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Effects of Reovirus Infection on the Spatial and Temporal Organization of DNA Replication in L Cells

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Abstract. DNA fiber autoradiography was used to analyze the spatial and temporal organization of activated initiation sites for DNA replication in mouse L929 cells infected with reovirus type 3 (Dearing strain) and in uninfected control cells. Cells were labeled for 10 min with ^{3}H-thymidine at high specific activity followed by 3 h of low specific activity labeling. Reovirus infection causes no change in the rate of replication fork progression, but increases both the mean distance between activated initiation sites by ~30% and the nonrandomness in the spatial distribution of the sites along the DNA fibers. Significant synchronization of initiation in adjacent activated sites was detected on DNA fibers from uninfected cells and from reovirus-infected cells. The mean relative initiation time for pairs of initiation events which had occurred prior to high specific activity labeling did not differ significantly between the infected and uninfected cells. The data are consistent with the interpretation that reovirus infection shuts off initiation sites in a coordinated fashion, possibly by preventing activation of entire clusters of potential initiation sites.

Introduction

Reovirus inhibits cellular DNA replication in L cells starting approximately 6 h after infection (Gomatos and Tamm, 1963). This inhibition does not appear to be due to an effect on DNA precursors or DNA polymerase activity, and does not represent a block either in the entry of cells into S phase or in the elongation of already initiated chains (Gomatos and Tamm, 1963; Ensminger and Tamm, 1969; Hand and Tamm, 1972, 1974). Cellular DNA is not degraded as late as 12 h after infection (Ensminger and Tamm, 1969). These results, and the observations that the mean distance between initiation sites and the fraction of long inter-initiation distances are both increased in reovirus-infected cells, indicate that it is the initiation process that is primarily affected (Hand and Tamm, 1974).

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We have studied the effects of reovirus infection on the spatial and temporal organization of DNA replication in mouse L cells using DNA fiber autoradiography in combination with sampling procedures and statistical tests which can resolve the replication process in fine detail (Jasny et al., 1978; Cohen et al., 1979; Jasny and Tamm, 1979). We find that in reovirus-infected L cells, an increase in distance between activated initiation sites is associated with detectable changes in the spatial organization of initiation sites in the direction of nonrandomness. These results indicate that reovirus infection turns off initiation sites in a coordinated fashion, rather than interfering with initiation sites for DNA replication at random along the fiber.

Materials and Methods

Cells and Virus. L929 cells, a continuous line derived from mouse fibroblasts, were grown in monolayer cultures in Eagle's minimal essential medium (Eagle, 1959) supplemented with 5% fetal calf serum.

Reovirus type 3, Dearing strain, was grown and assayed in L929 cells as previously described (Hand and Tamm, 1971).

Measurement of DNA Replication. Exponentially growing monolayer cultures of L cells were infected with reovirus type 3, 250–350 plaque-forming units per cell. The virus adsorption period was 1 h. Nine h after infection cultures of infected and mock-infected cells were labeled for 15 min with ^{3}H-thymidine (50–60 Ci/mmole, 100 μCi/ml). DNA replication in infected cells was markedly inhibited: the ratio of trichloroacetic acid-precipitable to total counts per min for infected cells was approximately 50% of the corresponding ratio for mock-infected cells. Replicate cultures were treated with 2×10^{-6} M FdUrd for 0.5 h starting at 8.5 h after infection to reduce the thymidine pool, and then labeled for 10 min with high specific activity ^{3}H-thymidine (50–60 Ci/mmole, 250 μCi/ml) followed by 3 h of low specific activity labeling (5–6 Ci/mmole, 250 μCi/ml). Processing of cells for DNA fiber autoradiography and scoring of high and low grain density stretches were carried out as previously described (Jasny et al., 1978). Only initiations that were located internally within strands were scored because such sites allow no ambiguity as to whether initiation preceded or followed the labelling pulse. The data in Table 1 represent pooled results from three experiments unless otherwise stated. For the statistical measures to be described, there was no significant difference among the mock-infected cell samples at the 0.01 level by the t test; the infected cell samples were similarly homogeneous.

Results

Characteristics of the System

We have confirmed previous observations (Hand and Tamm, 1972) that reovirus infection causes no change in the rate of replication fork progression (Table 1 A; $P > 0.5$ by t test). We have also confirmed the finding (Hand and Tamm, 1974) that the mean inter-initiation distance is increased in reovirus-infected cells (Table 1 B; $0.001 < P < 0.005$ by t test). In our measurements, the mean inter-initiation distance was approximately 30% greater in reovirus-infected than in control cells. Previous examination of inter-initiation distance in reovirus-infected cells relied on data pooled without regard to fragment length (Hand and Tamm, 1974). Such data do not permit precise quantitation because of

Table 1. Rate of fork progression, inter-initiation distance and relative time of initiation in reovirus-infected and mock-infected L cells

	L cells	Reovirus-infected L cells
A. Rate of fork progression[a]		
Number of halves of prepulse figures	233	221
Mean (μm per min)	0.553	0.549
Variance (μm per min)^2	0.067	0.047
B. Inter-initiation distance		
Number of distances	155	184
Mean (μm)	38.82	50.05
Variance (μm)^2	862.44	1,180.78
C. Relative time of initiation within pairs of prepulse figures		
Number of pairs of prepulse figures	178	152
Mean (min)	7.48	6.20
Variance (min)^2	80.48	51.02

[a] Data pooled from 2 experiments

the dependence of inter-initiation distance on strand length (Cohen et al., 1979; Jasny, 1978). For the data in Table 1, only strands ranging in length from 50 to 350 μm were examined and the mean inter-initiation distance was calculated separately for each strand. When strands were divided into 50 μm length categories (i.e., 50–100, 100–150, 150–200, etc.), there was no significant difference in the strand frequencies between reovirus-infected and mock-infected populations by χ^2 analysis ($P=0.47$). There was also no significant difference in mean length between the strands from the reovirus-infected cells (mean, 168 μm; standard deviation, 63; $N=89$) and the strands from the mock-infected cells (mean, 150 μm; s.d., 72; $N=97$) by t test ($P>0.5$).

To study the synchrony of initiation events as related to the proximity of initiation sites, Hand (1975) analyzed the frequency of occurrence of prepulse versus postpulse figures in pairs of adjacent activated initiation sites in DNA from reovirus-infected and control cells. Prepulse figures indicate initiation events that occurred before addition of radioactive label while postpulse figures indicate initiation activity that occurred during the 10-min hot pulse; see Jasny and Tamm (1979) for details. Immediately adjacent pairs of activated sites were scored as having two prepulse figures ("pre-pre"), one prepulse and one postpulse figure ("pre-post"), or two postpulse figures ("post-post"). The proportions of these three kinds of pairs of figures in DNA from the control sample were compared with the proportions in DNA from reovirus-infected cells using the chi-squared test of homogeneity. Hand (1975) interpreted an increase in the frequency of pre-post pairs as suggesting a decrease in the synchrony of initiation. In one of Hand's two experiments, the change in proportions of the three kinds of adjacent pairs was significant ($P<0.01$). The proportion of pre-post pairs of figures increased from 0.20 in the control sample to 0.37 in the sample of infected cells. In Hand's other experiment, the change in proportions of the three kinds of pairs was marginally significant ($P<0.025$).

The proportion of pre-post pairs of figures increased from 0.15 to 0.29. Based on these two experiments, Hand concluded that synchrony of initiation was decreased after reovirus infection.

To provide a frame of reference for our analysis, we repeated Hand's experiments and analysis. In one experiment, we found a marginally significant change in the proportions of the three kinds of adjacent pairs ($0.025 < P < 0.05$) and an increase in the proportion of pre-post pairs of figures in infected cells, from 0.29 to 0.38, as did Hand. In another experiment, we again found a marginally significant change in proportions ($0.01 < P < 0.025$), but this time reovirus infection decreased the proportion of pre-post pairs of figures from 0.30 in the control sample to 0.20. In these experiments, following Hand's procedures, we used all strands, whether or not they had free ends[1].

It is important to emphasize that in *both* control and infected samples of Hand's (1975) two experiments, the proportions of pre-pre, pre-post, and post-post pairs of figures differed significantly ($P < 0.01$) from the proportions expected if prepulse and postpulse figures had been randomly associated in these experiments. In our two experiments, when adjacent pairs were chosen from all strands without regard to whether they had two free ends, we also found significant differences ($P < 0.01$) from random association *both* in the control and in the infected samples. In Hand's and in our experiments, there were fewer pre-post pairs of figures than expected if the occurrence of pre- and postpulse figures adjacent to each other were randomly determined.

Thus, Hand's and our data together demonstrate a significant synchronization of initiation in adjacent activated sites on unselected strands of DNA from uninfected cells and from reovirus-infected cells. These data together also indicate that reovirus infection can, but may not always, cause a decrease in synchrony of initiation of replication in some stretches of DNA.

Effects of Reovirus Infection on Timing of Initiation of Cellular DNA Replication

A different aspect of synchrony in initiations of DNA replication in reovirus-infected cells is described by calculating the absolute values of the differences of the times of initiation for *every* possible pair of *prepulse* figures along a

[1] As a methodological aside, we note that the proportion of pre-post pairs of figures may in some circumstances not be a valid indicator of the lack of synchrony of initiation. Suppose in a control sample that the proportion of pre-post pairs were $2pq$, where p is the sample's proportion of prepulse figures and $q = 1-p$; $2pq$ is the proportion of pre-post pairs expected in the absence of synchrony. Suppose that viral infection had no effect on initiation except to change p to some value p' closer to 0.5. In the absence of synchrony, the proportion of pre-post pairs of figures in the infected sample would be $2p'q'$, where $q' = 1-p'$. If p' were closer to 0.5 than p, one may prove that $2p'q'$ would be larger than $2pq$, so there would appear to be a decrease in synchrony resulting from viral infection, even though we assumed initiation was unsynchronized in both control and infected samples. A better indicator of lack of synchrony would be the ratio of the observed proportion of pre-post pairs to the proportion predicted in the absence of synchrony. In Hand's data and ours, the direction of change in synchrony resulting from reovirus infection is the same using either indicator

given strand (Jasny and Tamm, 1979). The larger the relative initiation time, the further apart in time two activated sites initiated. These measurements give a quantitative description of coordinate control that differs from that of Hand (1975) in two respects: only prepulse figures are considered, and nearby sites, as well as immediately adjacent pairs, are included. For any slide, all strands containing at least two prepulse figures were examined to avoid sampling bias. For the results to be described, the strands did not necessarily have two free ends.

The mean relative time of initiation on strands with measurable length between 50 and 350 μm did not differ significantly between the infected and the uninfected cells (Table 1C; $0.1 < P < 0.2$ by t test). Approximately 90% of the recorded initiations in control and infected cells occurred within 30 min of the end of the hot pulse. In the strands examined, the mean distance between prepulse figures was 25% larger in reovirus-infected than in control cells, which was a marginally significant difference between the two populations by t test ($0.01 < P < 0.025$).

Although synchrony does decrease with increasing inter-initiation distance over large distances, the change in inter-initiation distance after reovirus infection was small enough so that the distance increase alone, from 39 to 50 μm, would not be expected to cause a detectable increase in the mean relative time of initiation within pairs of prepulse figures (see Fig. 4 in Jasny and Tamm, 1979).

Effects of Reovirus Infection on Spatial Pattern of Initiation of Cellular DNA Replication

To study the spatial pattern of activated initiation sites, we have used three statistical tests for randomness: the Durbin-Kolmogorov-Smirnov test, the Weibull test, and the Keiding test (Cohen et al., 1979). Each of these is sensitive to different kinds of nonrandomness. With these tests, we have previously demonstrated that initiation sites are nonrandomly distributed in L, MDBK, and muntjac cells, and that prolonged exposure of cells to FdUrd shifts the distribution of inter-initiation distances in MDBK cells toward randomness (Jasny, 1978; Cohen et al., 1979). It is necessary to analyze strands containing 9 or more inter-initiation distances per strand so that tests can be performed on each strand individually. The results of these tests were then pooled for the strands from reovirus-infected cells and for the strands from mock-infected cells (see Table 2).

For the Durbin-Kolmogorov-Smirnov test and the Keiding test, it was possible to determine whether the distributions of inter-initiation distances were random above a certain threshold α (which was taken as the lowest measurable inter-initiation distance). This estimation of α is already incorporated into the Weibull test. When α is estimated in the Durbin-Kolmogorov-Smirnov and Keiding tests there is a marked shift toward nonrandomness in the reovirus-infected cells. By contrast, when MDBK cells were exposed for a long period to FdUrd, the mean distance between initiation sites did not change, but the spatial organization became closer to random (Jasny, 1978; Cohen et al., 1979).

Table 2. The effect of reovirus infection on the spatial organization of activated initiation sites (two-tailed tests of exponentiality)

	L cells	Reovirus-infected L cells
Number of strands	20	20
Strand length (μm)		
Mean	512.9	561.4
Maximum	1319.3	1067.9
Minimum	136.8	153.9
Number of IIDs[a] per strand		
Mean	16	15
Maximum	52	24
Minimum	9	9
Durbin-Kolmogorov-Smirnov test		
$P_{\alpha=0}$	$<10^{-4}$	$0.0001 < P < 0.0005$
$P_{\alpha \text{ estimated}}$	$0.2 < P < 0.3$	$0.01 < P < 0.025$
Weibull test P	$<10^{-4}$	$<10^{-4}$
Shape parameter β		
Maximum	3.83	2.35
Minimum	0.97	0.70
Estimated median IID (Mean over strands) (μm)	29.8	31.8
Keiding test		
$P_{\alpha=0}$	$<10^{-4}$	$0.0005 < P < 0.001$
$P_{\alpha \text{ estimated}}$	0.39	0.025

[a] IID = inter-initiation distance

The Weibull test appears to be the most sensitive to the kind of nonrandomness detectable in L cells. The probability that inter-initiation distances are randomly distributed is so low in uninfected cells, according to the Weibull test, that no further nonrandomness in reovirus-infected cells is revealed (Table 2). The shape parameter β in the Weibull test indicates the extent to which inter-initiation distances cluster around the median value. In strands with β greater than 1, as observed here in some strands from both infected and mock-infected cells, activated initiation sites are more evenly spaced than expected from a random (exponential) model. In strands with β less than 1, long inter-initiation distances frequently appear to separate regions with several shorter inter-initiation distances.

Discussion

Statistical analysis of the spatial pattern of activated initiation sites on strands 137 to 1,320 μm long indicates a marked shift toward nonrandomness in the DNA of reovirus-infected L cells. The measurements underlying Table 2 suggest that the increased nonrandomness is due to an increased variability in inter-

initiation distances relative to the mean. This suggestion is compatible with the slightly lower range of values of the Weibull shape parameter β observed in reovirus-infected cells.

If reovirus infection randomly turned off potential initiation sites that would ordinarily be activated in uninfected cells, the spatial pattern of inter-initiation distances would move closer to randomness. Our observation of the contrary effect suggests that reovirus infection inactivates initiation sites in a coordinated way. Hand and Tamm (1974) had postulated that inactivation of clusters of replication units might explain the difference between the moderate increase in modal inter-initiation distance and the marked overall inhibition of DNA synthesis. Our data provide the first experimental evidence in support of this idea. Nonrandom inactivation of sites *within* localized regions or clusters is not ruled out, however.

The concept that adjacent initiation sites may be inhibited in a spatially coordinated way by reovirus infection is consistent with our finding of no striking effect of infection on the mean relative initiation time of strands 50–350 μm long. Our analysis of spatial patterning is based on measurements from strands with a mean length on the order of 500 μm (range, 137 to 1,320 μm). The measurements of timing of initiation derive from strands with a mean length on the order of 160 μm (range, 50 to 350 μm). Reovirus infection may not have a marked effect on timing of initiation on this spatial scale, because activated sites on strands $\sim$160 μm long are more likely to fall in a *single* (hypothetical) cluster of sites than sites distributed over longer distances.

Finally, there is suggestive evidence from Hand's (1975) and our experiments that at times a decreased synchrony of initiation in adjacent activated sites can be detected in DNA from reovirus-infected cells. This decrease is not consistently observed in all samples. If viral infection prevents activation of entire clusters of potential initiation sites, adjacent activated sites on sufficiently long DNA strands might belong to different clusters more frequently in infected than in control cells. A pair of activated sites from different clusters might be expected to be less synchronous in initiation than an adjacent pair within a single cluster.

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