J. Kelly, Jr.** 1980. Adenovirus DNA replication *in vitro*: characterization of a protein covalently linked to nascent DNA strands. Proc. Natl. Acad. Sci. U.S.A. **77**:5105–5109.
6. **Challberg, M. D., and T. J. Kelly, Jr.** 1979. Adenovirus DNA replication *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **76**:655–659.
7. **Challberg, M. D., and T. J. Kelly, Jr.** 1981. Processing of the adenovirus terminal protein. J. Virol. **38**:272–277.
8. **Challberg, M. D., J. M. Ostrove, and T. J. Kelly, Jr.** 1982. Initiation of adenovirus DNA replication: detection of covalent complexes between nucleotide and the 80-kilodalton terminal protein. J. Virol. **41**:265–270.

8a. **De Jong, P. J., M. M. Kwant, W. van Driel, H. S. Jansz, and P. C. van der Vliet.** 1983. The ATP requirements of adenovirus type 5 DNA replication and cellular DNA replication. Virology **124**:45–58.

9. **Enomoto, T., J. H. Lichy, J.-E. Ikeda, and J. Hurwitz.** 1981. Adenovirus DNA replication *in vitro*: purification of the terminal protein in a functional form. Proc. Natl. Acad. Sci. U.S.A. **78**:6779–6783.
10. **Ensinger, M. J., and H. S. Ginsberg.** 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. J. Virol. **10**:328–339.
11. **Galos, R. S., J. Williams, M.-H. Binger, and S. J. Flint.** 1979. Location of additional early gene sequences in the adenoviral chromosome. Cell **17**:945–956.
12. **Gingeras, T. R., D. Sciaky, R. E. Gelinas, J. Bing-Dong, C. E. Yen, M. M. Kelly, P. A. Bullock, B. L. Parsons, K. E. O'Neill, and R. J. Roberts.** 1982. Nucleotide sequences from the adenovirus-2 genome. J. Biol. Chem. **257**:13475–13491.
13. **Ginsberg, H. S., M. J. Ensinger, R. S. Kauffman, A. J. Mayer, and U. Lundholm.** 1974. Cell transformation: a study of regulation with types 5 and 12 adenovirus temperature-sensitive mutants. Cold Spring Harbor Symp. Quant. Biol. **39**:419–426.
14. **Horwitz, M. S.** 1978. Temperature-sensitive replication of H5ts125 adenovirus DNA *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **75**:4291–4295.
15. **Horwitz, M. S., and H. Ariga.** 1981. Multiple rounds of adenovirus DNA synthesis in vitro. Proc. Natl. Acad. Sci. U.S.A. **78**:1476–1480.
16. **Kaplan, L. M., H. Ariga, J. Hurwitz, and M. S. Horwitz.** 1979. Complementation of the temperature-sensitive defect in H5ts125 adenovirus DNA replication *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **76**:5534–5538.
17. **Klein, H., W. Maltzman, and A. J. Levine.** 1979. Structure-function relationships of the adenovirus DNA-binding protein. J. Biol. Chem. **254**:11051–11060.
18. **Klessig, D. F., and T. Grodzicker.** 1979. Mutations that allow human Ad2 and Ad5 to express late genes in monkey cells map in the viral gene encoding the 72K DNA binding protein. Cell **17**:957–966.
19. **Kruijer, W., F. M. A. van Schaik, and J. S. Sussenbach.** 1981. Structure and organization of the gene coding for the DNA binding protein of adenovirus type 5. Nucleic Acids Res. **9**:4439–4457.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lichy, J. H., J. Field, M. S. Horwitz, and J. Hurwitz.** 1982. Separation of the adenovirus terminal protein precursor from its associated DNA polymerase: role of both proteins in the initiation of adenovirus DNA replication.

Proc. Natl. Acad. Sci. U.S.A. **79**:5225–5229.

22. **Lichy, J. H., M. S. Horwitz, and J. Hurwitz.** 1981. Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and 5'-dCMP *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **78**:2678–2682.
23. **Linné, T., and L. Philipson.** 1980. Further characterization of the phosphate moiety of the adenovirus type 2 DNA-binding protein. Eur. J. Biochem. **103**:259–270.
24. **Maniatis, T., and A. Efstratiadis.** 1980. Fractionation of low molecular weight DNA or RNA in polyacrylamide gels containing 98% formamide or 7 M urea. Methods Enzymol. **65**:299–305.
25. **Nagata, K., R. A. Guggenheimer, T. Enomoto, J. H. Lichy, and J. Hurwitz.** 1982. Adenovirus DNA replication *in vitro*: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. U.S.A. **79**:6438–6442.
26. **Pincus, S., W. Robertson, and D. Rekosh.** 1981. Characterization of the effect of aphidicolin on adenovirus DNA replication: evidence in support of a protein primer model of initiation. Nucleic Acids Res. **9**:4919–4938.
27. **Rekosh, D. M. K., W. C. Russell, A. J. D. Bellett, and A. J. Robinson.** 1977. Identification of a protein linked to the ends of adenovirus DNA. Cell **11**:283–295.
28. **Robinson, A. J., H. B. Younghusband, and A. J. D. Bellett.** 1973. A circular DNA-protein complex from adenoviruses. Virology **56**:54–69.
29. **Schechter, N. M., W. Davies, and C. W. Anderson.** 1980. Adenovirus coded deoxyribonucleic acid binding protein. Isolation, physical properties, and effects of proteolytic digestion. Biochemistry **19**:2802–2810.
30. **Smart, J. E., and B. W. Stillman.** 1982. Adenovirus terminal protein precursor. J. Biol. Chem. **257**:13499–13506.
31. **Steenbergh, P. H., J. Maat, H. van Ormondt, and J. S. Sussenbach.** 1977. The nucleotide sequence at the termini of adenovirus type 5 DNA. Nucleic Acids Res. **4**:4371–4389.
32. **Stillman, B. W.** 1981. Adenovirus DNA replication *in vitro*: a protein linked to the 5' end of nascent DNA strands. J. Virol. **37**:139–147.
33. **Stillman, B. W., J. B. Lewis, L. T. Chow, M. B. Mathews, and J. E. Smart.** 1981. Identification of the gene and mRNA for the adenovirus terminal protein precursor. Cell **23**:497–508.
34. **Sussenbach, J. S., P. C. van der Vliet, D. J. Ellens, and H. S. Jansz.** 1972. Linear intermediates in the replication of adenovirus DNA. Nature (London) New Biol. **239**:47–49.
35. **Van der Vliet, P. C., A. J. Levine, M. J. Ensinger, and H. S. Ginsberg.** 1975. Thermolabile DNA binding proteins from cells infected with a temperature-sensitive mutant of adenovirus defective in viral DNA synthesis. J. Virol. **15**:348–354.
36. **Van der Vliet, P. C., and J. S. Sussenbach.** 1975. An adenovirus type 5 gene function required for initiation of viral DNA replication. Virology **67**:415–426.
37. **Van der Vliet, P. C., J. Zandberg, and H. S. Jansz.** 1977. Evidence for a function of the adenovirus DNA-binding protein in initiation of DNA synthesis as well as in elongation on nascent DNA chains. Virology **80**:98–110.
38. **Williams, J. F., M. Gharpure, S. Ustacelebi, and S. McDonald.** 1971. Isolation of temperature-sensitive mutants of adenovirus type 5. J. Gen. Virol. **11**:95–101.