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An Adenovirus Type 5 Gene Function Required for Initiation of Viral DNA Replication

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Adenovirus type 5 (Ad5) DNA replication was studied after infection of human or monkey cells with two DNA-negative temperature-sensitive mutants belonging to different complementation groups (H5ts125 and H5ts36). When infection was carried out at the permissive temperature (32°) followed by a shift to the nonpermissive temperature (39.5°) viral DNA synthesis in H5ts125-infected cells was reduced 90% within 1 hr after shift-up, while a decline in DNA synthesis in H5ts36-infected cells is only observed after 6 hr. Analysis of the various forms of DNA synthesized under conditions of inhibition showed a constant ratio of replicating to mature viral DNA for both mutants, while no accumulation of replicating molecules was observed.

When H5ts125-infected cells were pulse-labeled with [^{3}H]thymidine at 32 or 39.5° followed by a chase of the label at 39.5°, replicating DNA was converted into mature DNA at the same rate as in wild-type-infected cells. This indicates that chain propagation and termination could occur normally under nonpermissive conditions. The results of density labeling experiments performed at 39.5° are in agreement with an initiation block in H5ts125-infected cells at the nonpermissive temperature. It is concluded that the H5ts125 gene product and possibly also the H5ts36 gene product are required for the initiation of new rounds of replication. The potential role in initiation of the adenovirus-specific DNA binding protein, which is coded for by the H5ts125 gene, is discussed.

INTRODUCTION

Temperature-sensitive (ts) mutants of Ad5 have been divided into 17 complementation groups, which is one-third to one-half of the total number of groups expected on the basis of the genome size (Williams *et al.*, 1974). Most of the ts mutants characterized so far are defective in a late function, and only two groups of DNA-negative mutants have been isolated. Of these, one is represented only by a single mutant (H5ts125) and the other by three mutants, viz., H5ts36, H5ts37 and H5ts149.

Our knowledge concerning the phenotype of these mutants is limited. H5ts36 is defective in transformation at the nonpermissive temperature, while H5ts125 transforms rat cells at a frequency higher than wild type (Williams *et al.*, 1974; Ginsberg *et al.*, 1974). Genetic dominance studies have shown that the H5ts125 gene product is required in stoichiometric amounts for virus production, while for the H5ts36 gene product catalytic amounts are sufficient (Austin, Young and Williams, unpublished results). Recently, the H5ts125 gene product has been characterized as a DNA binding protein on the basis of the thermolability of this adenovirus-specific protein in H5ts125-infected cells (van der Vliet *et al.*, 1975). This DNA binding protein, with a molecular weight of 72,000, is made in large amounts, up to 10^7 copies per infected cell. Synthesis starts early in infection and is independent of viral or cellular DNA synthesis. *In vitro*, the protein binds preferentially to single-stranded, not to double-stranded, DNA (van der Vliet and Levine, 1973).

In order to investigate during which step of the viral DNA replication this protein is required, the phenotype of H5ts125 has been studied. It will be shown that initiation of new rounds of replication but not chain propagation or termination is blocked in H5ts125-infected cells at the nonpermissive temperature. In addition, evidence will be presented suggesting that the H5ts36 gene function may also be required for initiation.

MATERIALS AND METHODS

Growth of cells and viruses. Human KB cells were grown in monolayer as described earlier (Sussenbach and van der Vliet, 1972). Primary African green monkey kidney (AGMK) cells were cultivated in Dulbecco's modified Eagle's medium. H5ts125,[1] originally isolated by Ensinger and Ginsberg (1972), was kindly supplied by Dr. H. S. Ginsberg and H5ts36 by Dr. J. F. Williams. Mutant viruses were propagated at 32° and purified as described (van der Vliet and Sussenbach, 1972).

Pulse-chase experiments and the isolation of pulse-labeled viral DNA. KB cells (5×10^5/ml) in suspension were infected at a multiplicity of 2000 physical particles per cell in Eagle's medium containing 20 m*M* *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.2. In some cases, [^{14}C]thymine (0.5 μCi/ml) was added at 16 hr postinfection to prelabel viral DNA. The cells were pulse-labeled with [^{3}H]thymidine (30 μCi/ml) at a concentration of 5×10^6 cells/ml. For pulse-chase experiments, nonradioactive thymidine, up to 1 m*M*, was added, and the cells were washed three times at the indicated temperature with prewarmed medium containing 1 m*M* thymidine and suspended in medium containing 0.25 m*M* thymidine. At appropriate times, samples were mixed with an equal volume of phosphate-buffered saline containing 10 m*M* EDTA and 1 m*M* thymidine at 4°.

To isolate viral DNA, cells were washed three times with 0.01 *M* Tris-HCl (pH 8.1)/0.01 *M* EDTA and suspended in this buffer at a concentration of 3×10^6 cells/ml. Sodium dodecylsulfate (SDS) was added to 0.1% and Pronase to 0.5 mg/ml. After 15 min at 30°, the SDS concentration was raised to 2% and, after 2 min, NaCl was added to a concentration of 1 *M*. After 16 hr at 4°, the suspension was centrifuged for 30 min at 20,000 *g* and the supernatant fluid (called 1 *M* NaCl supernatant) was either dialyzed against 0.15 *M* NaCl/0.015 *M* Na citrate or used directly. Using this method, more than 90% of the total radioactivity incorporated during short (5 min) or long (up to 5 hr) pulses with [^{3}H]thymidine was found in the 1 *M* NaCl supernatant, indicating a high recovery of replicating as well as mature Ad5-DNA. DNA from purified Ad5 virions was isolated according to van der Eb *et al.* (1969).

Analysis of viral DNA. Band sedimentation was performed in 5–27% isokinetic sucrose gradients, containing 0.01 *M* Tris-HCl (pH 8.1)/0.001 *M* EDTA /1 *M* NaCl/0.1% Sarkosyl. Techniques for buoyant density equilibrium centrifugation in CsCl have been described (van der Vliet and Sussenbach, 1972). When indicated, fractions were spotted on Whatmann 3MM filter disks, and the DNA was precipitated with 10% trichloroacetic acid (TCA). The filters were washed twice with 10% TCA, once with acetone, dried and counted in a liquid scintillation counter.

The complementary strands of Ad5-DNA were separated using the poly(U, G) procedure as described by Tibbetts *et al.* (1974). A suitable batch of poly(U, G) was kindly supplied by Dr. L. Philipson. The light strand obtained after density centrifugation in neutral CsCl in the presence of poly(U, G) was identical to the strand with the higher density in alkaline CsCl density gradients. This strand will therefore be called the H-strand in this paper, in agreement with previously used nomenclature (Sussenbach *et al.*, 1973). DNA-DNA hybridization was performed according to Aloni *et al.* (1969).

RESULTS

When cells were infected at 39.5° with H5ts36 or H5ts125 and the cultures were kept at 39.5° for the entire period of

[1] Abbreviations have been used according to the proposed nomenclature for adenovirus mutants (Ginsberg *et al.*, 1973).

infection (16–20 hr), viral DNA synthesis was inhibited for more than 98% compared to wild-type-infected cultures (Ensinger and Ginsberg, 1972; Wilkie *et al.*, 1973; van der Vliet *et al.*, 1975). To study whether the two gene products are directly (or indirectly) involved in viral DNA replication, temperature shift-up experiments are required.

Kinetics of Shut-off of Viral DNA Synthesis After a Shift-up to the Nonpermissive Temperature

KB cells were infected at 32° for 40 hr with mutant or wild-type adenovirus, and the cultures were subsequently shifted to 39.5° or kept at 32°. At the same time [^{3}H]thymidine was added, and viral DNA synthesis was measured after various periods of time (Fig. 1). Wild-type- or H5ts36-infected cells continue to synthesize viral DNA for at least 4 hr after the shift, the rate of 39.5° being 1.6–2.1 times that at 32° (Figs. 1C and D). In H5ts125-infected cultures however, DNA synthesis declines rapidly after about 30 min (Fig. 1A). The inhibition occurs equally well whether studied early (20 hr) or late (44 hr) after infection. Prelabeled viral DNA was not broken down after the shift-up, indicating that the decline in incorporation was not caused by a combination of normal synthesis and extensive breakdown of synthesized DNA (data not shown).

Inhibition of protein synthesis by cycloheximide did not change the results obtained with H5ts125 (Fig. 1B). Viral DNA synthesis under permissive conditions was independent of concomitant protein synthesis, in agreement with previous reports (Horwitz *et al.*, 1973; Yamashita and Green, 1974). Similarly the decline in Ad5-DNA synthesis in H5ts125-infected cells after the shift to 39.5° occurred normally in the absence of protein synthesis. This suggests that the cessation of viral DNA synthesis results directly from inactivation of the thermosensitive H5ts125 protein. This is a strong indication that the H5ts125 gene product itself is required for viral DNA replication.

After incubation of KB cells at 39.5° for periods longer than 4 hr, we observed a decline in viral DNA synthesis in wild-type- and H5ts36-infected cells. Therefore,

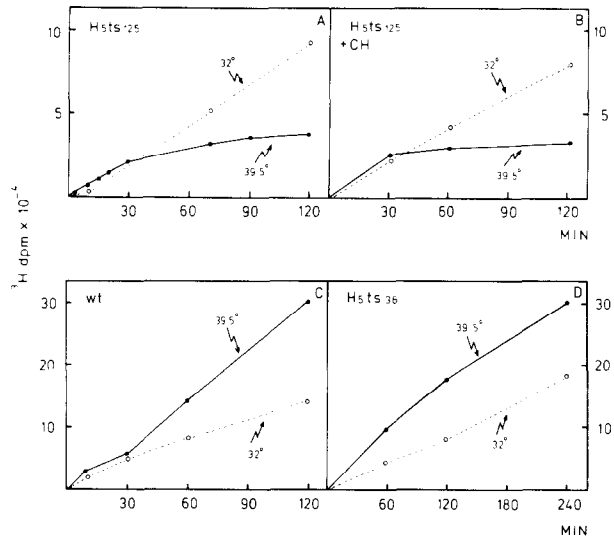

FIG. 1. Kinetics of viral DNA synthesis in infected KB cells after a shift from 32 to 39.5°. KB cells in suspension were infected with H5ts125, H5ts36 or wild type for 40 hr at 32°. Half of the suspension was brought to 39.5°, and the remaining cells were further incubated at 32°. [^{3}H]thymidine was added directly after the shift (t = 0), and viral DNA was isolated at the times indicated. Samples from the 1 *M* NaCl supernatant, containing DNA from 5 × 10^4 cells, were precipitated with TCA and counted. Separate analysis of the isolated DNA in CsCl density gradients showed that all radioactive label was present in viral DNA. (A), H5ts125-infected cells; (B), H5ts125-infected cells to which 100 μg/ml of cycloheximide was added at t = 0, causing a 95% inhibition of protein synthesis; (C), Wild-type-infected cells; (D), H5ts36-infected cells; note the difference in time scale.

we also studied AGMK cells using short pulses at different times after a shift to 39.5° (Fig. 2). As expected, the capacity to synthesize H5ts125 DNA slows down rapidly. In H5ts36-infected cells a decline is observed after 6 hr, while wild type still goes on. Similar results have been obtained by Levine *et al.* (1974). The slow response of H5ts36-infected cells to a shift-up to the nonpermissive temperature is a considerable disadvantage for detailed analysis of this mutant. Most of our further studies have therefore been limited to H5ts125.

Reversibility of the H5ts125 Block

In H5ts125-infected cultures, shift-down experiments were performed to obtain information on the reversibility of the inhibition. AGMK cells were incubated for 40 hr at 32° and subsequently shifted to 39.5°. Cycloheximide (100 μg/ml) was added, and at various times the cultures were brought back to 32° for 15 min, followed by a 15-min pulse with [^{3}H]thymidine at that temperature. At 1 hr after shift-up the 10% residual DNA synthesis at 39.5° could be restored to 83% by a shift-down, while 3 hr later a restoration from 6 to 39% was still possible. This means that the inactivation was readily reversible even in the absence of protein synthesis, suggesting an excess pool of thermolabile H5ts125 protein that could be easily reactivated at 32° and that, in the absence of protein synthesis, is only slowly diminished below the level required for viral DNA synthesis.

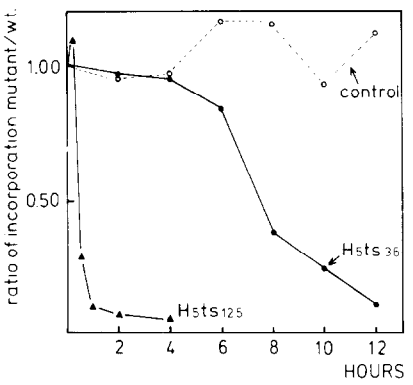

FIG. 2. Capacity of AGMK cells to synthesize viral DNA after a shift-up to the nonpermissive temperature. Mutant- or wild-type-infected AGMK cells in monolayer were incubated at 32° for 40 hr, followed by transfer to 39.5°. At the indicated times part of the cultures were pulse-labeled with [^{3}H]thymidine for 5 min (H5ts125) or 30 min (H5ts36). Pulse-labeled viral DNA was isolated and appropriate samples of the 1 *M* NaCl supernatant were precipitated with TCA and counted. The results are expressed as the ratio of incorporation of mutant to wild type and the ratio at $t = 0$ was set at 1.00. The control represents the ratio of incorporation of H5ts36 to wild type from cultures that were kept at 32° during the entire period.

Analysis of H5ts125 DNA Synthesized After a Shift-up to the Nonpermissive Temperature

Replicating adenovirus DNA contains extended regions of single-stranded DNA and can be separated from mature viral DNA using either sedimentation in neutral sucrose gradients or buoyant density equilibrium centrifugation in CsCl (Pearson and Hanawalt, 1971; Sussenbach and van der Vliet, 1972; Bellett and Younghusband, 1972; van der Eb, 1973; Pettersson, 1973). Pulse-labeled replicating DNA sediments faster than mature DNA in neutral sucrose gradients, with sedimentation values ranging from 31 S (mature Ad5-DNA) up to about 65 S. Its buoyant density is 5–10 mg/ml higher than mature viral DNA (ρ = 1.716 g/cm^3).

These properties of pulse-labeled DNA have been used to determine the amount of replicating intermediates in H5ts125-infected cells following a shift-up in temperature. AGMK cells, infected at 32°, were pulse-labeled for 5 min at 39.5° at various times after the shift-up. Viral DNA synthesis is reduced rapidly but does not stop completely even 2 hr after the shift-up (Fig. 2). A residual synthesis of about 3–5% is always observed. The viral DNA was isolated and subjected to neutral sucrose gradient sedimentation analysis and CsCl density centrifugation in the presence of ^{32}P-labeled mature viral DNA.

The results obtained with two samples, 15 and 60 min after the shift from 32 to 39.5°, respectively, are shown in Fig. 3. Although in the 60-min sample DNA synthesis is reduced tenfold, the gradient profiles of both samples are very much the same and the ratio of replicating to mature DNA does not differ significantly.

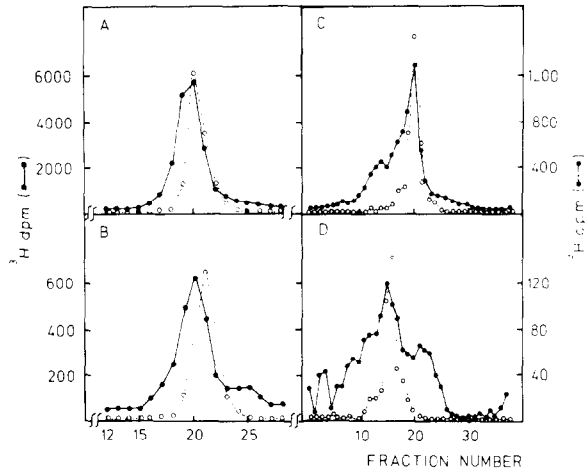

FIG. 3. Sucrose gradient sedimentation and CsCl equilibrium density centrifugation of H5ts125 DNA pulse-labeled under restrictive conditions. DNA samples from the experiment described in Fig. 2, labeled 15–20 min (A, C) or 60–65 min (B, D) after shift-up, were analyzed in the presence of ^{32}P-labeled mature Ad5-DNA (O——O). (A, B), CsCl density gradient profiles. The samples contained DNA isolated from 5×10^6 cells. The density difference was 0.005 g/cm^3 per fraction. Fractions were collected and counted directly (^{3}H dpm, ●——●). (C, D), Sucrose gradient profiles. The samples contained DNA isolated from 2×10^6 cells. Sedimentation was from right to left. Fractions were precipitated with TCA and counted (^{3}H cpm, ●——●).

A small amount of cellular DNA (ρ = 1.700 g/cm^3) was observed in the CsCl gradients (Figs. 3A and B, fractions 24–46), presumably due to the presence of low molecular weight cellular DNA which was not removed during the extraction procedure. Some slower sedimenting (20–25 S) DNA was observed during sucrose gradient analysis, most markedly in the 60-min sample (Fig. 3D). This slower-sedimenting DNA consisted partly (35%) of cellular and partly (65%) of viral DNA, as determined by CsCl equilibrium centrifugation of the 20–25 S DNA following preparative sucrose gradient centrifugation (data not shown). This indicates some breakdown of viral molecules at 39.5°.

The percentage of pulse-labeled replicating DNA for each sample was calculated from sucrose gradient sedimentation analysis and CsCl density gradient analysis and is summarized in Table 1. The two separation methods gave different amounts of replicating DNA, with lower values obtained in the case of CsCl gradients due to the lower sensitivity of this method in separating single-stranded and double-stranded DNA. It is clear, however, that accumulation of replicating DNA did not occur even when viral DNA synthesis was inhibited more than 90%.

These results suggest that chain propagation is not inhibited in H5ts125-infected cells at the restrictive temperature and that the cessation of viral DNA replication might be caused by a reduction in the number of molecules that initiate new replication rounds. The residual synthesis after shift-up may be due to the survival of a small amount of functional H5ts125 protein in the cell, even at 39.5°, which gives rise to a reduced amount of normal progeny H5ts125 DNA.

This notion is confirmed by hybridization experiments of newly synthesized H5ts125 DNA with the separated complementary strands of Ad5-DNA. The DNA synthesized in H5ts125-infected cells at the nonpermissive temperature hybridized to both the H- and L-strands, similar to DNA synthesized under permissive conditions, as illustrated in Table 2.

Completion of Replication Rounds in H5ts125-Infected Cells at the Nonpermissive Temperature

To test whether replication, once started at 32°, could be completed normally at

TABLE 1

AMOUNT OF REPLICATING DNA IN H5TS125-INFECTED CELLS PULSE-LABELED FOR 5 MIN AT VARIOUS TIMES AFTER A SHIFT-UP TO THE NONPERMISSIVE TEMPERATURE[a]

Time after shift-up to 39.5° (min)	Ratio of [^{3}H] thymidine incorporated, ts/wt	replicating DNA (%)	
		DNA >31 S	DNA >1.716 g/cm^3
0	1.00	66	36
15	1.10	60	29
30	0.31	53	35
60	0.10	67	32
120	0.08	63	39
240	0.06	66	28
Wild type (240)	–	65	31

[a] AGMK cells were infected at 32° for 44 hr and then shifted to 39.5°. Cells were pulse-labeled for 5 min with [^{3}H]thymidine at 39.5° at the indicated time. The DNA was centrifuged in neutral sucrose gradients in the presence of ^{32}P-labeled Ad5-DNA and the percentage of total [^{3}H]DNA sedimenting faster than the marker (>31 S) was calculated. In a similar way the percentage of [^{3}H]DNA with a density >1.716 g/cm^3 was calculated from centrifugation of the samples in CsCl gradients. The ratio of incorporation of mutant (ts) to wild type (wt) at time zero was set at 1.00.

39.5°, pulse–chase experiments were performed. Wild-type- or H5ts125-infected KB cells were incubated at 32° and labeled for 12 min at 32° with [^{3}H]thymidine during the period of active viral DNA replication. The radioactive label was then chased at 39.5°. DNA samples, obtained directly after the pulse and at different times during the chase were analyzed by sedimentation in neutral sucrose gradients (Fig. 4).

In DNA isolated immediately after the pulse, 50–60% of the label sedimented as replicating intermediates ahead of the marker. During the chase the replicating DNA disappears and the amount of mature 31 S DNA increased markedly. The flow of label from fast sedimenting to 31 S DNA occurred at an equal rate in wild-type- and H5ts125-infected cells (Fig. 5B). The samples were also analyzed in CsCl density gradients and the percentage of label in dense replicating intermediates was calculated (Fig. 5A). Also according to this method of analysis, replicating DNA in a mutant-infected cells was converted into mature viral DNA similarly to wild-type DNA.

The methods used in the experiments described above are based upon the single-stranded character of replicating adenovirus DNA. From these results it cannot be concluded that single-strand breaks or small gaps are still present in mutant-infected cells after the shift-up in temperature, due to incomplete ligation of 12 S "Okazaki"-pieces (Vlak *et al.*, 1975). To test this possibility, the viral DNA from cells 60 min after the chase was analyzed under alkaline conditions. Cells were mixed with ^{32}P-labeled Ad5-DNA and lysed on top of an alkaline sucrose gradient. The results show (Fig. 6) that the DNA was free of single-stranded breaks and sedimented at the same position as the genome-length marker DNA. A similar result was obtained with wild-type-infected cells. It is therefore concluded that the DNA completed at 39.5° in H5ts125-

TABLE 2

HYBRIDIZATION OF H5TS125 DNA SYNTHESIZED AT 32° OR 39.5° WITH SEPARATED VIRAL DNA STRANDS[a]

^{3}H-DNA in solution	Radioactivity (dpm input)	Hybridization with	
		H-strand (%)	L-strand (%)
H5ts125, 32°	13,025	11.2	10.7
H5ts125, 39.5°	4,175	12.9	9.8
Wild type, 39.5°	21,750	12.7	12.0

[a] [^{3}H]DNA was isolated from AGMK cells infected for 40 hr at 32° and subsequently labeled with [^{3}H]thymidine for 4 hr at 32° or for 2 hr at 39.5°, beginning half an hour after a shift-up in temperature. The amount of viral DNA synthesized at 39.5° in mutant-infected cells was 9% of that in wild-type-infected cells. The DNA was further purified by buoyant density equilibrium centrifugation in CsCl and 0.25 μg in 1 ml was incubated with nitrocellulose filters containing 0.25 μg of H- or L-strand Ad5-DNA. The H-strand is defined as the heavy strand in alkaline CsCl. The hybridization is expressed as percentage of input [^{3}H]DNA.

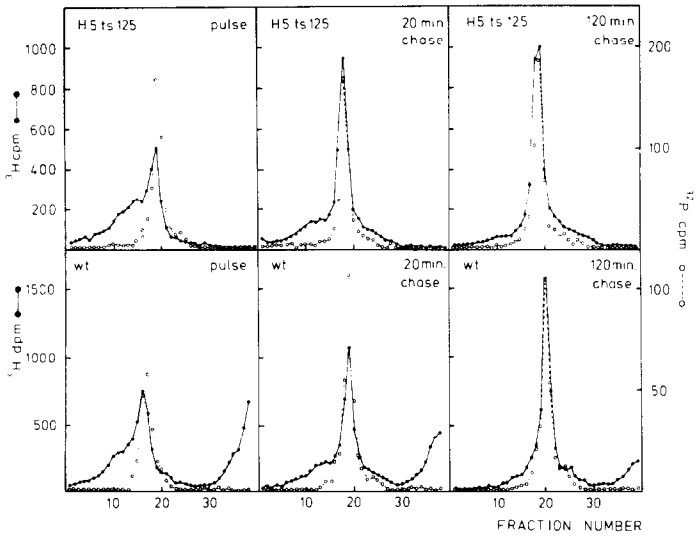

FIG. 4. Sucrose gradient sedimentation analysis of viral DNA pulse-labeled at 32° and chased at 39.5°. H5ts125 or wild-type-infected KB cells were incubated at 32° for 40 hr and labeled for 12 min at 32° with [^{3}H]thymidine. The radioactive label was then chased at 39.5° (see Materials and Methods), and DNA was isolated either immediately after the pulse or at different times during the chase. ^{32}P-labeled Ad5-DNA was added, and the samples were analyzed by sucrose gradient centrifugation for 16 hr at 25,000 rpm at 4° in an SW 41 rotor. Sedimentation was from right to left. In the case of H5ts125, fractions were precipitated with TCA and counted (cpm). The fractions from gradients containing wild-type DNA were counted directly and expressed as dpm. The total amount of label increased 1.7-fold between the pulse and the 120-min chase in H5ts125-infected cells and 1.6-fold in wild-type-infected (wt) cells.

infected cells is indistinguishable by these criteria from wild-type DNA.

Although these results strongly indicate that chain propagation is not impaired at the nonpermissive temperature, it might be argued that inactivation of the H5ts125 gene product is slow. It is possible that replication rounds are completed before inactivation of the protein has occurred. To investigate this possibility, cells were pulse-labeled for 10 min at 39.5°, 60 min after the shift to that temperature. At that moment the DNA synthesis is already strongly inhibited. The pulse-label was chased at 39.5°, and the DNA was analyzed as described above. The results, summarized in Figs. 5C and D, show that also under these conditions replicating DNA was converted to mature DNA at about the same rate in wild-type- and H5ts125-infected cells. Some slower-sedimenting DNA (18–22 S), consisting partly of viral and partly of cellular DNA, was also observed in this experiment, but the amount of this material did not change during the chase, in contrast to fast sedimenting DNA or 31 S DNA. It is concluded that even under conditions where the H5ts125 gene function was already inactivated, chain propagation could occur normally.

Density Labeling of H5ts125 Infected Cells with Bromodeoxyuridine at 39.5°

From the results presented above it appears that replicating molecules are completed at the nonpermissive temperature and that reinitiation of replicated molecules is a scarce event. The latter hypothesis was tested by density labeling experiments. H5ts125- or wild-type-infected cells were maintained at 32° for 40 hr followed by 8 hr at 39.5° in the presence of bromodeoxyuridine and [^{3}H]thymidine. Viral DNA was isolated and found to consist of more than 90% of 31 S molecules, with only a small amount of fast-sedimenting material. Analysis in CsCl density gradients (Fig. 7) showed that DNA of hybrid (H–L) and heavy (H–H) density was present in wild-type-infected cells at 39.5° and in

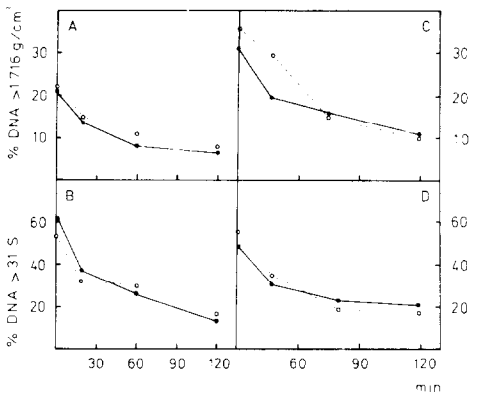

FIG. 5. Decrease in the relative amount of replicating intermediates during a chase at 39.5° of viral DNA, pulse-labeled at 32° (A,B) or 39.5° (C,D). H5ts125 (●——●)- or wild-type (O——O)-infected KB cells were incubated for 40 hr at 32°. The cultures were labeled with [^{3}H]thymidine for 12 min at 32° (A, B) or for 10 min at 39.5°, 1 hr after a shift from 32 to 39.5° (C, D). The radioactive label was chased, starting at $t = 0$ for various time periods. DNA was isolated and analyzed by CsCl density centrifugation (A, C) or sucrose gradient sedimentation (B, D) in the presence of ^{32}P-labeled marker DNA. From the gradient profiles the amount of replicating intermediates, expressed as percentage of total viral DNA sedimenting faster than 31 S or having a density higher than 1.716 g/cm^3, was calculated.

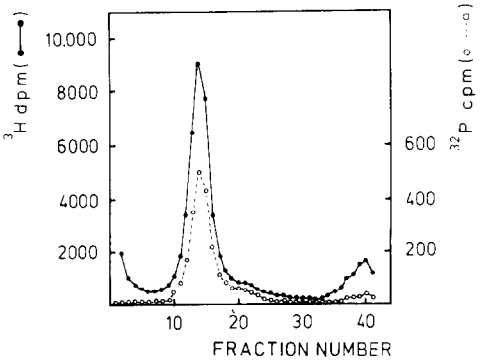

FIG. 6. Alkaline sucrose gradient analysis of H5ts125 DNA pulse-labeled at 32° and chased at 39.5°. H5ts125-infected AGMK cells were labeled for 12 min at 32° with [^{3}H]thymidine and chased at 39.5°. After 60 min the cells were washed and suspended in 0.2 ml of 0.01 *M* Tris/0.01 *M* EDTA, pH 7.5. ^{32}P-labeled Ad5-DNA was added and the cells were mixed with an equal volume of 0.3 *M* NaOH/1 *M* NaCl/0.01 *M* EDTA 2% SDS on top of a 5–27% isokinetic sucrose gradient containing 0.3 *M* NaOH and a 0.5-ml cushion of CsCl (1.700 g/cm^3). After 30 min the lysed cells were centrifuged for 16 hr at 10° at 25,000 rpm in an SW 41 rotor. Fractions were collected and counted.

H5ts125-infected cells at the permissive temperature. The first appearance of heavy DNA took about 3 hr to occur at 32°, which is much longer than the time required to replicate one Ad-DNA molecule. This indicates the existence of a pool of light (thymine-containing) DNA from which molecules were randomly picked to replicate and which was only slowly filled with H-L molecules.

No heavy DNA was observed in H5ts125-infected cells at 39.5°. Instead, the DNA banded between the light and hybrid position with a peak at 1.742 g/cm^3 (Fig. 7). This result is consistent with the notion that replicating molecules complete their replication round at 39.5°, thereby incorporating bromouracil in the remaining part of the daughter strands to be replicated. The absence of reinitiation in these replicated molecules, although in agreement with an initiation block in H5ts125 at 39.5°, might also be caused by the de-

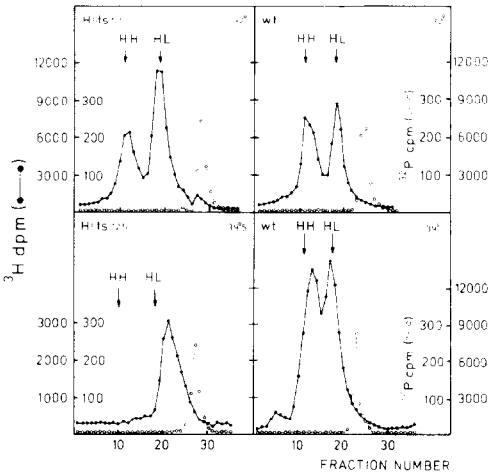

FIG. 7. CsCl density gradient centrifugation of H5ts125 or wild-type (wt) DNA labeled with 5-bromodeoxyuridine at 39.5°. H5ts125- or wild-type-infected AGMK cells were incubated for 40 hr at 32°, followed by a shift to 39.5° in half of the cultures. After 30 min the cells received 5-bromodeoxyuridine (10^{-5} *M*), 5-fluorodeoxyuridine (10^{-6} *M*) and [^{3}H]thymidine (2 μCi/ml, 4×10^{-8} *M*). Incubation was continued for 8 hr at 32 or 39.5°. Viral DNA was analyzed in CsCl density gradients in the presence of ^{32}P-labeled Ad5-DNA. The positions of viral DNA containing bromouracil in one strand (HL, $\rho = 1.752$ g/cm^3) or both strands (HH, $\rho = 1.788$ g/cm^3) were calculated from the refractive indexes.

creased chance of reinitiating an H–L molecule in the large pool of L–L molecules. Another possible explanation of these results is that the instability of the H5ts125 DNA binding protein in turn unstabilizes viral DNA, resulting in excision and repair of the viral genome later after the shift in temperature. This repair synthesis may thus account for the prolonged low level of DNA synthesis at 39.5°.

Characteristics of Viral DNA Synthesized in H5ts36-Infected Cells After Shift-up

As shown in Fig. 2, H5ts36 is a DNA-defective mutant that responds very slowly to a shift to the nonpermissive temperature. For a characterization of the viral DNA synthesized after a shift-up, we have determined the amount of labeled replicative intermediate in H5ts36-infected cells after 30 min of incubation in the presence of [^{3}H]thymidine at different times after the shift to the restrictive temperature. Wild-type-infected cells were pulse-labeled under identical conditions. The pulse-labeled DNA was isolated, subjected to CsCl density gradient centrifugation and the amount of labeled material banding heavier than mature Ad5-DNA was established (Table 3). No significant difference between the amounts of replicating DNA in wild-type- or H5ts36-infected cells was observed at any time after the shift-up. This indicates that no extensive inhibition of elongation occurs in H5ts36-infected cells, as was also found for H5ts125-infected cells. The absence of accumulation of replicating DNA suggests that H5ts36 may also be defective in initiation of replication. Pulse–chase experiments to confirm this notion are not appropriate in this case since the time to inactivate the H5ts36 gene product is far beyond the replication time. Density labeling experiments, similar to those described for H5ts125, showed the absence of material heavier than hybrid density under conditions where wild-type-infected cells synthesized H–H DNA (data not shown).

TABLE 3

AMOUNT OF REPLICATING DNA IN H5TS36- OR WILD-TYPE-INFECTED CELLS AT VARIOUS TIMES AFTER INCUBATION AT 32 AND 39.5°[a]

Time after shift-up to 39.5° (hr)	Percentage replicating DNA			
	wt		H5ts36	
	32°	39.5°	32°	39.5°
2	25	26	23	28
4	23	17	18	21
12	18	21	18	20

[a] AGMK cells were infected at 32° for 44 hr and then shifted to 39.5° or maintained at 32°. Cells were labeled for 30 min with [^{3}H]thymidine; at the time indicated DNA was isolated and centrifuged in CsCl density gradients in the presence of ^{32}P-labeled Ad5-DNA and the percentage of [^{3}H]DNA with a density greater than 1.716 g/cm^3 was calculated.

DISCUSSION

In this paper we have presented evidence showing that the H5ts125 gene product of Ad5 is required for the initiation of viral DNA replication and not for chain propagation or ligation of small "Okazaki-like" pieces (Okazaki *et al.*, 1968; Vlak *et al.*, 1975) to unit-length DNA. DNA synthesis in KB or AGMK cells, shifted from 32 to 39.5°, does not stop immediately, but inhibition is observed after 15–30 min, which is close to the time required for completion of one round of Ad5-DNA replication (Pearson and Hanawalt, 1971). Such a behavior is expected for an initiation mutant, assuming that the time required to inactivate the thermosensitive gene product is short. A similar conclusion was reached for H5ts125 and H5ts149 by Ginsberg *et al.* (1974).

From pulse–chase experiments we conclude that the rate of chain propagation is not decreased in H5ts125-infected cells at 39.5°. This conclusion has further been substantiated by the observation that viral DNA synthesis in isolated nuclei, obtained from H5ts125-infected cells, did not differ in thermosensitivity from wild type. In such a nuclear system, replicating Ad5-DNA molecules can be completed but reinitiation of new replication rounds does not occur (van der Vliet and Sussenbach, 1972). However, when H5ts125-infected cells were first shifted to 39.5° for 60 min, followed by measurement of viral DNA synthesis in isolated nuclei, a strong inhibition was observed (van der Vliet, Zand-

berg, and Jansz, manuscript in preparation). This confirms that the amount of replicating intermediates was largely reduced after a shift-up. Although all these data indicate that chain elongation occurs normally at 39.5°, it cannot be stated for sure that the H5ts125 gene product is not required for chain elongation since we cannot exclude that inactivation of the H5ts125 gene product occurs only after termination of replicating molecules. Another possibility is that the H5ts125 gene product, when involved in chain growth, is stabilized or much more slowly inactivated than the H5ts125 protein in an initiation complex.

The replication of adenovirus DNA is highly asynchronous. Based upon electron microscopical data, we have put forward the hypothesis that DNA replication starts at the right molecular end, copying the parental L-strand and at the same time displacing the H-strand. This so-called displacement synthesis leads to replicating intermediates containing a single-stranded H-strand, since the conversion of the H-strand to a duplex (called complementary-strand synthesis) is considerably delayed (Sussenbach *et al.*, 1972; Ellens *et al.*, 1974). Initiation of displacement synthesis apparently requires the H5ts125 gene product, but we have no evidence for a similar involvement of this protein in the initiation of the complementary-strand synthesis. A block in the initiation of the complementary-strand synthesis would lead to accumulation of single strands from parental origin. However, parental DNA, labeled for 2 hr at 32° followed by a chase at 39.5° up to 10 hr, did not remain single stranded but was normally converted to mature DNA (unpublished results). This shows that initiation of complementary-strand synthesis and displacement synthesis are not necessarily linked. An indication for such a different initiation of the H- and L-strand synthesis was obtained previously by studying viral DNA replication after release from inhibition by hydroxyurea (Sussenbach and van der Vliet, 1973). Under those conditions, displacement synthesis but not complementary-strand synthesis occurred.

The cessation of viral DNA replication after shift-up is readily reversible, even when the synthesis of new H5ts125 protein is blocked by addition of cycloheximide. This might be explained by the presence of a large excess of ts125 proteins, which are reversibly inactivated at 39.5° and, being nonfunctional, are slowly broken down by a proteolytic process. Such a process of inactivation has been proposed for the gene products of other Ad5 temperature-sensitive mutants (Russell *et al.*, 1974). A large excess of H5ts125 protein is in agreement with a stoichiometric role in virus replication (Austin, Young and Williams, unpublished observations).

Recently we have shown that, in H5ts125-infected cells, thermosensitive DNA binding proteins are synthesized (van der Vliet *et al.*, 1975). These proteins consist of a 72,000-MW component and a mixture of four proteins with MW varying from 45,000 to 50,000. The peptide maps of each of these proteins are most compatible with the notion that the 45,000–50,000-MW mixture consists of proteolytic breakdown products of the 72,000-MW protein (Rosenwirth and Levine, personal communication). Thus, it is plausible that the H5ts125 gene product is identical to the 72,000-MW DNA binding protein.

This early adenovirus-specific protein binds preferentially to single-stranded DNA and is synthesized in large amounts in the infected cells (van der Vliet and Levine, 1973). It is also able to bind to replicating intermediates of adenovirus DNA *in vitro*, presumably to the single-stranded DNA that originates as a result of chain growth. Therefore, it is somewhat surprising to find that chain propagation is not inhibited under conditions that inactivate the DNA binding properties of the 72,000-MW protein. We can not exclude that binding to single-stranded DNA occurs *in vivo* without an effect on the rate of chain elongation, serving only to protect single-stranded DNA against nuclease action. The small amount of viral 20–22 S DNA observed during pulse-labeling after shift-up might be an indication for such a protection.

The role of the adenovirus DNA binding

protein in initiation has still to be elucidated. It might serve to unwind the double helix at the origin since proteins that bind specifically to single-stranded DNA, like the T_4 gene-32 protein, have "unwinding" properties (Alberts and Frey, 1970). The adenovirus DNA binding protein binds equally well to single-stranded adenovirus DNA as to ϕX174 DNA indicating the absence of sequence specificity *in vitro*. Although recognition of the base sequence in the origin *in vivo* cannot be ruled out completely, it seems more likely that specific binding to the origin is dictated by interaction with other initiation proteins. A possible candidate for this function is the H5ts36 gene product. The density labeling experiments with H5ts36 show the absence of a second round of replication, while no accumulation of replicating DNA was observed at the nonpermissive temperature. These results are in agreement with a block in initiation. The slow inhibition of viral DNA synthesis after a shift-up might be explained either by a pool of stable complexes of H5ts36 protein and DNA or by slow inactivation of the protein. The latter hypothesis seems more likely since another mutant from this group, H5ts149, starts to shut off immediately after shift-up, but with slower kinetics than H5ts125. (Ginsberg *et al.*, 1974). Analysis of this mutant or other mutants from this group will be needed to clarify this point.

DNA-minus mutants have also been isolated for Ad12 (Shiroki *et al.*, 1972; Ledinko, 1974; Ginsberg *et al.*, 1974) and Ad31 (Suzuki *et al.*, 1972). These mutants may also be required for the initiation of Ad12- and Ad31-DNA synthesis (Shiroki and Shimojo, 1974). Further analysis of the specific role of these gene products in the initiation of Ad5-DNA replication is now in progress.

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