REPLICATION OF MAMMALIAN DNA IN VITRO

Evidence for Initiation from Fiber Autoradiography

ROGER HAND and JOHANNES R. GAUTSCHI

From the Department of Medicine, McGill University, Montreal, Quebec H3A 2B4, Canada, and the Pathology Institute, University of Bern, Bern 3010, Switzerland

ABSTRACT

We have used fiber autoradiography to examine the DNA product made in vitro in a lysed cell system. CHO cells were treated with 0.01% Brij-58 and the lysates were incubated at 30°C in a complete reaction mixture for in vitro DNA synthesis with [3H]thymidine triphosphate ([3H]TTP) as the radioactive tracer. Fiber autoradiograms prepared from the DNA showed that it was synthesized on tandemly arranged replication units that were of average size of 20 μm, very similar to the size of units found in vivo. The rate of replication fork movement was 25-50% of the in vivo rate. More than 80% of forks stopped functioning by 15 min, and 95% stopped by 60 min. This suggests that synthesis is halted by premature terminations. Evidence for new initiations was provided by replication units with labeled origins in DNA synthesized in an in vitro reaction in which radioactivity was omitted for the first 10 min of incubation. This, plus the observations that the distance between initiation points (replication unit size) is not increased and that premature termination accounts largely for the cessation of synthesis, suggest that significant initiation takes place in this in vitro replication system.

KEY WORDS
in vitro DNA synthesis
DNA fiber autoradiography
in vitro initiation
replication unit size
replication fork movement rate

In vitro synthesizing systems have been of immense value in the study of DNA replication in prokaryotic cells (25) and papovavirus-infected mammalian cells (21). Mammalian cellular DNA replication has also been studied in vitro but not to the same extent. A variety of systems have been developed in which exogenous deoxynucleoside triphosphates are used as the direct precursors of cellular DNA; these include permeabilized cells (1, 2, 5, 20), lysed cells (7, 19), isolated nuclei (14, 18, 23), and chromatin (17, 22). In most of these, DNA synthesis has been shown to be semiconservative and a continuation of the in vivo process. However, it rarely persists for more than 2 h and <10% of the genome replicates. There is also little evidence for initiation of new synthesis. Therefore, a legitimate question to ask is how faithfully do the in vitro systems reflect in vivo DNA replication?

One approach to answer this is the use of fiber autoradiography. This technique, invented by Cairns (3) and applied to intact mammalian cells by him (4) and by Huberman and Riggs (16), has allowed the demonstration of individual replication units in chromosomal DNA, of bidirectional replication on these units, and of synchronous initiation of synthesis on neighboring units. In addition, it has provided the most direct measurements of unit size and rate of replication fork movement (6).

In the present work, we have used fiber au-
radiography to study in vitro DNA replication in a lysed cell system. The DNA product in this system has been characterized by biochemical techniques (9, 19). Synthesis is semiconservative and a continuation of the in vivo process. Okazaki pieces are made and incorporated into DNA the size of replication units. The initial rate of synthesis is ~30% of the in vivo rate, and up to 3% of the genome is replicated within a 60-min incubation period. In addition, there is some evidence for initiation of new chain synthesis.

The results reported here provide additional evidence for initiation, and show as well that in vitro synthesis involves the progressive elongation of nascent chains on tandemly arranged replication units that are the same size as those found in vivo. The factor limiting the continuation of synthesis in this subcellular system appears to be premature termination of chain growth.

MATERIALS AND METHODS

Cells

The CHO cell line provided by Dr. Robert Painter (University of California, San Francisco) was grown and maintained in monolayer as previously described (8).

In Vivo Labeling Conditions

The cells were treated with fluoro-deoxyuridine (2 × 10⁻⁶ M) for 30 min before in vivo labeling with [³H]-thymidine. Fluoro-deoxyuridine remained in the medium during the radioactive pulses. The concentrations of radioactivity during the pulse labeling periods were adjusted to correspond to those used for labeling in vitro, and are indicated in the legends for the figures and tables.

Preparation of Lysed Cells and In Vitro Labeling Conditions

The procedure for the preparation of the in vitro DNA synthesizing system from lysed cells has been described (19). In brief, the cells were lysed by treatment with 0.01% Brij-58. The nuclei with large amounts of attached cytoplasm were centrifuged through a buffer solution to free them from detergent and resuspended in a complete reaction mixture for sustaining DNA synthesis that included four deoxynucleoside triphosphates, four ribonucleoside triphosphates, and an energy source. Radioactive tracer was provided as [³H]thymidine triphosphate ([³H]TTP) of high specific activity at the concentrations indicated in the legends for the figures and tables.

Preparation and Analysis of Autoradiograms

The autoradiograms were prepared from lysed cells or whole cells as previously described (12). After an exposure of 4 mo, they were analyzed by light microscopy. For those in vitro experiments in which the lengths of DNA fibers were measured, autoradiograms from the in vitro preparations and the in vivo controls were traced on clear acetate sheets from projections on a frosted glass screen at a magnification of 680. In experiments in which autoradiographic patterns were scored, preselected microscope fields were examined at a magnification of 400. The only exception to the above is the in vivo experiment reported in Table I in which the measurements were made with an eyepiece micrometer at a magnification of 400. This was done so that a direct comparison could be made between these data and those from other cell lines collected in this way and reported previously (10, 11).

Details as to the number of measurements made are reported with the individual experiments.

RESULTS

In Vitro Incorporation of [³H]TTP

The incorporation of TTP into DNA at 30°C in CHO cells lysed with low concentrations of Brij-58 is shown in Fig. 1. It is nearly linear for the first 30 min and continues at a rapid rate through 60 min. There is then a progressive decrease in incorporation through 120 min. These results are very similar to those from earlier studies (19).

In Vivo Replication at 30°C

The incubation temperature of 30°C was chosen...
for these experiments because the earlier work on the biochemical characterization of the DNA product had been at this temperature. Since this is lower than the 37°C that has been used in all other fiber autoradiographic analyses in mammalian cells, it was worthwhile to examine the patterns of autoradiograms in CHO cells in vivo at 30°C.

Cells were labeled for 30 or 60 min with [3H]-thymidine of high specific activity and then for an additional 45 min with [3H]thymidine of low specific activity. The cells were then lysed and the DNA was spread and fixed for autoradiography. In general, the appearance of the fiber autoradiograms prepared from cells labeled at 30°C was the same as from cells labeled at 37°C (not illustrated). Replication initiated at the centers of tandemly arranged units and proceeded bidirectionally toward outlying termini where fusion with the daughter chains on neighboring units took place.

The step-down labeling protocol resulted in two distinct replication patterns: prepulse, in which a central gap was flanked by two high-density tracks leading to low-density tracks indicating a unit that initiated replication before the radioactive pulse, and postpulse, in which a central high-density track was flanked by two low-density tracks indicating a unit that initiated after the beginning of the pulse of high specific activity. These patterns were used to make the measurements of DNA replication presented in Table I. The rate of replication fork movement is <0.3 μm/min. This is lower than the rates that have usually been found in mammalian cells at 37°C (6) and is most probably a direct result of the lowered temperature. The median length of replication units is between 30 and 35 μm, in good agreement with previous estimates for CHO cells (15, 16). The larger values for the mean lengths reflect the usual asymmetric distribution of replication unit size with the skew toward larger sizes (13). The degree of synchrony of initiation of adjacent replication units found in these cells is also similar to that found in other mammalian cells (10, 11). Neither replication unit size nor initiation synchrony appears altered by lower temperature.

The difference in the values found with pulses of 30 and 60 min deserves comment. With the longer high specific activity pulse, there is more fusion of adjacent tracks. Since the fused tracks have not had the chance to elongate during the entire pulse, they yield an apparent lower rate of fork progression. The 60-min pulse also gives longer tracks allowing recognition of fibers with more widely spaced origins. This accounts for the larger apparent size of replication units. The higher expected proportion of pairs of units with like patterns results from an increase in postpulse units relative to prepulse units, since the 60-min pulse gives more time for new initiations; this also causes the higher observed proportion of units with like patterns.

### In Vitro Replication

In the first set of experiments in which we prepared fiber autoradiograms from DNA synthe-

<table>
<thead>
<tr>
<th>Duration of high specific activity pulse</th>
<th>Rate of fork movement, μm/min</th>
<th>Replication unit length, μm</th>
<th>Initiation synchrony: pairs of units with like patterns, proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>SEM</td>
<td>xi ± SEM</td>
<td>Observed</td>
</tr>
<tr>
<td>30</td>
<td>0.28 ± 0.011</td>
<td>31.5 ± 1.14</td>
<td>0.75</td>
</tr>
<tr>
<td>60</td>
<td>0.23 ± 0.012</td>
<td>35.0 ± 4.31</td>
<td>0.84</td>
</tr>
</tbody>
</table>

* Cells were labeled with [3H]thymidine of high specific activity (50 Ci/mmol, 500 μCi/ml) for the indicated times and then with [3H]thymidine of low specific activity (5 Ci/mmol, 500 μCi/ml) for 45 min.

† High-density tracks from prepulse autoradiograms were measured and the mean length was divided by the duration of the high specific activity pulse. Each sample contained 100 tracks.

§ Center-to-center intervals between adjacent replication units were measured. Each sample contained 50 measurements.

‖ To obtain the observed proportions, autoradiograms containing two units were scored as to whether they showed like patterns (both prepulse or both postpulse), or unlike patterns (one prepulse and one postpulse). Expected proportions were calculated using the formula (p + q)^2 = 1, where p = proportion of prepulse units in the sample and q = proportion of postpulse units. Each sample contained 50 measurements, and the observed values are significantly different from the expect values (P < 0.005, χ² for goodness of fit).
sized in vitro, we used a protocol that called for pulses with [3H]TTP of high specific activity. The photomicrographs in Fig. 2 show that short tandemly arrayed stretches of DNA are labeled in vitro and that the size of the labeled stretches increases with longer pulse times.

The high background in the photomicrographs is probably due to unincorporated radioactive nucleotides enmeshed in the DNA gel that were not removed during the washing and fixing of the autoradiograms. The high background was noted only in areas of the microscope slides near the spread fibers; in areas of the slides without DNA fibers, the background was extremely low.

To determine whether there was progressive elongation of the DNA chains, we measured size of the labeled segments. As a control, we also measured the size of segments from intact CHO cells labeled in vivo with [3H]thymidine at 30°C according to the same protocol. The cumulative frequency distributions of these measurements are presented in Fig. 3. The distributions for all measurements are asymmetrical with skews towards larger sizes. As the pulse time increases from 15 to 60 min, the median size of the labeled stretches increases in vitro although not so rapidly or to so large a size as in vivo. From these results, it would appear that there is progressive elongation of nascent DNA chains in vitro.

**Rate of Replication Fork Movement In Vitro**

We next estimated the rate of DNA replication fork movement. This was done by plotting the mean or median values of each of the distributions in Fig. 3 vs. the time of the pulse and determining the line of best fit between the points. The slope gives the rate of increase in the size of the fragments, and this may be used, with certain reservations, as an estimate of the rate of fork move-
ment. These results are presented in Fig. 4. For in vitro DNA synthesis, there is a linear increase in the means and medians from 15 through 45 min, but with little further increase at 60 min. The estimated rate from the linear portion of both curves is 0.07 μm/min. In vivo, the increase in size is linear through 60 min. The estimated rate from the means is 0.17 μm/min and from the medians, 0.14 μm/min.

These values almost certainly underestimate the true rate of fork movement. With a protocol that has a radioactive tracer of a single specific activity, the labeled segments may be produced either by a single replication fork, if initiation began before the pulse, or by two replication forks moving in opposite directions from a common origin, if replication began after the start of the pulse. In the segments in which replication began after the start of the pulse, elongation will not have taken place during the entire pulse, and measurement of the segment length will underestimate the rate of fork progression. The degree of underestimation in vivo may be seen by comparing the values obtained from the slopes in Fig. 4b with the values in Table I, in which the estimates were made by measuring labeled segments produced by single replication forks from prepulse units identified by the step-down labeling protocol. The degree of underestimation will vary with the amount of initiation that takes place during the radioactive pulse. In experimental situations in which there is no initiation during the radioactive pulse, the slope is an accurate measure of the rate.

The slopes in Fig. 4a underestimate the rate of fork progression in vitro as well. Evidence will be presented below suggesting that some initiation takes place in vitro. We can still conclude from these data that there is a significant reduction in the rate of fork movement when DNA replication takes place under in vitro conditions; it is 50% of the in vivo rate if we compare it with the values from the slopes from Fig. 4b, and 25% of the in vivo rate if we compare it with the values in Table I.

**Replication Unit Size In Vitro**

We were also able to estimate the size of replication units from this experiment. We used the “half-replicon” method of Van't Hof and Bjerkness (24) in which the length of DNA fiber is measured and divided by the number of labeled segments in the fiber. This will estimate the average distance between replication forks and therefore give the size of half a replication unit. With longer pulse times, there is a progressive overestimation because of the increased number of single tracks produced by two replication forks, either from initiation at a common origin or fusion at a common terminus. This overestimation can be reduced by plotting the values and extrapolating back to zero pulse time to minimize the contribution of fused segments.

This calculation is presented in Fig. 5. There is a linear increase in the interval between segments for pulses of up to 60 min in vivo and up to 45 min in vitro. The lack of increase from 45 to 60 min in vitro most probably reflects the slowing down of the synthetic process that takes place by 60 min after lysis. Extrapolation back to zero pulse...
FIGURE 5 Replication unit length of DNA synthesized in vitro (■) and in vivo (○). From the tracings collected for the experiments in Fig. 3, the total length of DNA fiber in each of the samples was measured and divided by the number of segments in the sample. This was plotted vs. pulse time, and the line of best fit was calculated by regression analysis using the method of least squares. For the in vitro calculation, the 60-min point was not included, since the linear increase did not extend beyond 45 min. Extrapolation back to zero time gives an estimate of the average distance between replication forks or the size of half a replication unit (24).

Extrapolation back to zero time yields a half-unit estimate of 13 μm for in vivo replication and 10 μm for in vitro replication, and therefore unit sizes of 26 and 20 μm, respectively. The difference is not statistically significant. The in vitro value falls within the 95% confidence limits of the in vivo value, and the in vivo value falls within the 95% confidence limits of the in vitro value when student's t distribution is used. We conclude that the size of replication units is the same in vitro as in vivo.

The estimates obtained by this method are less than those obtained by the more standard center-to-center measurements from autoradiograms produced by the step-down protocol in Table I. We believe that the increased length of the labeled segments in the autoradiograms in the experiments in Table I allows the recognition of larger units as adjacent. Both methods yield reproducible results, and comparisons between control and experimental values with either are valid so long as it is realized that the results give relative rather than absolute sizes.

FIGURE 6 Fiber autoradiograms of DNA labeled in vitro with [3H]TTP of high then low specific activity. CHO cells were lysed and allowed to synthesize DNA as in Fig. 2 except that the pulses of [3H]TTP of high specific activity (50 Ci/mmol, 400 μCi/ml) of (a) 15 min, (b) 30 min, and (c) 60 min were followed by a pulse of [3H]TTP of low specific activity (4 Ci/mmol, 400 μCi/ml) for 45 min. The DNA was then spread and prepared for fiber autoradiography. The arrowheads indicate transitions from heavy to light grain density caused by the step-down in [3H]TTP specific activity. Bar, 50 μm. × 480.

Premature Termination In Vitro

In another group of experiments, we used a step-down protocol to label DNA synthesized in vitro. The lysed cells were pulsed sequentially with [3H]TTP of high then low specific activity. Replication forks operating during both pulses produced segments with heavy grain density tracks attached to tracks of lighter grain density. Examples are shown in Fig. 6. With high specific activity pulses of 15 min followed by low specific activity pulses of 45 min, segments containing both heavy and light stretches were frequent. As the time of the high specific activity pulse was increased to 60 min, the fraction of segments containing both heavy and light labels decreased. This suggested to us that many units stopped operating before 60 min elapsed and that some measure of the time that replication forks were active in vitro could be...
obtained by scoring the fraction of segments with both heavy and light density tracks. Using microscope fields in which the DNA fibers were well spread and separated, we scored labeled segments for whether they had uniform heavy density or both heavy and light density regions. Those with both were considered to have resulted from replication forks in operation beyond the end of the high specific activity pulse. The results are presented in Table II. In control cells labeled in vivo, >70% of the replicating forks were functional beyond 15 min and >60% operated beyond 60 min. In vitro, <20% were functional beyond 15 min, and this decreased to <5% with a 60-min pulse of high specific activity. The high proportion of tracks with just heavy density labeling cannot be explained by retarded fork movement since retardation would have produced shorter tracks that contained both high- and low-density regions. Rather, it seems likely there is premature termination of synthesis on the majority of replication units.

No attempt was made to quantitate the fraction of units with bidirectional replication. As shown in Fig. 6a, segments with heavy and light tracks were often adjacent to segments with heavy tracks. This type of pattern could arise from unidirectional replication or from bidirectional replication on a unit in which one of the forks terminated replication prematurely. If the mode of replication in vitro is bidirectional, this was much less obvious compared with in vivo replication, but it does not necessarily indicate that unidirectional replication was more common in vitro.

Since some of the in vitro segments with uniform heavy grain tracks could have been produced by fusion of chains from two adjacent replication forks, the fraction of forks operating for longer than the high specific activity pulse could be even lower than indicated in Table II. The results here suggest that premature termination of synthesis is a major cause of the shut-off of DNA synthesis in this in vitro system.

**In Vitro Initiation**

We also attempted to determine whether initiation of synthesis occurs in vitro. In these experiments, in vitro synthesis was allowed to proceed in the absence of radioactivity for 10 min. A pulse of [³H]TTP of high specific activity was then administered for 60 min and immediately followed with a low specific activity pulse for an additional 60 min. We reasoned that if we observed radioactive segments that contained a heavy grain density track flanked on both ends by tracks of lighter grain density (postpulse pattern), we could be reasonably sure that initiation occurred on those units in vitro. Autoradiograms with this pattern were relatively easy to find and several examples are shown in Fig. 7. We quantitated this by scoring the proportions of postpulse, prepulse, and indeterminate patterns. The results are shown in Table III. A small fraction of units showed patterns consistent with in vitro initiation. This suggests that there may be some new initiations taking place in vitro, although by this measure the number is low. We have no way of knowing what fraction of the indeterminate patterns represent in vitro initiation, since replication terminated on them before light-density tracks were produced.

**DISCUSSION**

We have examined in vitro DNA synthesis in mammalian cells by fiber autoradiography to determine whether the in vitro product resembles that synthesized in vivo. Our results show that it does insofar as daughter chains are synthesized on tandemly arranged replication units that are ~20 μm long and that these chains undergo progressive elongation. There are certain striking differences.
FIGURE 7 Fiber autoradiograms of replication units showing patterns consistent in vitro initiation. CHO cells were lysed and allowed to synthesize DNA as in Fig. 2 except that radioactivity was omitted from the reaction mixture for the first 10 min of the incubation; it was then added as [3H]TTP for 60 min at high specific activity (20 Ci/mmol, 200 μCi/ml), and for an additional 60 min at low specific activity (1.6 Ci/mmol, 200 μCi/ml). The large arrowheads indicate the presumed origins of the units and the small arrowheads, the transitions from heavy to light grain density caused by the step-down in [3H]TTP specific activity. Bar, 50 μm. x 600.

Table III

DNA Replication Unit Initiation In Vitro

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of tracks scored</th>
<th>Postpulse</th>
<th>Prepulse</th>
<th>Indeterminate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysed</td>
<td>825</td>
<td>3</td>
<td>5</td>
<td>92</td>
</tr>
<tr>
<td>Intact</td>
<td>339</td>
<td>47</td>
<td>28</td>
<td>25</td>
</tr>
</tbody>
</table>

3H concentrations during the [3H]TTP pulses in lysed cells and [3H]thymidine pulses in control intact cells were the same, 20 Ci/mmol and 200 μCi/ml during the pulse of high specific activity and 1.6 Ci/mmol and 200 μCi/ml during the pulse of low specific activity.

50 consecutive microscope fields from five slides in which the autoradiograms were well spread and separated were selected at a magnification of 100. The fields were subsequently examined at a magnification of 400, and autoradiograms were scored for the three types of patterns. Indeterminate patterns were tracks of uniform high grain density. Tracks suggesting unidirectional replication were <5% of the total and were not scored. Since they are also indeterminate with respect to initiation during the pulse of high specific activity, they would serve to lower slightly the percentage of pre- and postpulse patterns.

Additional evidence for initiation is provided by the results of this autoradiographic study. First, the size of replication units is the same in vitro and in vivo. Although adjacent units tend to initiate synthesis at the same time in vivo (10), there is a significant number of units initiating replication during the pulse interspersed among those initiating before the radioactive pulse, and the average size of replication units is in part determined by the distance between adjacent prepulse and postpulse units. If there were no initiations during the pulse in vitro, then the distance between origins would increase and there would be an apparent increase in the size of replication units. Since this was not found, it suggests that some in vitro initiation took place so that the interval between unit origins (and therefore the size of units) was maintained.

Second, over 80% of replication forks that were initially active cease operation in <15 min in vitro, and >90% have stopped by 30 min. Since incorporation in vitro at 30 min is still >10% of the initial rate, at least part of the synthesis at this time is due to in vitro initiation.

Third, postpulse initiation patterns were found in autoradiographic preparations in which the DNA had been incubated in vitro for 10 min in the absence of radioactivity before long radioactive pulses with [3H]TTP. The simplest explanation...
for these patterns is that they represent units on which initiation took place 10 min after the in vitro incubation began. The argument against this—that initiation took place in vivo and that the gap representing DNA replicated during the first 10 min in vitro is below the resolution of the autoradiographic technique—is probably not valid. The estimate of rate fork movement of 0.07 μm/min is a minimum, and the actual rate in vitro is probably somewhat higher. Using this minimum estimate and taking into account that the patterns indicate bidirectional replication, we calculate that the minimum gap at the center of these patterns after a 10-min incubation in the absence of isotope should be 1.4 μm. Gaps this size can be recognized easily at the magnification used to examine the autoradiograms in these experiments.

These results from the centrifugation studies of Gautschi et al. (9) and those of the autoradiographic studies presented here provide good evidence that significant initiation takes place in this in vitro system.

We believe then that the stoppage of in vitro synthesis does not result from lack of new initiations but rather from premature terminations. Another deficiency in the in vitro reaction is retarded fork movement. The slower increase in the size of in vitro labeled segments as the pulse time increases suggests that fork movement is retarded, but the estimate of only 25% of the in vivo rate may be too low. First, the method used to calculate the rate underestimates it in proportion to the amount of initiation that occurs. Second, segments that have prematurely terminated, and therefore have a zero rate during part of the radioactive pulse undoubtedly bias the result towards a lower calculated figure. Thus, the actual rate is probably closer to 50% of the in vivo rate. This is similar to the estimate of Fraser and Huberman (7) obtained by fiber autoradiographic analysis in a lysed cell system.

We cannot draw any conclusions as to whether the frequency of initiation is normal in vitro. The incidence of premature termination is so high under our experimental conditions that unambiguous postpulse patterns were relatively rare. Additional experiments are needed on this point under optimal conditions for in vitro synthesis.

These experiments were begun when R. Hand was a Visiting Scientist in the laboratory of J. R. Gautschi. Dr. Gautschis tragic death before the project was completed makes R. Hand solely responsible for analysis and interpretation.

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REFERENCES