Characterization of Virus DNA Synthesized in KB Cells Infected with Two Temperature-sensitive Mutants of Adenovirus Type 5

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SUMMARY

KB cells were infected with H5ts36 or H5ts125, two adenovirus type 5 (Ad5) mutants with a temperature-sensitive synthesis of virus DNA. Infection was started at the nonpermissive temperature and at 16 h p.i. the temperature was shifted down to the permissive temperature. Shortly after the shift-down H5ts125-infected cells showed an accumulation of purely single-stranded DNA of virus origin, which was not observed in H5ts36-infected cells. This single-stranded DNA has been characterized by hybridization and sedimentation analysis. It was found that the single-stranded DNA was derived from both complementary strands and consisted of short fragments. The observation that the single-stranded DNA accumulates in H5ts125-infected cells under conditions in which the amount of DNA binding protein is reduced, suggests that the DNA-binding protein is not only involved in initiation, but also in elongation of nascent strands.

INTRODUCTION

Although the mechanism of replication of adenovirus DNA has been elucidated to a large extent (for a review see Levine et al. 1976), our knowledge of the proteins involved in DNA replication is still very poor. The only well-characterized protein important for DNA replication is the virus coded DNA binding protein (BP; van der Vliet & Levine, 1973; Linné et al. 1977; Sugawara et al. 1977). Analysis of mutant H5ts125, which shows a temperature-sensitive DNA replication, has revealed that its BP is thermolabile (van der Vliet et al. 1975) and that in its initiation of DNA replication is affected (Ginsberg et al. 1974; van der Vliet & Sussenbach, 1975). Recently, evidence was obtained suggesting that this protein is also involved in chain elongation (van der Vliet et al. 1977).

Besides the class of mutants including H5ts125, another complementation group of DNA negative mutants has been found of which H5ts36 is a representative (Wilkie et al. 1973). Preliminary evidence was obtained that this mutant is also affected in its initiation of replication (van der Vliet & Sussenbach, 1975). A third class of early mutants is represented by H2ts206. This mutant is affected in a very early function in virus reproduction (Kathmann et al. 1976). In Ad12 three classes of temperature-sensitive mutants have been isolated; all of them probably have a temperature-sensitive initiation of DNA replication (Shiroki & Shimojo, 1974). Except for H5ts125 and one of the classes of the Ad12 mutants, it is not known which virus protein has the temperature-sensitive character in any of the other mutants. This communication describes experiments in which virus DNA was synthesized in H5ts125- as well as in H5ts36-infected cells shortly after a shift from the nonpermissive to the permissive temperature. In contrast to H5ts36-infected cells, under these conditions...
H5tsI25-infected cells produce high amounts of purely single-stranded virus DNA. Further analysis of the newly synthesized DNA leads to the suggestion that BP is not only involved in initiation but is also required for appropriate chain elongation.

METHODS

Growth of cells and virus. Human KB cells were grown in monolayer as described earlier (Sussenbach & van der Vliet, 1972). H5tsI25, originally isolated by Ensinger & Ginsberg (1971), was kindly supplied by Dr H. S. Ginsberg and H5ts36 by Dr J. F. Williams. These mutants were propagated at 32 °C (van der Vliet & Sussenbach, 1975).

Conditions of labelling and isolation of virus DNA. KB cells were infected in suspension at a concentration of 5 x 10^5 cells/ml with H5tsI25 or H5ts36 at a multiplicity of infection (m.o.i.) of 2000 to 3000 physical particles per cell (Sussenbach et al., 1973). After 16 h incubation at 40 °C, cells were concentrated to 10^7 cells/ml and incubated further at 32 °C for various periods of time in the presence of radioactive thymidine (methyl-3H-thymidine, 52 Ci/mmol, 100 µCi/ml, Radiochemical Centre, Amersham, England). In a control experiment, cells were maintained at 40 °C in the presence of radioactive thymidine. Virus DNA synthesized was selectively extracted according to a modified Hirt procedure (van der Vliet et al., 1977). The DNA was further purified by sucrose gradient and CsCl gradient centrifugation as described below. This purified DNA was used for the analysis of the single-stranded DNA as well as for the restriction enzyme analysis described in the Results section. The incorporation of radioactivity in DNA was determined by precipitation with 5% trichloroacetic acid (TCA) as described by van der Vliet & Sussenbach (1972).

Centrifugation and chromatography. Neutral and alkaline sucrose gradient velocity centrifugation as well as CsCl equilibrium density centrifugation were performed as described before (van der Vliet & Sussenbach, 1972). Hydroxylapatite (HAP) chromatography was performed according to Tolun & Pettersson (1975). Hybridization was performed according to Sussenbach et al. (1976) with purified r- and l-straands, kindly provided by U. Pettersson.

Restriction enzyme analysis. A restriction enzyme from Haemophilus suis (HsuI) was kindly provided by R. Schiphof. HsuI recognizes the same nucleotide sequence as Hind III (Sambrook et al., 1975). Digestions with HsuI were performed in 0.006 M-tris-HCl, 0.006 M-MgCl₂, 0.006 M-β-mercaptoethanol and 0.04 M-NaCl (pH 7–5). Further details on the purification of DNA fragments, on gel electrophoresis and the HsuI restriction enzyme map of Ad5 DNA are described by Sussenbach & Kuijk (1977).

Determination of DNA-binding protein. Three x 10^7 cells were washed with phosphate buffered saline, suspended in RSB buffer (20 mM-tris-HCl, pH 7.6, 0.01 M-NaCl, 1.5 mM-MgCl₂ and 2 mM-β-mercaptoethanol) to a concentration of 2 x 10^7 cells/ml, sonicated for 3 to 4 min and made to 0.6 M-NaCl. The suspension was centrifuged for 20 min at 10000 rev/min and to the supernatant EDTA was added to a concentration of 10 mm and glycerol to a concentration of 10%. These extracts were used in a radioimmuno-inhibition assay to determine the amount of BP. For this assay BP and rabbit anti-BP-antiserum were prepared as described by van der Vliet et al. (1977) and purified BP was labelled in vitro with 3H-NaBH₄ (10 Ci/mmol, INCO) as described by Rice & Means (1971) to a specific activity of 2 x 10^8 d/min ³H/µg. About 1 to 50 µl of an unlabelled extract or of a reconstruction mixture of a known amount of purified unlabelled BP was added to an extract of uninfect KB cells (1 mg protein/ml) to a final vol. of 100 µl. To these solutions 25 µl of phosphate buffered saline (5x), Triton 5%, desoxycholate 2.5% and SDS 0.5% [PBSTDS (5x)] and 250 ng of rabbit anti-BP-γG in phosphate buffered saline were added. After 1 h at 4 °C, 12 ng of the highly labelled BP were added and the incubation was continued overnight.
**Adenovirus DNA replication**

![Graph of Adenovirus DNA replication](image)

Fig. 1. Incorporation of \(^{3}H\)-thymidine in mutant-infected KB cells after a shift-down of the temperature to the permissive temperature. The conditions of labelling are described in Methods. ○ --- ○, H5ts36, 32 °C; ●—●, H5ts125, 32 °C; □, H5ts36, 40 °C; ■, H5ts125, 40 °C.

Next day, 10 μg of rabbit γG and 200 μl of goat anti-rabbit antiserum were added. After incubation for 2 h at 4 °C, the immunoprecipitates were collected by centrifugation for 15 min at 10000 rev/min, washed twice with PBSTDS (1x), dissolved in 0.1 M-NaOH and counted for radioactivity. Without inhibitor 30 to 35% of the highly labelled BP was precipitated. The inhibition of the extracts was compared with the inhibition of the reconstruction mixtures. Controls were included for non-specific reactions.

**RESULTS**

**Kinetics of virus DNA synthesis**

KB cells were infected in suspension with either H5ts36 or H5ts125 and incubated at 40 °C for 16 h as described in Methods. The cells were then shifted to the permissive temperature (32 °C) and were further incubated in the presence of tritiated thymidine. A part of the suspension was kept at 40 °C in the presence of tritiated thymidine to determine the degree of leakage of virus DNA synthesis at the nonpermissive temperature. Samples were taken at various times after shift-down and the amount of incorporation is shown in Fig. 1. In both mutant-infected cells DNA synthesis starts immediately after shift-down with almost the same rate, while cells kept at the non-permissive temperature show a highly reduced incorporation.
Fig. 2. Neutral sucrose gradient centrifugation of $^3$H-labelled DNA synthesized after shift-down of H5ts36-infected cells to the permissive temperature. Samples containing equal amounts of label were analysed after 10, 22, 45 and 75 min of synthesis, respectively. Centrifugation was as indicated in Methods and is from right to left. The arrows indicate the 31S position (mature molecules).

Fig. 3. Neutral sucrose gradient centrifugation of $^3$H-labelled DNA synthesized after shift-down of H5ts125-infected cells to the permissive temperature. Samples were analysed after 10, 45, 75 and 90 min of synthesis, respectively. Centrifugation was as indicated in Methods. The arrows indicate the 31S position (mature molecules). In contrast to the other samples, the DNA synthesized during a 10 min-pulse after shift-down was not dialysed prior to centrifugation. The crude DNA preparation therefore still contained unincorporated $^3$H-thymidine leading to high amounts of radioactivity at the top of the gradient.
Adenovirus DNA replication

Fig. 4. CsCl gradient centrifugation of ³H-labelled DNA synthesized after shift-down of H5ts36-infected cells to the permissive temperature. Samples were taken at 10, 22, 45 and 75 min after shift-down. The dashed lines indicate the density gradient; V and C refer to the positions of virus and cellular DNA, respectively. Conditions of infection, isolation of ³H-DNA and conditions of centrifugation are indicated in Methods. The control consists of material isolated from infected cells which were maintained at 40 °C and labelled for 10 min.

Isolation and sedimentation analysis of new DNA

At various times after shift-down virus DNA was isolated according to a modified Hirt procedure and this DNA was analysed by sucrose and CsCl gradient centrifugation. The results of the sucrose gradient centrifugations are shown in Fig. 2 and 3. Comparison of the sedimentation profiles of new DNA from H5ts36- and H5ts125-infected cells, respectively shows a marked difference. All profiles show a major peak at 31S, the position of mature virus DNA molecules and faster sedimenting DNA representing virus replicative intermediates. However, there is far more fast-sedimenting material in H5ts125-infected cells than in H5ts36-infected cells, especially at 45 min after shift down. Further, the sedimentation profiles of DNA isolated from H5ts125-infected cells show a considerable amount of radioactive material sedimenting more slowly than 31S (Fig. 3), especially at 45 min after shift-down. DNA samples were also examined by CsCl gradient centrifugation (Fig. 4 and 5). Again, a striking difference between the banding profiles of the two mutants was observed. Almost all DNA synthesized after shift-down is of virus origin. However, while the H5ts36-infected cells contain material that bands at the virus position (1·716 g/ml) or somewhat heavier (0·002 to 0·005 g/ml), the H5ts125-infected cells contain material banding at the virus position, but also a considerable amount of DNA banding at the position of single-stranded virus DNA (1·732 g/ml). The DNA synthesized at 40 °C is almost completely of cellular origin.

These results lead to the surprising conclusion that at the start of the first round of replication in H5ts125-infected cells, after a shift to the permissive temperature, a considerable amount of purely single-stranded labelled DNA is produced, even during periods of
replication considerably shorter than the replication time of a DNA molecule at 32 °C which is about 60 min (Sussenbach & Kuijk, 1978). This led us to a further characterization of the new DNA synthesized under the conditions of shift-down in H5ts36- and H5ts125-infected cells.

**Synchronization of initiation of replication**

To determine whether the shift-down procedure leads to a real synchronous initiation of the first round of replication, the new DNA synthesized at various times after shift-down was isolated and subjected to restriction enzyme analysis employing the restriction endonuclease Hsul. This type of analysis allows us to test whether the incorporated ³H-thymidine is indeed present predominantly in the terminal fragments which contain the origins of replication (Ariga & Shimojo, 1977; Sussenbach & Kuijk, 1978). The previously described analysis of the H5ts125-infected cells has shown that the above procedure of a shift-down does lead to initiation of the first round of replication (Sussenbach & Kuijk, 1978). Similar analysis of the DNA synthesized in H5ts36-infected cells may lead to the same conclusion for the latter mutant. Fig. 6 shows the relative labelling of the Hsul fragments of DNA labelled for various periods after shift-down of H5ts36-infected cells to the permissive temperature. This figure indicates that shortly after the shift-down the termini are preferentially labelled, while at later times the differences between the termini and the centre of the molecule decrease. These data on H5ts36 and the results obtained previously with H5ts125 (Sussenbach & Kuijk, 1978) show that both mutants are in the first round of DNA replication during the first 60 min after the shift-down.
Adenovirus DNA replication

Fig. 6. Specific radioactivity of *H*, fragments of Ad5 DNA labelled for various periods of time (min) after shift-down of H5ts36-infected cells to the permissive temperature. 3P-labelled marker DNA was used as a reference. The specific activity is expressed as the ratio *H*/3P, while the ratio of the I fragments is arbitrarily set at 1.0. ●—●, 10 min sample; □—□, 45 min sample; △—△, 75 min sample. Conditions of isolation of the DNA and of the restriction enzyme analysis are indicated in Methods.

Table 1. Percentage of *H*-labelled single-stranded DNA in virus DNA synthesized in mutant-infected cells after shift-down*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Time (min)</th>
<th>Percentage prior to sonication</th>
<th>Percentage after sonication</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5ts125</td>
<td>10</td>
<td>26</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>ND†</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>46</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>25</td>
<td>43</td>
</tr>
<tr>
<td>H5ts36</td>
<td>10</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>

* Virus DNA was isolated at various times after shift-down as described in Results. Samples were sonicated for 2 min with a Branson sonicator at maximal output and subjected to HAP chromatography as described in Methods. The fraction eluting with 0.15 M-phosphate represents the purely single-stranded DNA, while the double-stranded DNA-containing fraction is eluted with 0.4 M-phosphate.
† ND: not determined.

Characterization of the single-stranded DNA accumulated in mutant-infected cells

Virus DNA isolated from mutant-infected cells was purified by sucrose gradient and CsCl gradient centrifugation as described above. The amounts of purely single-stranded new DNA as well as the total amount of exposed labelled single-stranded DNA in replicative intermediates were determined by hydroxylapatite (HAP) chromatography. Samples of new DNA isolated at various times after shift-down were analysed immediately on HAP or first sonicated and then fractionated on a HAP column. The percentages of labelled single-stranded DNA before and after sonication are shown in Table 1. These results show that in H5ts125-infected cells labelled single-stranded DNA accumulates and reaches a maximum
Table 2. Strand specificity of labelled single-stranded DNA*

<table>
<thead>
<tr>
<th>Strand added</th>
<th>10 min</th>
<th>22 min</th>
<th>45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>47</td>
<td>51</td>
<td>53</td>
</tr>
<tr>
<td>l</td>
<td>64</td>
<td>58</td>
<td>67</td>
</tr>
<tr>
<td>—</td>
<td>22</td>
<td>28</td>
<td>28</td>
</tr>
</tbody>
</table>

* The purely single-stranded DNA obtained after HAP chromatography of non-sonicated DNA samples from H5ts125-infected cells 10, 22 and 45 min after shift-down, respectively, was hybridized in solution with an excess of the r- or l-strand, respectively (0.5 μg in a final volume of 100 μl) for 16 h at 68 °C as described by Sussenbach et al. (1976). After hybridization, the percentage of hybridization of the radioactive DNA was determined by HAP chromatography. After binding of the DNA to the HAP column, the single-stranded fraction was eluted with 0.15 M-phosphate, while the double-stranded fraction was eluted with 0.4 M-phosphate (see Methods).

Fig. 7. Alkaline sucrose gradient centrifugation of 3H-labelled purely single-stranded DNA isolated from H5ts125-infected cells 10 and 75 min after shift-down to the permissive temperature. After a Hirt extraction, the DNA was fractionated by HAP chromatography and the purely single-stranded fraction was layered on top of an alkaline sucrose gradient (see Methods). Centrifugation is from right to left.

Fig. 7. Alkaline sucrose gradient centrifugation of 3H-labelled purely single-stranded DNA isolated from H5ts125-infected cells 10 and 75 min after shift-down to the permissive temperature. After a Hirt extraction, the DNA was fractionated by HAP chromatography and the purely single-stranded fraction was layered on top of an alkaline sucrose gradient (see Methods). Centrifugation is from right to left.

value 45 min after shift-down. At that time 65% of the new DNA behaves as single-stranded DNA after sonication, while at the time before sonication, 46% of the label is present in a purely single-stranded form. On the other hand H5ts36-infected cells show a relatively low percentage of material which behaves as single-stranded DNA and remains almost constant. The origin of this single-stranded DNA is unclear; the amount is close to the background (5%) observed in this procedure with purely double-stranded DNA.

Analysis of the single-stranded DNA in H5ts125-infected cells

The exposed single-stranded DNA present at 10, 22 and 45 min after shift-down respectively was further characterized by hybridization to separated r- and l-strands (Table 2). The exposed DNA hybridizes with almost equal efficiency with the two strands, indicating that the single-stranded DNA is derived from both complementary strands. The DNA shows a very limited self-hybridization suggesting that the single-stranded r- and l-sequences are derived from different parts of the genome.

The purely single-stranded DNA isolated prior to sonication was also analysed by
**Adenovirus DNA replication**

Table 3. Presence of DNA binding protein in wild type and H5ts125-infected cells

<table>
<thead>
<tr>
<th>Time after shift-down (min)</th>
<th>DNA binding protein (μg/5 × 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>60</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* The amount of DNA binding protein was determined as indicated in Methods.

alkaline sucrose gradient centrifugation (Fig. 7). It appears that at 10 min after shift-down the single strands are considerably shorter (average 13S) than strands of genome size (34S). The size distribution does not change considerably at longer times, although the absolute amount of purely single-stranded DNA decreases.

**Presence of DNA binding protein after shift-down of H5ts125-infected cells**

From earlier communications it was known that H5ts36 produces normal amounts of the adenovirus-specific DNA binding protein at the non-permissive temperature, while H5ts125-infected cells contain very reduced amounts of binding protein (Van der Vliet et al. 1975). To determine how fast the synthesis of BP starts after the shift-down, samples of cells were analysed for the presence of the DNA binding protein at 0 and 60 min after shift-down respectively. The amount of BP in H5ts125-infected cells was compared with the amount in wild type-infected cells. The results are shown in Table 3. It appears that the pool of immunologically reactive DNA binding protein is very low at the non-permissive temperature and that it takes several hours to return to wild type level after shift-down (see also Ginsberg et al. 1977). It should be emphasized that this immunological determination of BP does not allow conclusions on the amount of functional BP.

**DISCUSSION**

One of the typical features of the replication of Ad5 DNA is the formation of single-stranded DNA (Sussenbach et al. 1973; Sussenbach et al. 1976). Flint et al. (1976) have shown that, although all regions of the adenovirus DNA are represented in the single-stranded DNA, not all regions are represented at equal frequencies. In contrast to the studies mentioned above, which describe the analysis of single-stranded DNA synthesized late in infection, we have investigated the DNA in the first rounds of replication using two mutants ts in their DNA replication, namely H5ts36 and H5ts125. Analysis of new DNA synthesized in H5ts125-infected cells after a shift-down to the permissive temperature revealed that shortly after shift-down within the first round of replication, purely single-stranded molecules are produced, which are derived from both strands and have a length of about 10% of genome size. The production of these short single strands is specific for mutant H5ts125 and is not found in cells infected under similar conditions with another initiation mutant, H5ts36. The low level of BP in H5ts125-infected cells in the first 60 min after shift-down is probably the major cause of the production of these single strands. The present results can be explained by assuming that the very low pool of DNA binding protein does allow initiation of replication but leads to a destabilization and malfunctioning of the replication machinery, which is demonstrated by premature displacement of growing single strands. This assumption is in agreement with recent studies with antisera against the BP which have provided evidence that this protein is probably involved in elongation of nascent strands (Van der Vliet et al. 1977). The question remains how the growing strands are displaced prematurely. From previous studies (for a review, see Levine et al. 1976) it
was concluded that Ad5 DNA replication starts at both ends of the DNA molecule and proceeds by displacement of parental strands (displacement synthesis). The displaced parental strands are replicated at a later stage during so-called complementary strand synthesis. One of the proteins required for initiation of the displacement synthesis is the DNA binding protein (Van der Vliet & Sussenbach, 1975).

Since initiation might be performed with a single BP molecule, the frequency of this process is probably not affected by a low pool of the BP, while a low pool apparently disturbs the process of elongation. The most likely explanation for the accumulation of purely single-stranded DNA in H5ts125-infected cells after a shift-down to the permissive temperature is that the low pool of BP leads to branch migration during the displacement synthesis, resulting in release of newly synthesized short strands. At longer times after shift-down the pool of BP reaches its normal level and replication can proceed normally without the accumulation of single-stranded DNA. The above explanation is in agreement with the general notion that DNA binding proteins stabilize the replication fork and protect exposed single-stranded DNA.

In conclusion, the experiments with H5ts36 and H5ts125 have suggested that low levels of BP lead to an aberrant elongation of nascent strands, indicating that this protein is not only involved in initiation, but also in chain elongation.

We wish to thank R. Schiphof and Dr U. Pettersson for providing HsuI and separated strands, respectively, and Dr P. C. van der Vliet for providing DNA binding protein and antiserum against the DNA binding protein. We gratefully acknowledge stimulating discussions with Drs H. S. Jansz and P. C. van der Vliet. This investigation was supported in part by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organization for the Advancement of Pure Research.

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