In Vitro Polyoma DNA Synthesis: Studies on an Early Temperature-Sensitive Mutant

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Received for publication 9 July 1973

The polyoma ts-a function was investigated by using an in vitro DNA-synthesizing system. A comparison of systems derived from ts25 (a ts-a group mutant) and ts1260 (a late group mutant)-infected cells showed that the activation energies for DNA chain elongation and the mechanisms of discontinuous growth were identical for both mutants.

The function of one class of early mutants of polyoma has been shown to be continuously required for viral DNA synthesis (3). This class of 14 temperature-sensitive mutants includes the ts-a mutant (4), as well as ts25 (1; W. Eckhart, unpublished observations). Phenotypically, this class displays a shut-off in viral DNA synthesis upon a shift from permissive (32°C) to nonpermissive (39°C) temperature, although the virus-induced cellular DNA synthesis is unaffected by the shift-up (3). The ts-a function appears to be necessary for initiation of each new round of viral DNA replication (3). Such conclusions are inferred from the negative evidence that the overall rates of processing of mutant replicative DNA to mature viral DNA are similar to that of wild-type replicative DNA. Minor differences in the detailed mechanism of chain growth would not have been detected by these in vivo studies. We have therefore used an in vitro DNA-synthesizing system from polyoma-infected BALB/3T3 cells (6) to examine possible differences between ts25 (ts-a class) and ts1260 (a late mutant which makes normal amounts of viral DNA at the nonpermissive temperature). Since viral DNA synthesis in this system occurs predominantly on pre-existing replicative intermediates (6), the results reported here bear on the steps involved in chain elongation during one single round of DNA replication.

Figure 1 shows the time course of incorporation of [3H]TTP into viral and cellular DNA in vitro at 32 and 39°C by lysates derived from ts1260- and ts25-infected BALB/3T3 cells. The cells were either maintained at 32°C or shifted to 39°C for 2 h before preparation of the system. In the case of ts25 infection, it is clear that the shift to 39°C drastically reduced incorporation into the viral DNA fraction in vitro. This was confirmed by sucrose gradient analysis of the in vitro product (Fig. 2). The lysate from cells maintained at 32°C made 20S viral DNA, whereas the viral DNA fraction from the lysate of cells shifted to 39°C showed radioactivity only in very low-molecular-weight DNA with no evidence of 20S viral DNA. On the other hand, lysates from both shifted and nonshifted ts1260-infected cells showed good incorporation into 20S viral DNA (Fig. 2). There was no indication of a shut-off in viral DNA synthesis in vitro when lysates from ts25-infected cells maintained at 32°C were incubated at 39°C. The extent of cellular DNA synthesis in lysates from ts25-infected cells was the same whether or not a shift to the nonpermissive temperature had been made, which corresponds with the situation in vivo. Analysis of the in vitro product synthesized at 32°C by ts25- and ts1260-infected cell lysates showed that approximately half (50 and 60%, respectively) was mature form I viral DNA.

The majority of the replication cycle of polyoma DNA is probably carried out by cellular functions, with a virus-specific event required only for the initiation step (3, 8). For this reason, one would expect the temperature dependence of DNA chain elongation to be the same in ts25- and ts1260-infected cell lysates. We have confirmed this in two ways. First, from the initial rates of DNA synthesis in ts25- and ts1260-infected cell lysates at four temperatures, we determined the activation energy of both viral and cellular DNA synthesis (Table 1). The activation energies for viral DNA synthesis with both ts25 and ts1260 and for cellular DNA synthesis in both cases were all very similar. This result implies that the ts-a muta-
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Fig. 1. Time courses of in vitro incorporation of [3H]TTP into viral and cellular DNAs. BALB/3T3 cells were infected at 32°C with either ts25 or ts1260 at a multiplicity of approximately 20. At 41 h postinfection in the case of ts25 or 36 h in the case of ts1260, half of the plates were shifted to 39°C for 2 h (32°→39°C), while the other half were left at 32°C (32°). Lysates were prepared as described (6) from all four sets of cells, and 50-μl samples were incubated in vitro under conditions optimal for DNA synthesis (6) at either 32°C (●) or 39°C (■) for the times shown. Viral and cellular DNAs were fractionated by the selective extraction technique of Hirt (5) and were assayed separately for incorporation of [3H]TTP (6).

**TABLE 1. Determination of the activation energies for viral and cellular DNA synthesis**

<table>
<thead>
<tr>
<th>Cell lysate infected with</th>
<th>Activation energy (Cals)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Viral DNA synthesis</td>
</tr>
<tr>
<td>ts1260</td>
<td>-19</td>
</tr>
<tr>
<td>ts25</td>
<td>-20</td>
</tr>
</tbody>
</table>

* Duplicate 50-μl samples of lysates from ts25- and ts1260-infected cells maintained at 32°C were incubated at 22, 28, 32, and 37°C for 3 min as described (6). [3H]TTP incorporation into viral and cellular DNA was measured. The energy of activation was determined from the slope of a plot of log (rate of incorporation) against 1/T°K according to the Arrhenius equation $K = A e^{-E_r/RT}$.

Fig. 2. Sucrose gradient analysis of in vitro products. Samples of the Hirt supernatant from the lysates of the four sets of cells (described in Fig. 1) that had been incubated at 32°C for 60 min were subjected to neutral sucrose gradient sedimentation (6). The arrows indicate the positions of [3P]-marker viral DNAs (form I and form II). Sedimentation was from right to left.

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does not result in an altered activation energy for elongation-type DNA synthesis. Second, viral DNA synthesis has been shown to occur at least partly in a discontinuous fashion (2, 7; B. Francke and T. Hunter, unpublished observations) by a process which is thought to reflect the general mechanism of DNA replication in eukaryotic cells. By using short pulses of [α-32P]dCTP together with a chase of unlabeled dCTP, we examined the generation of short DNA pieces (S-S) and their joining into longer DNA chains in ts25-infected cell lysates. Both processes appear to proceed equally well at 32°C and 39°C (Table 2). These findings confirm that the lesion in the ts-a class of mutants does not affect any of the processes thought to be involved in discontinuous chain growth. In pulse experiments of this kind, the viral DNA fraction (Hirt supernatant) for ts25 contained, independent of the temperature of incubation, up to fivefold more labeled, free, single-stranded DNA pieces of cellular origin than labeled replicative intermediate. It is unlikely that this was due to a ts25-specific effect. Rather, it probably was a consequence of the lower amount of viral DNA replication for this mutant in relation to cellular DNA synthesis.

Initiation of new rounds of viral DNA synthesis occurs to a very limited extent in vitro, if at all (6). The complete shut-off of viral DNA synthesis in ts25-infected cells takes 30 to 60 min at 39°C (3), so that the failure to observe the shut-off in vitro is attributable not only to the lack of initiation, but also to the inability of the lysate to maintain viral DNA synthesis for an extended period. The time required to inactivate the ts-a function at 39°C is not known, and therefore it cannot be excluded that during in vitro incubation at 39°C an active gene product was present. Yet the failure to detect differences
in the activation energy and the pattern of discontinuous synthesis at 39 C extends the negative evidence of the in vivo results (3, 8) to the detailed mechanism of chain elongation.

Attempts to inactivate the ts-a gene product by preincubation of the ts25-infected cell lysates at 39 C in the absence of DNA synthesis (e.g., in the presence of 1-β-d-arabinofuranosyl cytidine triphosphate or EDTA) followed by reversal of the block have failed because of the extreme sensitivity of the system to preincubation in the absence of DNA synthesis (6). Lysates from ts25-infected cells shifted to 39 C for 2 h did not spontaneously recover and synthesize viral DNA when incubated at 32 C in vitro. However, because the recovery of measurable viral DNA synthesis for this mutant takes at least 4 h in vivo (T. Hunter and B. Francke, unpublished observations), this is not a surprising result. Since the shut-off state is relatively stable in vitro, we have attempted to complement such lysates with extracts from ts1260-infected cells without success. We attribute this failure to the deficiency of initiation in the in vitro system.

Since the majority of viral DNA synthesis observed in vitro represents completion and maturation of pre-existing replicative intermediates, our results show that, for the first cycle of viral DNA synthesis after exposure to the non-permissive temperature in vitro, there is no detectable impairment in the processes involved in DNA chain elongation and viral DNA maturation. At present, we are trying to obtain an in vitro system which will initiate new rounds of viral DNA synthesis in order to further our studies of the ts-a gene function.

**LITERATURE CITED**