Initiation Points for DNA Replication in Nontransformed and Simian Virus 40-Transformed BALB/c 3T3 Cells

ARIELLA OPPENHEIM AND ROBERT G. MARTIN*

Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Maryland 20014

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The number of initiation points for DNA synthesis per unit length of DNA in rapidly growing cells is greater for simian virus 40-transformed than for nontransformed BALB/c 3T3 cells.

BALB/c 3T3 cells, like other nontransformed cells, stop growing at confluence, enter a resting state in depleted medium, and fail to grow in soft agar (2, 6, 7, 12, 14, 19–21, 24, 25, 28). These cells are nonpermissive for the replication of simian virus 40 (SV40), but become "transformed" when SV40 DNA integrates into their DNA (5). The transformed cells form dense foci on a normal monolayer, continue growing in depleted medium, and form clones in soft agar. If the integrated SV40 contains a temperature-sensitive mutation in the A gene, which encodes the T-antigen (1, 13, 22, 23, 26–28), then the transformed cells lose their transformed phenotype when incubated at the temperature that is nonpermissive for the taA mutation. The T-antigen is thus required for maintenance of the transformed phenotype. The T-antigen is also required for the formation of viral replicative intermediates during lytic infection of permissive monkey kidney cells; i.e., T-antigen is also required for the initiation of each round of viral DNA synthesis (9, 24). It has therefore been proposed that transformation is related to the aberrant initiation of host DNA synthesis (6, 7, 15, 17, 18, 20, 21, 24, 25).

Our model for this aberrant initiation (15, 17, 18) suggests that the T-antigen starts host DNA synthesis at new sites, "T-sites"; therefore, in rapidly growing transformed cells, DNA synthesis is initiated both at T-sites and at "N-sites," sites that normally act as initiation points for DNA synthesis. Support for this model has been obtained by the demonstration that the initiation of DNA synthesis occurs at more sites in SV40-transformed than in nontransformed Chinese hamster lung (CHL) cells (6). We report here further experiments with transformed BALB/c 3T3 cells that show that our previous findings are not limited to CHL cells.

Because there is no technique for the direct measurement of the number of initiation sites for mammalian DNA synthesis, we used the method of DNA fiber radioautography of Hand and Tamm (10) and Huberman and Riggs (11), which allows the direct visualization of replication forks radioactively labeled during a short pulse. From the radioautographs, we estimated the average distance between replication forks in rapidly growing and starved cells. Since DNA replication in mammalian cells is bidirectional (3, 8, 10, 11), the average distance between initiation sites is twice the distance between forks.

From these data the number of initiation points can be determined, since the number of initiation points per unit length of DNA is inversely proportional to the distance between initiation points.

Asynchronous BALB/c 3T3 cells at 34°C, growing rapidly in Dulbecco-Vogt medium or starved in isoleucine-depleted medium for 48 h, were treated with $2 \times 10^{-6}$ M 5-fluoro deoxyuridine for 30 min prior to pulse-labeling with $[^3H]thymidine (50 mCi/µmol; 500 µCi/ml) for 10 min. Cells were grown and labeled both on slides and in flasks. The slides were fixed and covered with photographic emulsion to determine the percentage of labeled nuclei. The cells in flasks were removed from the monolayer by treatment with trypsin-EDTA, lysed, spread on slides in triplicate for radioautography, and exposed for 6 months at -20°C. To avoid bias in scoring the radioautographs, the analysis was performed in a double-blind manner. The slides were coded for us by Carol A. F. Edwards. One of us then took 24 photomicrographs of each slide of transformed and nontransformed cells in complete and depleted medium (i.e., 28 microscopic fields were photographed). The slide code was recorded on the backs of the photographs. The other one of us measured the distance between the center of the tracks in each photograph and, after recording the distances, turned over the photograph and recorded the
code letter. The criteria for choosing tracks to measure and the method of analysis of the intertrack distances have been presented in detail (16). The data were decoded only after completion of the recording and analyses.

Approximately half of the cells were in the S-phase when the pulse-labeling was performed on rapidly growing cells (Table 1). A similar percentage of the transformed cells were in the S-phase even after 48 h of starvation for isoleucine. This was expected since transformed cells are unable to enter a resting state in depleted medium (19). On the other hand, less than 10% of the nontransformed cells were in the S-phase after 48 h of isoleucine depletion. We had expected even a lower percentage of S-phase cells than the 9.7% found because 3T3 cells presumably enter a resting state in isoleucine-depleted medium and because only 3% of CHL cells are in the S-phase after only 24 h of isoleucine depletion (16, 19). We have subsequently learned that 3T3 cells do not readily enter a resting state upon depletion for isoleucine (A. Yen and A. Pardee, personal communication). We have not yet determined why the percentage of S-phase cells in the 3T3 line was not lower or whether the cells undergoing DNA synthesis were randomly distributed in the S-phase.

Intertrack distances were analyzed as previously described (16). As previously observed (3, 8, 10, 11, 16), the distributions of the frequencies of intertrack distances were not Gaussian; rather, the distributions were skewed to longer intertrack distances. The data were therefore analyzed by log-normal (geometric) distributions as previously described (16). The results are given in Table 2.

The results are similar, but not identical, to those obtained with CHL cells (16). Specifically, the geometric mean distances between replicating forks in SV40-transformed cells in depleted medium were long: 16 µm in 3T3 cells versus 22 µm in CHL cells. The geometric mean distances between forks in rapidly growing nontransformed cells were of intermediate lengths: 14 µm in 3T3 cells versus 17 µm in CHL cells. The geometric mean distances between forks in rapidly growing SV40-transformed cells were short: 12 µm in 3T3 cells versus 14 µm in CHL cells.

One difference between the two cell types concerned nontransformed cells incubated in isoleucine-depleted medium. The geometric mean intertrack distance in the nontransformed CHL cells in depleted medium was the same as in complete medium, whereas the average intertrack distance in the nontransformed 3T3 cells in depleted medium was greater than in complete medium. This observation may be related to the fact that only 3% of the nontransformed CHL cells were in the S-phase after 24 h in isoleucine-depleted medium, whereas 9.7% of the nontransformed 3T3 cells were in the S-phase even after 48 h. Two possible explanations are that: (i) 3T3 cells are already "partially" transformed (4), and (ii) 3T3 cells are partially synchronized by treatment with isoleucine-depleted medium for 48 h and intertrack distances vary during the S-phase. Whatever the reason for the long mean intertrack distance in nontransformed 3T3 cells in isoleucine-depleted medium, the results clearly indicate (P < 0.005) that the geometric mean intertrack distance, and hence average replicon size, in rapidly growing SV40-transformed cells is less than that for rapidly growing 3T3 cells.

### Table 1. Percentage of nuclei labeled in 10 min by [³H]thymidine

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Growth condition (medium)*</th>
<th>% Nuclei labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3</td>
<td>Complete</td>
<td>42.2</td>
</tr>
<tr>
<td></td>
<td>Depleted</td>
<td>9.7</td>
</tr>
<tr>
<td>3T3WTB1a</td>
<td>Complete</td>
<td>55.2</td>
</tr>
<tr>
<td></td>
<td>Depleted</td>
<td>40.5</td>
</tr>
</tbody>
</table>

* Complete medium is Dubelco-Vogt medium supplemented with 10% fetal bovine serum. Depleted medium contains no isoleucine and is supplemented with 2.5% serum.

** The nontransformed BALB/c 3T3 cells and the wild-type SV40-transformed cell line 3T3WTB1a were obtained from William Brockman.

### Table 2. Geometric mean intertrack distances and pairwise t tests between means for nontransformed and SV40-transformed 3T3 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Growth condition (medium)</th>
<th>Geometric mean intertrack distance (µm)</th>
<th>No. of intertrack distances measured</th>
<th>3T3 (depleted medium)</th>
<th>3T3WTB1a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Complete</td>
<td>Depleted</td>
</tr>
<tr>
<td>3T3</td>
<td>Complete</td>
<td>14.1 ± 2.9</td>
<td>388</td>
<td>&lt;0.001</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>Depleted</td>
<td>16.5 ± 3.0</td>
<td>448</td>
<td>&lt;0.001</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>3T3WTB1a</td>
<td>Complete</td>
<td>12.3 ± 3.1</td>
<td>483</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Depleted</td>
<td>16.0 ± 3.3</td>
<td>334</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
growing nontransformed cells. Thus, there are more initiation points for DNA synthesis per unit length of DNA in randomly growing SV40-transformed than in nontransformed BALB/c 3T3 cells.

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LITERATURE CITED