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Evidence for an altered adenovirus DNA polymerase in cells infected with the mutant H5ts149

(DNA synthesis/temperature-sensitive mutation)

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ABSTRACT **The N complementation group of adenovirus (Ad) serotype 5 mutants, which are temperature sensitive for viral DNA synthesis *in vivo*, has been used to study a 140,000-dalton DNA polymerase (Pol) that copurified with the 80,000-dalton terminal protein precursor (pTP). Extracts prepared from HeLa cells infected with the N group mutant H5ts149 at nonpermissive temperature were unable to synthesize viral DNA. The defect in these extracts was specifically reversed by addition of the Pol purified from wild-type Ad-infected cytosol. Addition of the pTP, free of the Pol, did not restore replicative activity to H5ts149 extracts. The reactions studied depend on the presence of the DNA template and include the initiation reaction (the covalent attachment of dCMP to the pTP) and the selective replication of Ad DNA restriction endonuclease fragments containing the origin sequences. Glycerol gradient sedimentation showed that a replicative activity representing the pTP–Pol complex was greatly reduced in H5ts149 extracts as compared with wild-type extracts, suggesting some alteration in the mutant. A pool of pTP free of Pol was detected on these gradients in extracts from both wild-type and H5ts149-infected cells. In addition, the initiation and elongation of Ad DNA catalyzed by H5ts149 extracts prepared from cells grown at permissive temperatures was more labile to urea inactivation than extracts prepared from cells infected with wild-type virus. These results, considered together with the mapping of the H5ts149 mutation within an open reading frame approximately large enough to code for the 140,000-dalton DNA polymerase [Gingeras, T. R., Sciaky, D., Gelinas, R. E., Bing-Dong, J., Yen, C. E., Kelly, M. M., Bullock, P. A., Parsons, B. L., O'Neill, K. E. & Roberts, R. J. (1982) *J. Biol. Chem.* 257, 13475–13491; Alestrom, P., Akusjarui, G., Pettersson, M. & Pettersson, U. (1982) *J. Biol. Chem.* 257, 13492–13498], suggest that the Pol is a virally encoded protein, as is the pTP.**

Current *in vitro* systems for studying adenovirus (Ad) DNA replication have led to the identification of a number of proteins involved in both initiation and elongation (1, 2). The template required in these systems is Ad DNA covalently linked at each 5′ end to a 55-kilodalton (kDa) terminal protein (Ad DNA-pro). The crude *in vitro* system contained a nuclear extract from infected or uninfected HeLa cells and a cytosol extract from Ad-infected HeLa cells (3). Fractionation of the uninfected nuclear extract has identified two host proteins that can replace the nuclear extract in the *in vitro* system (4). These two proteins are free of DNA polymerase activity. The Ad cytosol has been fractionated into three polypeptides required for replication: the 72-kDa Ad DNA binding protein (Ad DBP), the 80-kDa precursor to the terminal protein (pTP), and a 140-kDa protein possessing DNA polymerase activity (Pol) (5). The latter two proteins are found associated in a complex that has been separated into 80- and 140-kDa subunits by sedimentation through glycerol gradients in the presence of urea. Studies with separated subunits have shown that both the pTP and Pol subunits were necessary for the initiation step, the covalent addition of dCMP to the pTP, and for the elongation of Ad DNA-pro (6).

Previous results suggest that the 140-kDa Pol might be a viral gene product. The Pol was not detected in extracts from uninfected HeLa cells. In addition, its properties differed from those of the known host DNA polymerases, α, β, and γ with respect to template preference and sensitivity to inhibitors (6). The Ad Pol was the only polymerase that used the pTP as a primer and supported replication of Ad DNA-pro *in vitro*. Ad serotype 5 (Ad5) mutants in the N complementation group, which are unable to synthesize viral DNA at nonpermissive temperature (7, 8), define a region of the Ad genome that may code for the viral Pol gene. The mutants in this group, which include H5ts149 and H5ts36, map between coordinates 18 and 22 from the left end of the Ad genome, within an open reading frame extending from coordinate 22.9 leftward to coordinate 14.2 in the early region E2B transcription unit (9–11). This region is adjacent to the coding region for pTP, which has recently been located between coordinates 28.9 and 23.5 (11, 12). There appears to be no overlap between the pTP region and the region that encodes the N group mutants (12).

To further define the nature of the N group mutation, we have prepared cytosol extracts from H5ts149-infected cells. In this report, we describe the effect of adding the isolated wild-type pTP or Pol subunits to extracts prepared from cells infected with H5ts149. Activity was restored to H5ts149 extracts prepared at nonpermissive temperature by addition of the 140-kDa Pol subunit but not by the 80-kDa pTP.

A free pool of the 80-kDa subunit has been observed in cytosol fractions isolated from cells infected with wild-type Ad serotype 2 (Ad2) or H5ts149; however, the ratio of intact pTP/Pol complex to free pTP is much greater for Ad2 than for H5ts149 grown at either 33° or 39°C. In addition, we have shown that H5ts149 extracts from cells grown at permissive temperature were more sensitive to inactivation by urea than were wild-type Ad2 extracts. These results suggest that the H5ts149 defect is in the viral DNA polymerase gene.

MATERIALS AND METHODS

Cells and Viruses. The growth of HeLa S3 cells and purification of the Ad2 and H5ts149 viruses have been described (13).

Preparation of Components for Ad DNA Synthesis *in Vitro*. Infected cytosol extracts were prepared from HeLa cells in-

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Abbreviations: Ad, adenovirus; Ad2 and Ad5, Ad serotypes 2 and 5; Ad DNA-pro, Ad DNA covalently linked to a 55,000-dalton terminal protein at each 5′ end; Ad DBP, 72,000-dalton Ad-encoded DNA binding protein; Pol, 140,000-dalton Ad DNA polymerase; pTP, 80,000-dalton precursor to the terminal protein; kDa, kilodalton(s).

fected with 4,000–8,000 virions per cell of either Ad2 or H5ts149. Infected cells were incubated for 41 hr at 33°C with addition of 0.4 mM hydroxyurea 16 hr after infection (permissive conditions) or for 21 hr at 39°C with addition of 0.4 mM hydroxyurea 2 hr after infection (nonpermissive conditions). Infected cytoplasmic extracts and uninfected HeLa nuclear extracts were prepared as described (14, 15). Ad DBP and the complex of pTP and Ad DNA polymerase (pTP–Pol) were purified as described (14, 15); the pTP–Pol complex was separated into 80- and 140-kDa subunits on glycerol gradients containing urea as described (6). Urea (Fisher) was dissolved in water, stirred with Amberlite MB-3 (Mallinkrodt; 2 g/10 ml of 8 M solution) for 30 min at room temperature, and filtered prior to use. Ad DNA-pro was purified from Ad2 virion, which was sedimented in sucrose gradients containing 4 M guanidine hydrochloride (16).

Assays: Synthesis of DNA Using Ad DNA-pro Cut with *Xba* I. Reaction mixtures (50 μl) contained 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 μM dATP/dGTP/dCTP, 1.5 μM [α-^{32}P]dTTP (20,000–40,000 cpm/pmol), 3.75 mM ATP, uninfected nuclear extract (4–8 μg of protein), 0.5 μg of Ad DBP, 0.07 μg of Ad DNA-pro that had been cleaved at 37°C with 2.5 units of *Xba* I, and various cytosol extracts or isolated subunits. Reactions were terminated after 2 hr at 30°C by the addition of 0.2% $NaDodSO_4$. (The reaction remained in the linear range at this time.) After addition of Pronase (10 μg) and a 1-hr incubation at 37°C to remove covalently bound terminal protein, the DNA was precipitated by addition of 2.5 vol of ethanol/0.3 M ammonium acetate for 16 hr at −20°C. The DNA was dissolved in 50 μl of 40 mM Tris·HCl/1 mM EDTA/5 mM NaOAc, pH 7.8, and electrophoresed in 1.4% agarose slab gels containing the same buffer until the bromophenol blue dye reached the bottom edge of the gel.

Synthesis of the pTP–dCMP Complex. Reaction mixtures (25 μl) containing 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 3 mM ATP, 0.5 mM [α-^{32}P]dCTP (410 Ci/mmol; 1 Ci = 37 GBq), nuclear extract from uninfected HeLa cells (2–5 μg of protein), Ad DNA-pro (0.07 μg), and various cytosol extracts or isolated subunits were incubated for 2 hr at 30°C, and reactions were terminated as described (15). (Reactions were in the linear range at this time.) pTP–dCMP was detected by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (15).

Synthesis with Ad DNA-pro as Template. Ad DNA replication was measured in reaction mixtures (50 μl) containing 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 3 mM ATP, 40 μM dATP/dCTP/dGTP, 4 μM [^{3}H]dTTP (3,000 cpm/pmol), 0.5 μg of Ad DBP, uninfected HeLa nuclear extract (10 μg of protein), 0.07 μg of Ad DNA-pro, and various cytosol extracts or isolated subunits. In some reactions, extracts were incubated with urea prior to addition of the reaction components. Acid-insoluble radioactivity was determined after incubation at 30°C for 60 min.

For sedimentation studies, 0.1-ml aliquots of cytosol extracts were adjusted to a final NaCl concentration of 0.5 M and a pH of 6.0 in a volume of 0.2 ml. After 1 hr at 0°C, the solution was layered onto a 4.8-ml gradient of 15–35% glycerol in 25 mM sodium phosphate, pH 6.0/1 mM dithiothreitol/1 mM EDTA/0.01% Nonidet P-40/0.5 M NaCl. Gradients were centrifuged at 49,000 rpm for 24 hr in an SW 50.1 rotor. Fractions were collected from the bottom of the tube.

RESULTS

Cytosol Extracts of Cells Infected with H5ts149 at 39°C Are Deficient in the Ad DNA Polymerase. Cytosol extracts prepared from cells infected with H5ts149 at both permissive (33°C) and nonpermissive (39°C) temperatures *in vivo* were assayed for their replicative activity using *Xba* I restriction fragments of Ad DNA-pro (Fig. 1). In this assay, replication was measured by the incorporation of radioactivity specifically into the terminal fragments C and E, which contain the origins of Ad DNA replication, and the C′ and E′ bands, which represent displaced single strands produced during multiple rounds of *in vitro* replication (17). This assay was totally dependent on the presence of an uninfected HeLa nuclear extract and cytosol from Ad-infected cells that contained the Ad-encoded 72-kDa DBP, 80-kDa pTP, and 140-kDa Pol. The pTP and Pol activities are typically found as a complex (pTP–Pol). The cytosol extract prepared from H5ts149-infected cells grown at permissive temperature (33°C) was nearly as active as wild-type Ad2 extracts prepared from cells grown at either 33°C or 39°C. However, extracts prepared from H5ts149-infected cells grown at 39°C were inactive. Such extracts, which contained functional Ad DBP, could be activated by addition of the purified pTP–Pol complex. To further explore the nature of the defect, separated 80-kDa pTP and 140-kDa Pol subunits were added to the inactive extracts. Addition of the 140-kDa Pol subunit restored the activity of the extracts to normal levels, indicating that mutant extracts were deficient in active 140-kDa Pol protein. Addition of the pTP subunit to the H5ts149 (39°C) extract did not appreciably stimulate DNA synthesis. Each subunit had little effect when added to wild-type Ad2 extracts or to extracts of cells infected with H5ts149 grown at the permissive temperature (33°C). The observed effect was not due to the presence of an inhibitor in the 80-kDa-subunit preparation because addition of both the 80- and 140-kDa subunits to reaction mixtures did not block stimulation due to the 140-kDa subunit but further enhanced the reaction (unpublished data).

Extracts of cells infected with H5ts149 were also tested for their ability to form the 80-kDa pTP–dCMP initiation complex. Addition of the 140-kDa Pol subunit stimulated formation of the 80-kDa–dCMP complex about 15-fold in H5ts149 nonpermissive extracts as compared with a 2.5-fold stimulation of H5ts149 permissive extracts (Fig. 2*A*). Addition of the 80-kDa pTP subunit to H5ts149 permissive and nonpermissive extracts had little effect. Addition of either the 80-kDa pTP or the 140-kDa Pol subunit also had little effect on wild-type Ad2 extracts prepared at either 33° or 39°C. This further suggested a defect in the 140-kDa Pol activity in the extracts from cells infected with H5ts149 at 39°C.

Since the H5ts149 permissive extracts used in the experiment shown in Fig. 2*A* were less active than control wild-type Ad2 extracts, the amounts of extracts used were normalized to similar activity levels rather than to equal protein concentrations. However, in Fig. 2*B*, the effects of added subunit on extracts prepared at the nonpermissive temperature were compared at equivalent protein concentrations. Addition of the Pol subunit to H5ts149 (39°C) cytosol restored the activity to the level seen with wild-type extracts. A small enhancement by the Pol subunit was also seen in control cytosol prepared from cells infected with Ad2 at 39°C. This may be explained by the presence of a pool of free 80-kDa pTP present in the cytosol that can react with added 140-kDa subunit, as described below. Mixing experiments were also done to eliminate the possibility of nonspecific inhibition of extracts by the addition of isolated subunits (data not shown). The combination of Ad2 or H5ts149 cytosol with both subunits showed an additive effect. [The activity of extracts prepared from cells infected at 33°C with H5ts149 varied. In some experiments, cytosol fractions were found to be less active. However, supplementation of these extracts with the 140-kDa Pol subunit restored the activity to the level observed with wild-type Ad2-infected cytosol.]

Inactivation of H5ts149 Permissive Extracts in the Presence of Urea. Ad DNA replication in cells infected with H5ts149 at

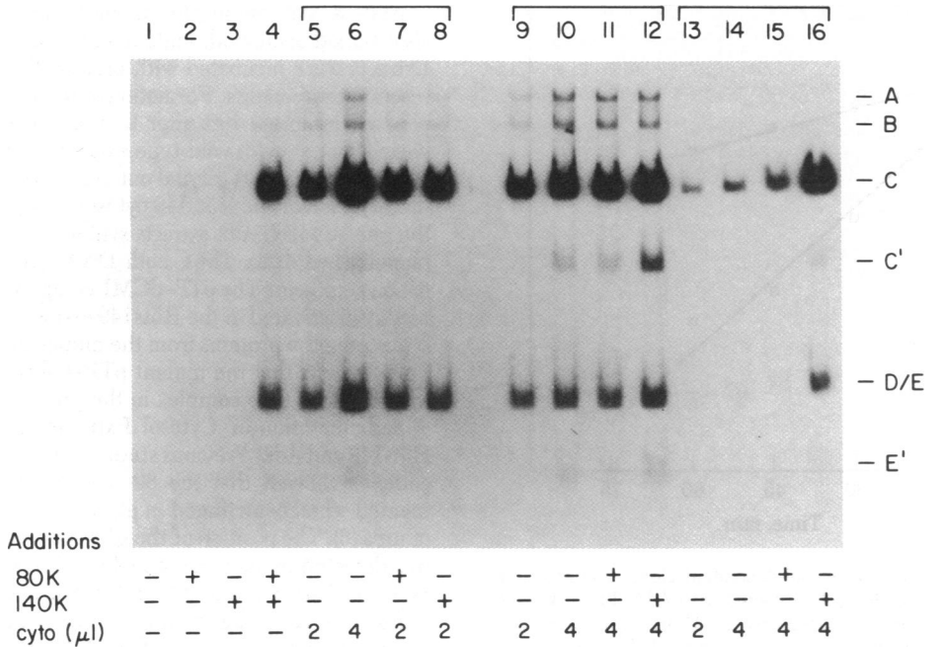

FIG. 1. Complementation of H5ts149 cytosol extracts with isolated wild-type subunits (pTP or Pol) in the elongation reaction. Replication of *Xba* I restriction fragments was carried out using cytosol extracts (cyto) of wild-type Ad2-infected cells grown at 33°C (lanes 5–8) and H5ts149-infected cells grown at either the permissive (33°C) (lanes 9–12) or the nonpermissive (39°C) (lanes 13–16) temperature. All reaction mixtures contained uninfected nuclear extract (8.2 μg of protein); cytosol extracts and the purified 80- and 140-kDa subunits were added as indicated. In lanes 1–4, in which no cytoplasmic extract was present, 1.0 μg of Ad DBP was added to the reaction mixtures. Ad2, H5ts149 (33°C), and H5ts149 (39°C) extracts were used at protein concentrations of 10.4, 10.2, and 8.9 mg/ml, respectively; the 80- and 140-kDa subunits were used at 10 ng. Reaction mixtures were incubated for 120 min at 30°C.

33°C gradually diminished beginning approximately 4 hr after shift to 39°C. This difficulty of inhibiting DNA synthesis was also encountered *in vitro*. H5ts149 extracts grown at 33°C were not inactivated at elevated temperatures *in vitro*. To investigate possible differences between mutant and wild-type DNA synthesis activity, we studied the influence of urea on the stability of each extract. Wild-type Ad2 and H5ts149 extracts were preincubated at 30°C for various times with 2.2 M urea; reaction components essential for DNA synthesis were added and DNA synthesis was allowed to proceed for 1 hr at 30°C. With increasing time of exposure to urea, extracts of cells infected with H5ts149 at permissive temperature were inactivated more rapidly than wild-type DNA extracts (Fig. 3). To show that no inhibitor was present in the H5ts149 extracts, mixing exper-

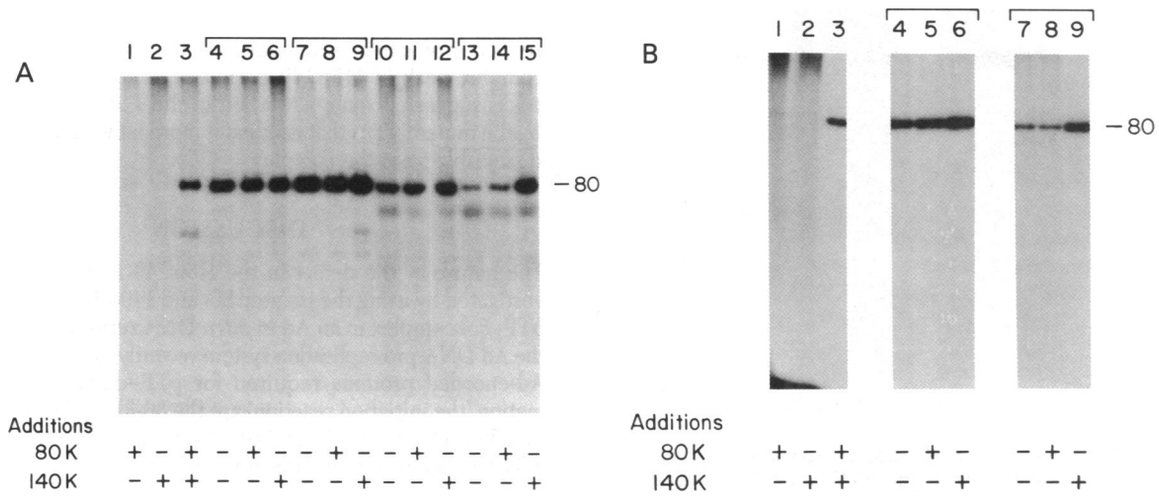

FIG. 2. Complementation of H5ts149 cytosol extracts with isolated wild-type subunits (pTP or Pol) in the initiation reaction. (*A*) Cytosol extracts were prepared from cells infected with Ad2 or H5ts149 and incubated at either 33°C or 39°C. Synthesis of the pTP–dCMP complex was assayed with incubation for 120 min at 30°C. All lanes contained uninfected nuclear extract (2.0 μg of protein); in addition, lanes 4–6 contained Ad2 (33°C) cytosol (4.8 μg of protein); lanes 7–9, Ad2 (39°C) cytosol (5.0 μg of protein); lanes 10–12, H5ts149 (33°C) cytosol (36.8 μg of protein); lanes 13–15, H5ts149 (39°C) cytosol (42.0 μg of protein). Results of quantitation of the 80-kDa band ("80") in arbitrary units (U) on a Transidyne scanning densitometer were as follows: lanes 1 and 2, <2 U; lane 3, 26 U; lanes 4–6, 67, 81, and 102 U, respectively; lanes 7–9, 137, 158, and 188 U, respectively; lanes 10–12, 34, 59, and 90 U, respectively; lanes 13–15, 8, 14, and 116 U, respectively. (*B*) Reaction mixtures were assayed as in *A* except that Ad2 (39°C) cytosol (lanes 4–6) containing 25 μg of protein and H5ts149 (39°C) cytosol (lanes 7–9) containing 26 μg of protein were used. Densitometer readings were as follows: lanes 1 and 2, 2 U; lane 3, 108 U; lane 4, 136 U; lane 5, 155 U; lane 6, 244 U; lane 7, 37 U; lane 8, 35 U; lane 9, 117 U.

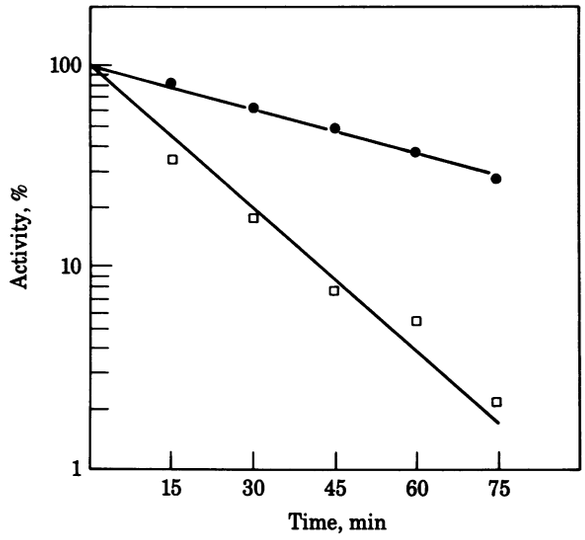

FIG. 3. Inactivation of the cytosol-dependent elongation reaction by urea. Cytosol extracts from cells infected with Ad2 (33°C) (●) and H5ts149 (33°C) (□) were preincubated with 2.2 M urea at 30°C for the times indicated; then, the reaction components needed for DNA synthesis using Ad DNA-pro as template were added to 5 μl of the preincubation mixture and DNA synthesis was allowed to proceed for 1 hr at 30°C. Both Ad2 and H5ts149 cytosol extracts contained 50 μg of protein per 50 μl of reaction mixture. Activity is expressed as pmol of [^{3}H]-dTMP (6,050 cpm/pmol) incorporated; 100% values for Ad2 (33°C) cytosol and H5ts149 (33°C) cytosol were 1.92 and 0.92, respectively. Control reactions lacking enzyme had an activity of 0.02 pmol; this value was subtracted from the data presented.

iments were done; addition of cytosol from cells infected with H5ts149 to the Ad2 cytosol did not inhibit the wild-type DNA complementing activity (unpublished data).

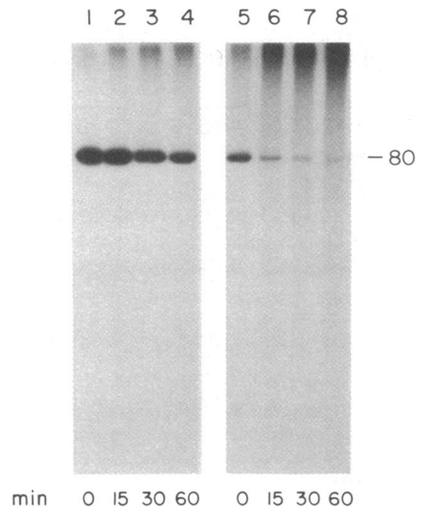

FIG. 4. Inhibition of pTP–dCMP complex formation by urea. Cytosol extracts of cells infected with Ad2 (33°C) (lanes 1–4) or H5ts149 (33°C) (lanes 5–8) were incubated at equal protein concentrations with 2.0 M urea at 30°C for the times indicated; then, components necessary for pTP–dCMP complex formation were added and the mixtures were incubated at 30°C for 1 hr. Reactions were terminated and products were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis as described for detection of the 80-kDa–dCMP complex. Densitometer tracings in arbitrary units (U) of the 80-kDa pTP–dCMP band were quantitated as in Fig. 2. Densitometer readings were as follows: lanes 1–4, 165 U (100%), 147 U (89%), 96 U (58%), 78 U (47%), respectively; lanes 5–8, 67 U (100%), 21 U (31%), 13 U (19%), 8 U (13%), respectively.

pTP–dCMP complex formation in the presence of urea was also studied using both mutant and wild-type extracts (Fig. 4). Extracts were incubated with urea at 30°C before addition of reaction components. Formation of the H5ts149 initiation complex was inhibited 70% after 15 min of exposure to urea; complex formation with wild-type extract was virtually unaffected. Mixing experiments carried out in the presence of urea showed that addition of the H5ts149 cytosol to Ad2 cytosol did not alter the rate at which Ad2 extracts synthesized pTP-dCMP complex (unpublished data). Thus, both DNA synthesis activity and initiation as measured by pTP–dCMP complex formation were more readily inactivated in the H5ts149 extracts, indicating some differences in the proteins from the mutant and wild-type viruses. This suggests that the mutant pTP–Pol complex is more labile than the wild-type complex in the presence of urea.

Sedimentation of Cytosol Extracts of Cells Infected with H5ts149 and Ad2. Previous studies using purified Ad2 pTP–Pol complex showed that the 80- and 140-kDa proteins cosedimented when centrifuged in glycerol gradients in the absence of urea (6). The position of the pTP–Pol complex in the gradient was detected by measuring DNA synthesis in the presence of HeLa nuclear extract and Ad DBP. Ad2 and H5ts149 cytosol extracts were centrifuged under similar conditions and the gradient fractions were assayed for DNA synthesis in the presence and absence of added 140-kDa subunit. Complementing activity for DNA synthesis was detected in the Ad2 gradient (Fig. 5*A*) in the absence of free 140-kDa subunit. This peak of pTP–Pol complex was greatly reduced in H5ts149 permissive extracts (Fig. 5*B*) and virtually absent in cytosol fractions prepared from cells infected with H5ts149 at nonpermissive conditions (Fig. 5*C*). When glycerol gradient fractions were supplemented with the free 140-kDa Pol subunit, a second peak of DNA complementing activity was detected in gradients containing cytosol from Ad2-infected cells and in gradients containing H5ts149 cytosol prepared at either 33° or 39°C (Fig. 5). However, when glycerol gradient fractions were supplemented with the 80-kDa pTP subunit, no further stimulation of DNA synthesis was observed (unpublished data). These results suggested that the cytosol extracts contained a pool of 80-kDa pTP free of the 140-kDa polymerase. In gradients containing Ad2 extracts, the 80-kDa pTP was well separated from the pTP–Pol complex. The reduction of activity sedimenting in the position of the pTP–Pol complex peak in H5ts149 permissive and nonpermissive extracts suggested some alteration in the 140-kDa Pol produced by the mutant virus that renders it more labile and more readily dissociable from the complex.

DISCUSSION

The nature of the defect in the H5ts149 mutant has been investigated by using the isolated 80- and 140-kDa subunits of the pTP–Pol complex in an Ad *in vitro* DNA replication system. In the Ad DNA-pro replication system recently developed, the only Ad-encoded proteins required for pTP–dCMP complex formation (the initiation reaction) are the 80-kDa pTP and the 140-kDa Pol (14, 18, 19). In addition to these two proteins, the 72-kDa Ad DBP is required for the elongation reaction (13, 18, 19). In the experiments described in this report, we used uninfected nuclear extract and partially purified cytosol extracts of Ad2 and H5ts149, which contain all the Ad-encoded components necessary for Ad DNA replication. Ad2 and H5ts149 (permissive) preparations initiated and elongated Ad DNA-pro while extracts prepared from cells infected with H5ts149 at the nonpermissive temperature showed very low levels of activity in both initiation and elongation. These inactive H5ts149 extracts contained the wild-type 72-kDa Ad DBP since they could be complemented

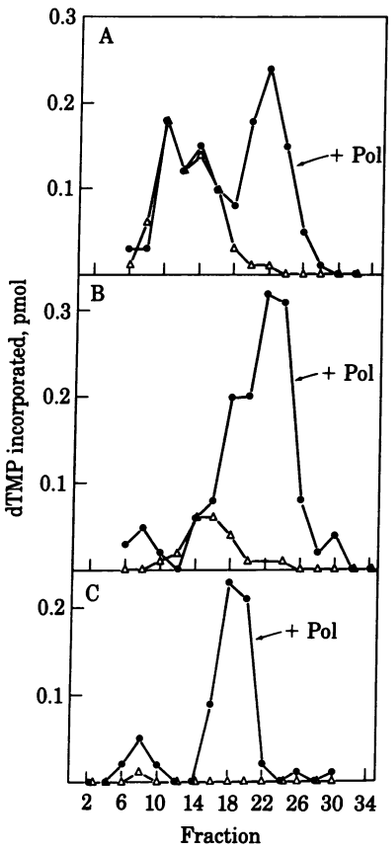

FIG. 5. Glycerol gradient centrifugation of cytosol extracts of cells infected with H5ts149 and Ad2. Cytosol extracts were centrifuged in glycerol gradients, and gradient fractions were assayed for their ability to synthesize DNA using Ad DNA-pro as template in the presence (●) and absence (△) of added Pol subunit. Acid-insoluble radioactive material was assayed after incubation at 30°C for 90 min. Gradients formed with Ad2 (33°C) cytosol (*A*) and H5ts149 (33°C) cytosol (*B*) contained 37 fractions. Previous results showed no activity in the fractions at the bottom (nos. 2–4) and top (nos. 32–37) of the gradients and therefore these fractions were not assayed in this experiment. H5ts149 (39°C) cytosol (*C*) centrifuged under identical conditions in a separate experiment contained 34 fractions.

with purified pTP–Pol complex in assays dependent on the three polypeptides found in Ad cytosol: pTP, Pol, and Ad DBP. The nature of the lesion was determined by distinguishing between the effects of adding either the pTP or Pol purified polypeptide; inactive H5ts149 extracts were activated by addition of the 140-kDa Pol subunit while the 80-kDa pTP had no effect. This provided evidence that the 80-kDa pTP present in the mutant extracts was functional since both subunits are needed for Ad DNA replication. These data strongly suggest that the defect in the H5ts149 mutant lies in its polymerase gene.

Previous studies showed that the pTP–Pol complex could be separated in the presence of urea (6). Comparison of Ad2 and H5ts149 extracts that had been incubated with urea provided evidence that the mutant was inactivated more rapidly than the wild type when assayed both for pTP–dCMP complex formation and for elongation. These results suggest that the mutant pTP–Pol complex may dissociate more rapidly than the wild-type complex. This might be due to an altered polymerase that is not as strongly associated with the pTP as is the wild-type polymerase. Alternatively, a reduced amount of Pol molecules may be present in the mutant, causing a more rapid rate of inactivation of DNA synthesis in the presence of urea.

Partially purified H5ts149 pTP–Pol complexes were prepared and assayed along with wild-type controls in a DNA polymerase assay using nicked calf thymus DNA. In these assays, the H5ts149 preparations were more labile than wild-type preparations at elevated temperatures (2- to 3-fold; unpublished data). The marked instability of the 140-kDa Pol may have hampered our further attempts to purify the H5ts149 gene product.

Variability in the activity levels of various preparations of H5ts149 permissive extracts was noted. Some extract preparations lost activity on storage at −80°C. These differences may reflect an instability of the H5ts149 Pol both *in vivo* and *in vitro*. Wild-type preparations prepared simultaneously under similar conditions remained stable. Sedimentation studies using wild-type and mutant extracts led to the discovery of a free cytosol pool of 80-kDa pTP. This probably accounted for the small increase in activity sometimes observed on addition of the 140-kDa subunit to wild-type extracts. The properties of the unbound 80-kDa pTP appear to be the same as those of the pTP isolated from the pTP–Pol complex, although this has not been studied in detail.

Note Added in Proof. Stillman *et al.* (figure 4 in ref. 20) have recently also complemented the H5ts149 defect in the specific elongation of Ad terminal restriction fragments. However, they used the pTP–Pol complex and thus did not distinguish between the two viral gene products in their complementation assay.

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