

## TEMPORAL ORGANIZATION OF REPLICATION IN DNA FIBERS OF MAMMALIAN CELLS

BARBARA R. JASNY and IGOR TAMM. From The Rockefeller University, New York 10021. Dr. Jasny's present address is the Department of Microbiology, State University of New York at Stony Brook, Stony Brook, New York 11794

### ABSTRACT

The extent of coordinate control over the multiple initiation events in DNA replication has been investigated in three mammalian cell lines by DNA fiber autoradiography. Quantitative estimates have been obtained of the degree of synchrony among initiations occurring on stretches of DNA. Synchrony decreases markedly with increasing distance between initiation sites in MDBK (bovine) and L929 (mouse) cells, but only slightly in muntjac cells. Possible control mechanisms for the initiation process are discussed.

KEY WORDS DNA replication · initiation sites · temporal synchrony · DNA fiber autoradiography

Evidence for temporal programming of DNA replication in eucaryotic cells at the level of the chromosome was first obtained by Taylor (39) in the roots of *Crepis capillaris*. Studies in several systems, including Chinese hamster (15, 40) and human cells (8, 24, 34), have revealed a regional distribution of DNA replication along the chromosome (reviewed by Hand, reference 10). The activated regions appear to be related one to another in a characteristic temporal pattern. Latt (23) and Stubblefield (32, 33) have suggested that structural chromosome bands may be units of replication. The possibility of sub-bands, each containing a smaller number of origins, has been noted by Willard (41). Instead of the ~350 bands in the Paris nomenclature, there are 2,000–3,000 or more bands or chromomeres (reviewed by Comings, reference 4). As replication of DNA in mammalian cells in S phase is initiated at ~50,000–100,000 sites (37, 38), this would give an estimate of ~30 replication units for the mean number of coordinately controlled units/band.

We have previously demonstrated by DNA fiber autoradiography that the physical location of the initiation sites is determined in a nonrandom

manner (3, 17). DNA fiber autoradiographic studies have also suggested that the time of initiation of adjacent replication units on the double-stranded fiber is regulated in mammalian cells (16). Hand and Tamm (12) observed that light-microscope fields of autoradiographs mostly contained either units which had initiated before pulse labeling or units which had initiated during the pulse, but generally not both. Further evidence was presented by Hand (9), who demonstrated that the probability of finding two adjacent units which had initiated before addition of label (two adjacent prepulse figures) was greater than could be explained by a random distribution. It was concluded that adjacent units tend to initiate replication synchronously, but exceptions were noted (9). In a preliminary study of the temporal pattern of initiation events in L-cell DNA (18), we used the mean rate of replication fork progression to arrive at estimates of the timing of initiation events on stretches of DNA. The end of labeling with [<sup>3</sup>H]thymidine at high specific activity provides a reference point in time. We concluded that there was indeed a high degree of synchrony along DNA fibers in an exponentially growing population of L cells (18).

The rate of fork progression has been found to vary over a wide range in exponentially growing cells (11), which may in part be explained by

differences in the rate of fork progression during different stages of the S phase (13, 26). The mean rate of fork progression also varies among cell lines; the rate in muntjac cells is approximately one-half of that in L or MDBK cells (17). In the present study, we have calculated the rate separately for each replication unit activated before the beginning of hot pulse labeling (prepulse figures). This permits a more accurate determination of the time of initiation of replication at the multiple sites distributed over segments of DNA. We have thus been able to compare synchrony in three cell lines.

## MATERIALS AND METHODS

### *Cell Culture*

Three mammalian cell lines were used in monolayer cultures (3, 17, 18). Mouse L929 cells were grown in Eagle's minimal essential medium (6) supplemented with 5% fetal calf serum, whereas muntjac and bovine MDBK cells were grown in reinforced Eagle's medium (1) with 10% fetal calf serum.

### *Light-Microscope DNA*

#### *Fiber Autoradiography*

The autoradiographic procedure has been described in full (3, 17, 18). In summary, exponentially growing, asynchronous monolayer cultures were first treated with  $2 \times 10^{-6}$  M 5-fluoro-2'-deoxyuridine for 0.5 h to deplete the thymidine pool. Cells were then exposed to high specific activity [ $^3$ H]thymidine (50–60 Ci/mmol,  $4\text{--}6 \times 10^{-6}$  M) for 10 min. After this, the specific activity was lowered 10-fold by the addition of  $2 \times 10^{-4}$  M thymidine and incubation was continued for an additional 3 h. The cells were then lysed and the fibers were spread across slides with a glass rod. After drying, the DNA was precipitated with 5% TCA and the slides were processed for autoradiography.

## RESULTS

The labeling protocol used (10-min exposure to [ $^3$ H]thymidine, 50–60 Ci/mmol, followed by 3-h exposure to [ $^3$ H]thymidine, 5–6 Ci/mmol) gives rise to two types of figures: postpulse figures, in which replication was initiated during the period of high specific activity labeling, and prepulse figures, which initiated before labeling began. A prepulse figure is readily recognized as consisting of a central unlabeled stretch bordered by high grain density regions, and then by lightly labeled regions. An example of a prepulse figure is shown in Fig. 1. Prepulse initiation is assumed to have occurred at the center of the unlabeled stretch.

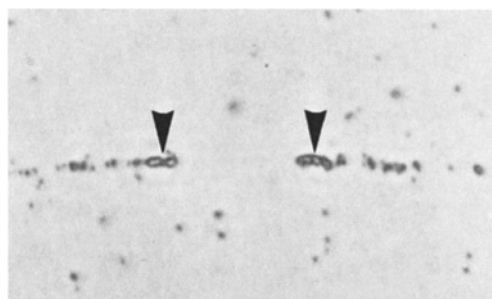


FIGURE 1 Prepulse figure. Initiation of bidirectional DNA replication occurred before the beginning of high specific activity labeling (note two high grain density regions indicated by arrows).  $\times 950$ .

The rate of chain elongation is determined by measuring the lengths of the internal hot pulse-labeled halves of each prepulse figure, and dividing the mean of the two values by the duration (10 min) of the high specific activity pulse. Only internal hot pulse lengths (i.e. at least  $3.4 \mu\text{m}$  from the ends of the strands) were included to avoid the possibility of breakage artifacts.

For each prepulse figure, it is also possible to determine the mean distance from the middle of the gap (the presumed initiation site) to the distal ends of the hot-labeled stretches. Dividing this distance by the rate of fork progression, measured as micrometers/minute, gives an approximation of the time interval between initiation and the end of the hot pulse. We shall refer to this interval as the initiation time of prepulse figures. The times calculated are limited only by the size of the gap which can be recognized as connecting two parts of the same strand.

The frequency distributions of the initiation times of prepulse figures in DNA from muntjac, MDBK, and L cells are shown in Fig. 2. For all three cell lines,  $>70\%$  of the initiations occurred within 30 min of the end of the hot pulse, with 90% of the initiations observed in each cell line occurring within 60 min of the end of the hot pulse.

For each strand, all the pairs of adjacent and nonadjacent prepulse figures were then examined. The absolute value of the difference in time of initiation within any pair is defined as the relative initiation time for this pair. The larger the relative initiation time, the farther apart in time the two sites initiated. Initiations occur with a high degree of synchrony when relative initiation time is low. Fig. 3 shows the frequency distributions of such synchrony determinations. Initiations occur most

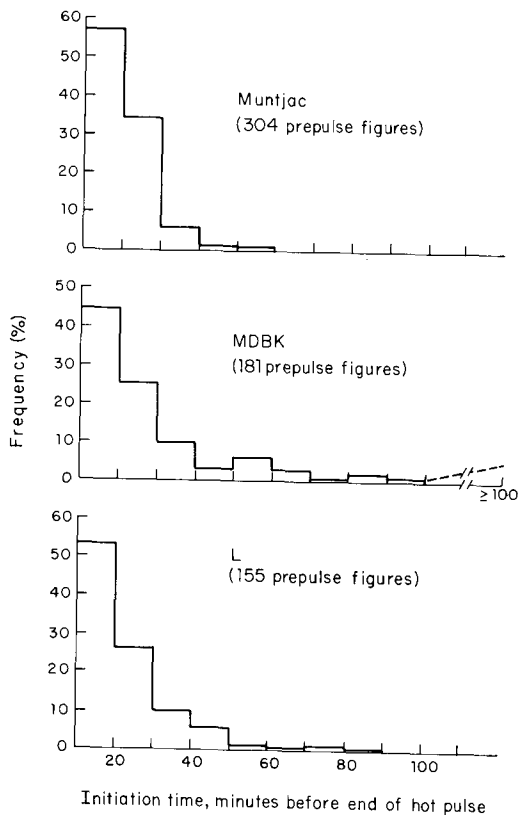


FIGURE 2 Frequency distribution of the time of initiation measured in strands with two or more prepulse figures. Data were obtained from three experiments/cell line (at least one slide/experiment) on strands at least 50  $\mu\text{m}$  long with two free ends. Time of initiation was estimated as minutes before the end of hot pulse labeling.

frequently within 10 min of each other. It was possible to observe such tight synchrony when the origins were separated by as much as 275  $\mu\text{m}$ . In muntjac cells initiations possessed the highest degree of synchrony, with 90% of the initiations occurring within 20 min of each other. In MDBK cells initiation sites appeared to be activated with a lesser degree of synchrony, whereas L cells showed an intermediate pattern. No clear differences were observed between adjacent and nonadjacent prepulse figures within individual strands.

Relative initiation time is plotted against the center-to-center distance between prepulse figures in Fig. 4. In MDBK cells, and to a somewhat less pronounced extent also in L cells, the synchrony diminishes with increasing distance between initi-

ations. In both cell lines, prepulse figures initiated  $\sim 6$  min apart when they were separated by distances of no greater than 17  $\mu\text{m}$ ; they initiated on the average 25–35 min apart when the mean distance between prepulse figures was  $\sim 170 \mu\text{m}$ . This four- to sixfold difference is statistically significant at the 0.001 level by *t* test.

It is important to note, however, that on fairly short stretches of DNA relative to the unbroken fiber, some initiations appear to have occurred quite asynchronously. Two examples of strands containing asynchronously firing units are shown in Fig. 5. Possible fusion of very closely spaced, adjacent units cannot be ruled out; however, it appears very possible that in at least some sections of the fiber, one lone initiation event may occur and that there is no other initiation activity in that region for a considerable period of time. Differences in time of initiation within a strand have

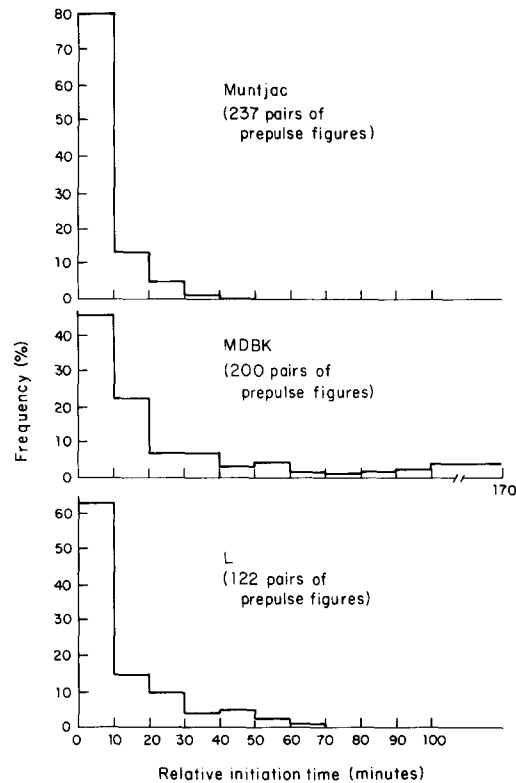


FIGURE 3 Frequency distribution of the relative initiation time. For the strands in Fig. 2, the absolute values of the differences in time of initiation between adjacent and nonadjacent prepulse figures were determined. For muntjac cells, 237 pairs were measured, 200 for MDBK, and 122 for L cells.

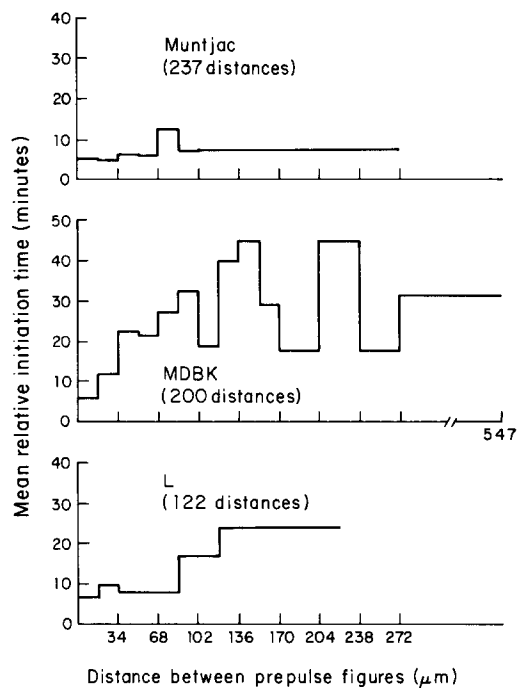


FIGURE 4 Relationship between mean relative initiation time and distance between prepulse figures. The center-to-center distances between the pairs of prepulse figures described in Fig. 3 were measured, and the mean relative initiation time for 17- $\mu\text{m}$  intervals was calculated.

also been suggested previously (7, 12), based on observations of varying prepulse figure gap sizes or ratios of prepulse and postpulse figures on the same strand.

#### DISCUSSION

The existence of synchrony as evidence of some form of coordinate control over the time of initiation along regions of the DNA fiber has been noted previously (see review by Hand, reference 10). The analyses we present for muntjac, MDBK, and L cells provide the first estimates of synchrony in terms of the actual times of initiation. Within stretches of DNA whose length was short compared to the unbroken fiber, initiations occur most frequently within 10 min of each other. This suggests a very high degree of synchrony in localized areas of the genome. It seems probable that these clusters or arrays of synchronously firing initiations form the "replication bands" observed on chromosomes.

As the distance between origins increases, the

initiations tend to occur less synchronously; however, differences were observed among cell lines. Initiations in muntjac DNA occurred with the greatest synchrony; there was only a small change over a 270- $\mu\text{m}$  range of interinitiation distances. The decay of synchrony with increasing distance was more striking in L- and MDBK-cell DNA than in muntjac. Hand (9) concluded, after examining the frequency of adjacent prepulse and postpulse figures, that there is synchrony in the timing of initiation of replication units within segments of L-cell DNA. The method of analysis used in the present study provides the first quantitative estimates of the degree of synchrony operating over defined interinitiation distances.

Independent evidence of a control mechanism at the level of clusters of 25 or more replication units has been obtained in studies of the effects of x-rays on DNA replication in mammalian cells (27, 28). Because inhibition of replication is ob-

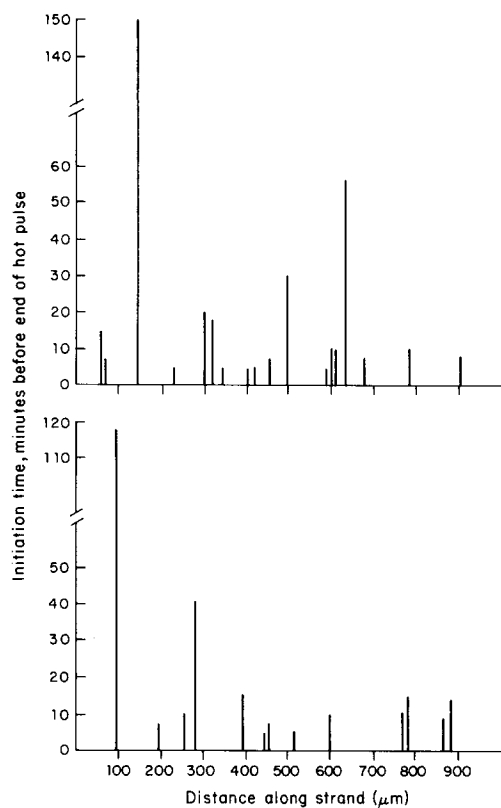


FIGURE 5 Relationship between time of initiation and the spatial location of individual initiation events. Two strands from MDBK cells which had never been treated with FUdR were examined.

served at doses too low to affect individual replication units, it has been proposed that a single hit in a cluster of replication units may prevent initiation of all units in that cluster (27). A possible mechanism would be a strand break causing a change in the conformation of a segment of DNA containing a cluster of replication units (29, 30).

Coordinate control over initiation of replication in stretches of DNA may involve both the time of appearance of appropriate initiator proteins and changes in chromatin. It has been proposed that homologous inverted complementary sequences possess a number of properties that would be expected of recognition sites for proteins that function in the initiation of bidirectional DNA replication in mammalian cells (37, 38). Inverted complementary sequences have been described at or near initiation sites in several viral (14, 19, 32, 35), plasmid (5), and bacterial (25, 36) systems. There are enough inverted complementary sequences in mammalian DNA to account for the total number of initiation sites activated in the course of the S phase (reviewed in Tamm et al., reference 38). Although the inverted complementary sequences show considerable homology, they do not show sequence identity (20–22, 31). If all inverted complementary sequences at all sites of initiation of DNA replication are not exactly alike, then the possibility arises that there may be functionally significant differences in the inverted repeat regions among clusters activated at different times in S phase, with clusters activated at the same time sharing a common inverted repeat region. Because more than one cluster is commonly operating at the same time, a particular activator protein might be shared by replication units in several synchronously replicating clusters. Other mechanisms for activation of given initiation sites in a cell traversing S phase have also been proposed (2).

If clusters of initiation sites correspond to chromosome bands, it is possible that long stretches of DNA, which had been inaccessible to initiation factors, "open up" at a particular time in S phase by changes in DNA-associated proteins and in the organization of the chromatin. The occasional highly asynchronously initiations may be associated with changes in chromatin occurring over very small segments of the fiber.

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