Effect of Simian Virus 40 on the Temporal and Spatial Organization of DNA Replication in Muntjac Cells

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We examined the effects of simian virus 40 infection on the temporal and spatial organization of initiation sites for DNA replication in Muntjac cells by means of light microscopic DNA fiber autoradiography. Initiation at multiple sites along the DNA fiber in virus-infected confluent Muntjac cells was more nearly synchronous than in serum-deprived controls, although temporal control in the infected cells did not reach the level observed in cells incubated in serum-enriched medium. Initiation sites in virus-infected cells appeared to be spatially closer together than in either uninfected serum-deprived or uninfected serum-enriched cells. This change did not appear to be the result of the induction or repression by simian virus 40 of clusters of replication units with new and different organizations.

Simian virus 40 (SV40) infection induces DNA synthesis in permissive and nonpermissive mammalian cell systems (22, 24, 32, 44). According to several models for transformation, SV40 alters the process of initiation of DNA replication at multiple sites along the host cell DNA fiber, and it has been proposed that T antigen, which is required for the initiation of each round of viral DNA synthesis (6, 33, 47), also acts as an initiator of cellular DNA synthesis (3, 36, 47). Evidence for the initiation of cellular DNA synthesis by T antigen comes from studies of temperature-sensitive early SV40 mutants (6, 26, 47), and microinjection into mammalian cells of RNA complementary to the early SV40 coding region (19), early-coding viral DNA sequences (38), or purified T antigen (49). Alterations in the initiation process might affect the synchrony of events in clusters of replication units, the spatial organization of activated initiation sites, or both.

Very little information is available concerning the effect of SV40 infection on the temporal organization of DNA replication. SV40 infection does not change the overall length of the S phase in Muntjac cells which are nonpermissive for SV40 and can be abortively transformed (17, 18), but in permissive cell lines of African green monkey origin, such as AGMK and CV1, cells appear to be arrested after one round of cellular DNA synthesis (16). Similar observations have been made by Lehmann et al. (34) and Hiscott and Defendi (25). No changes have been observed in the timing of satellite DNA replication in mouse kidney cells infected with polyoma

virus (1, 23) or in the replication of component α DNA in primary African green monkey kidney cells (AGMK) induced by SV40 (40).

Using DNA fiber autoradiography, Martin and Oppenheim (37) observed that for Chinese hamster lung cells, the geometric mean inter-track distance is significantly shorter in SV40-transformed cells than in nontransformed cells in complete medium, but longer in isoleucine-deprived medium. Similar results were obtained with BALB/c 3T3 cells (39). It was suggested that T antigen provides new and different initiation sites. Kapp et al. (30) found a twofold increase in replication unit size upon transformation of normal and of *Xeroderma pigmentosum* cells of human origin and of mouse 3T3 cells by SV40. It is possible, however, that results obtained with SV40-transformed cells reflect secondary changes occurring during the establishment of a stable transformed state and are not indicative of early events in the infection process.

We used DNA fiber autoradiography to determine whether SV40 infection affects the synchrony of the initiation process or the spatial organization of DNA replication in Muntjac cells. We were especially interested in the Muntjac system because SV40 infection induces Muntjac DNA replication (17) and because of the finding that T antigen binds nonuniformly to Muntjac chromosomes (8, 10). Moreover, the absence of viral replication in these cells (18) simplifies the interpretation of results. We elected to examine DNA replication 24 h after infection rather than in stably transformed cell lines

in which a broad range of pleiotropic changes has already occurred. To examine DNA replication that is predominantly SV40 induced, we infected confluent serum-deprived cells and as controls used uninfected cells deprived of serum or incubated in 10% serum.

MATERIALS AND METHODS

Virus and cells. SV40 used in these experiments was strain VA4554, isolated and propagated as described by Diacumakos and Gershey (11). Muntjac cells are a fibroblastic strain derived from a male Indian deer, *Muntiacus muntjak*. They were obtained from the American Type Culture Collection, Rockville, Md. (ATCC 157). The cells were maintained as logarithmically growing monolayer cultures in Eagle minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), potassium penicillin G (500,000 U/liter), streptomycin sulfate (100 mg/liter), and mycostatin (25,000 U/liter) as previously described (9,10). Cell cultures were free of mycoplasma contamination on the basis of fluorescence staining (5) and scanning electron microscopy.

Serum deprivation and infection. For each of three experiments, a separate ampoule of cells was thawed and passaged. Cells of equal population doubling levels were seeded into petri dishes of 100-mm diameter at approximately 7×10^5 cells per dish. Each dish contained a section of a glass slide and two cover slips. Cells were allowed to grow until saturation density was reached, at which point the cell number no longer increased, as determined by cell counts. After reaching confluence, the cultures were maintained for 72 h in medium lacking FBS. The medium was changed daily.

On day 3 of serum deprivation, one set of cell cultures was infected with SV40 in Eagle MEM at a multiplicity of 200 PFU/cell in a volume of 1 ml/plate (virus infected). Another set of cells, serum deprived, was mock infected with Eagle MEM, and the remaining set received Eagle MEM containing 10% FBS (serum enriched). After incubation for 2 h at 37°C, 10 ml of medium per plate lacking FBS was added to serum-deprived and virus-infected cells. A 10-ml amount of medium containing 10% FBS was added per plate of serum-enriched cells, and incubation was continued for 24 h. The cover slips were then removed and stained for T antigen (see below).

Radioisotope labeling and light microscope DNA fiber autoradiography. At 24 h after infection, 5-fluoro-2'-deoxyuridine (FUDR) was added to the culture dishes to a final concentration of 2 μ M for 30 min to deplete the intracellular thymidine pool, after which cells were labeled with [3 H]thymidine (New England Nuclear Corp.; 50 to 56 Ci/mmol, 250 μ Ci/ml) for 10 min. Sufficient unlabeled thymidine was then added to lower the specific activity 10-fold. The glass slides were removed, washed four times in phosphate-buffered saline (PBS), and fixed in 100% ethanol at -70°C.

The cells in the petri dishes were incubated for 3 more h, washed in PBS deficient in calcium and magnesium (PBS def; pH 7.2), and removed from the dishes by brief exposure to trypsin. Cells were lysed with 1% sodium dodecyl sulfate-0.01 M EDTA in PBS def and spread down glass slides. The DNA was precipitated in 10% trichloroacetic acid followed by

dehydration in graded solutions of ethanol (see reference 23 for details). Slides with either spread DNA or labeled cells were dipped in melted Kodak NTB2 emulsion in total darkness. They were stored at -20°C for 6 months and developed in a 1:1 dilution of Kodak D19 developer. With this protocol, labeled nuclei in whole cells were covered with grains and were easily recognizable over the background.

T antigen staining. SV40 T antigen was detected by means of indirect immunofluorescence staining (9). Cells fixed on cover slips in absolute ethanol at -70°C at 60 h after infection were stained for 60 min at 37°C with hamster anti-T serum (National Cancer Institute 79x-137) diluted 1:20 in PBS. The percentage of fluorescent nuclei remains essentially constant for the period from 24 to 72 h after infection (18). The anti-T serum had previously been adsorbed on subconfluent monolayers of Muntjac cells and clarified by centrifugation at $9,000 \times g$. Cells were then washed with PBS and incubated for 60 min at 37°C with fluorescein isothiocyanate-conjugated goat anti-hamster immunoglobulin G (IgG) (Antibodies Inc., Davis, Calif.) diluted 1:10 in PBS. Fluorescence was detected at a final magnification of 160 $\times$ with an inverted microscope equipped for epifluorescence excitation at 495 nm and suppression at 525 nm (Leitz).

Analysis of autoradiograms. In examining autoradiograms, fragments of the intact DNA fiber, which are defined as strands, are readily identifiable. Initiation sites are located within either prepulse or postpulse figures, which are recognized by the characteristic changes in grain density. A prepulse figure, which indicates the occurrence of initiation before the addition of radioactive label, consists of a central gap bordered on either side by a length of high-specific-activity labeling (high grain density) in autoradiograms. In postpulse figures, a central region of high-specific-activity labeling is bordered on both sides by stretches of low-specific-activity labeling.

The calculation of initiation times from prepulse figures has been described fully (27). To summarize, the rate of fork progression was calculated for each prepulse figure as the mean of the lengths of the high-specific-activity-labeled halves divided by the time of high-specific-activity labeling (10 min). The mean distance from the origin (taken as the center of the prepulse figure) to the two ends of the prepulse figure was divided by the rate of fork progression to generate the time interval between initiation and the end of the high-specific-activity labeling. This interval is defined as the initiation time.

The term *interinitiation distance* is defined as the distance between two adjacent initiation sites, with the location of a site taken as the center of the prepulse or postpulse figure. For studies of spatial organization, strands containing nine or more interinitiation distances were selected. The theoretical basis for selection of this lower limit has been previously described (7). A total of 13 to 15 strands were selected for each experimental variable, pooled from three experiments. For the temporal organization studies, strands containing eight to nine interinitiation distances which had at least two prepulse figures were used. Measurements were made with an ocular micrometer at a final magnification of 640 $\times$. There was no significant difference in mean strand length among the experimental conditions by Student's *t* test ($0.4 < P < 0.5$).

FIG. 1. Cumulative frequency of initiation times expressed as the absolute values of the numerical differences between the median time of initiation on a strand and the time of initiation for each prepulse figure on that strand. Symbols: —, uninfected, serum enriched; ----, virus infected, serum deprived;, uninfected, serum deprived. Frequencies were plotted at the center of 10-min intervals.

RESULTS

In Muntjac cells deprived of serum for 72 h and then incubated in the presence of serum for 24 h, 35% of the nuclei were labeled after 10 min of labeling with high-specific-activity [^3H]thymidine. The percentage of labeled nuclei was reduced 5.8-fold (to 6%) in cells deprived of serum for 96 h. The infection of cells with SV40 resulted in an 11.6-fold increase in DNA synthesis (70% of nuclei were labeled) when compared with serum-deprived cells and a 2-fold increase when compared with cells which had been incubated with serum (35% of nuclei were labeled). Approximately 50% of the Muntjac cells exposed to SV40 contained T antigen as monitored by immunofluorescence staining.

Temporal organization. Our hypothesis was that SV40 infection altered the coordinate control over the timing of initiation at sites along the DNA fiber. As described above, it is possible to calculate the time of initiation for prepulse figures relative to the end of the high-specific-activity labeling period. The numerical differences between the median time of initiation on a single strand and the time of initiation for each prepulse figure on that strand were calculated. The frequency distributions for these differences are shown in Fig. 1. Although it is true under all three conditions that most of the initiations occur at approximately the median times, it is apparent that in uninfected serum-deprived cells, there is a higher frequency of outlying values. The distribution of initiations in the

virus-infected cells appears to occupy an intermediate position between those in serum-deprived and serum-enriched cells. To determine the significance of these differences, the following approach was used. The data are not normally distributed; thus, a standard t test is not appropriate. Therefore, for each pair of variables, we converted our data into the form of a two-sample quantile function (to be described below) and compared the data with the Fourier transform that would be observed in the case of two identical distributions. This test was originally described by Parzen (41). It has the advantages of making no assumptions concerning the distribution of the data (i.e., it does not require a normal distribution) and of being very sensitive to differences in the frequencies in the tails of the distributions. Comparisons of data were made by means of the computer program TWO-SAM as described by White (50).

The normalized initiation times were pooled for serum-deprived, serum-enriched, and virus-

FIG. 2. Quantile analysis of initiation times: virus infected versus serum deprived, serum deprived versus serum enriched, and virus infected versus serum enriched. The abscissa represents the cumulative frequencies for individual initiation times in the second variable in each panel. The ordinate represents the cumulative frequencies for initiation times in the first variable corresponding to the initiation times on the abscissa. The dashed line is the theoretical curve of identity between the two variables.

TABLE 1. Square modulus of Fourier coefficients

Square modulus term	Virus infected vs serum deprived	Serum deprived vs serum enriched	Virus infected vs serum enriched
1	0.0005	0.0278	0.0122
2	0.0288	0.0638	0.0227
3	0.0140	0.1031	0.0318
4	0.0194	0.0340	0.0236
5	0.0048	0.0356	0.0180
6	0.0199	0.0786	0.0187
7	0.0325	0.0720	0.0044
8	0.0062	0.0025	0.0013
9	0.0024	0.0629	0.0207
Critical value	0.0286	0.0291	0.0272

infected samples. For any two sets, if the distributions of the normalized initiation times are the same, then the medians of the two sets should occur at approximately the same value. We determined the quantiles of initiation times and compared them, two variables at a time. For example, in serum-deprived cells, 95% of all observations are at or below 20 min from the median [called $\hat{D}(U)$]. The fraction of all serum-enriched initiations below 20 is 0.90 (called U). The plot of $\hat{D}(U)$ versus U for serum-deprived versus serum-enriched initiations will pass through the point (0.95, 0.90). The plots of $\hat{D}(U)$ versus U for each pair of variables are shown in Fig. 2 and have been "smoothed" with an autoregressive smoother (41). If the two samples are identical, $\hat{D}(U)$ versus U will run from (0, 0) to (1, 1) along the diagonal. For a real sample, some oscillation around the line would be expected, but this should resemble Brownian motion, i.e., lack an obvious pattern of deviation. Visually, it is apparent that the data for serum-deprived versus serum-enriched initiations do not lie along the diagonal, whereas virus-infected versus serum-deprived or virus-infected versus serum-enriched initiations lie either on or near the diagonal.

We now have one $\hat{D}(U)$ curve for each pair of variables. We next determined the Fourier transform of the raw estimate $\hat{D}(U)$. Essentially, the Fourier transform is a mathematical analysis of the curve as the sum of sine and cosine wave components (see references 41 and 45 for details). Our question is whether the Fourier transform of the experimental $\hat{D}(U)$ curve looks like the Fourier transform of a straight line (which would mean the two samples had identical distributions). We examined the sequence of Fourier coefficients to see whether they resembled a line with only random variations (called a Brownian Bridge process; 41). It is known that the square

of a coefficient (called the square modulus) from a Brownian Bridge process would have asymptotically the distribution of $y/2n$, where y has a chi-square distribution with 2 degrees of freedom and n is the total number of observations in the combined sample. If the square modulus terms of the coefficients in our curve are greater than $CV/2n$, where CV is the appropriate critical value from the chi-square distribution, then the two curves are significantly different. The squared modulus terms of the first nine Fourier coefficients and the critical values at the 0.01 level are shown in Table 1. For initiation in serum-deprived versus serum-enriched groups, all except two of the values were greater than the critical value; therefore, we can conclude that there are significant differences between the two variables at the 0.01 level. For comparisons between virus-infected versus serum-deprived and for virus-infected versus serum-enriched initiations, the square modulus terms were less than or only marginally greater than the critical value. Thus, virus infection of serum-deprived cells decreased the difference between serum-deprived and serum-enriched cells and resulted in at least a partial restoration of the tight control over the timing of initiation observed in serum-enriched cells.

Spatial organization. We analyzed the spatial organization of activated initiation sites in pooled samples, as well as within individual strands. We first pooled the interinitiation distances from different strands as a way of comparing our system with those of previous investigators. The cumulative frequency distributions of pooled interinitiation distances in Muntjac cells (Fig. 3) suggest that initiations are physically

FIG. 3. Cumulative frequency of interinitiation distances. Symbols: —, uninfected, serum enriched; ---, virus infected, serum deprived; ····, uninfected, serum deprived. Frequencies were plotted at the mid-points of 10- μ m intervals.

TABLE 2. Significance of differences in interinitiation distance distribution by the Mann-Whitney rank sum test

Category	No. of interinitiation distances	Level of significance ^a
Serum deprived	186	
Serum enriched	219	
Virus infected	190	
Virus infected vs serum deprived		0.0155
Serum deprived vs serum enriched		0.4682
Virus infected vs serum enriched		0.0119

^a Assuming chi-square distribution with 1 degree of freedom.

closer together in virus-infected cells than in serum-deprived cells and that in serum-enriched cells their distribution occupies an intermediate position. Interinitiation distances were shortened on the average by 20% in DNA replicating 24 h after SV40 infection of serum-deprived Muntjac cells as compared with uninfected serum-deprived cells. Interinitiation distances in cells incubated in 10% serum were 17% longer than those in virus-infected serum-deprived cells.

The distribution of pooled interinitiation distances for each experimental variable differed markedly from a statistically normal distribution, making it invalid to apply the typical *t* tests of significance. Although the geometric transformation (as described by Martin and Oppenheim [37]) brought the data closer to statistical normality (data not shown), we felt it would be preferable to use a nonparametric statistical test of significance which does not require a normal distribution of data points. In the Mann-Whitney

rank sum test, also known as the Wilcoxon test (35, 51), all data points for any two of the experimental variables are pooled, after first tagging them so that they can be distinguished. This new pooled set is then ranked according to size. Using the computer program package P3S of the Biomedical Computer Program P Series (1977), we asked whether low-ranking data points occur more frequently in one or the other set or whether they are intermingled. The sensitivity and efficiency of the Mann-Whitney rank sum test are very high relative to the standard *t* test for both normal and nonnormal data (46). The results of this test for the interinitiation distances are shown in Table 2. Virus infection of Muntjac cells resulted in significant differences in the pooled interinitiation distance distribution when compared with serum-deprived cells ($P = 0.0155$) or with serum-enriched cells ($P = 0.0119$). There was no significant difference between serum-deprived and serum-enriched cells by this test ($P = 0.4682$).

Previous investigations have indicated that interinitiation distances along the mammalian DNA fiber are not randomly (i.e., exponentially) distributed and that the distribution is most closely satisfied by a Weibull formulation (7). The Weibull distribution of interinitiation distances for the pooled strands (Table 3) was clearly not exponential ($P \ll 0.005$ in all cases). Engelhardt and Bain (14) devised a procedure for estimating the parameters α and β for the Weibull distribution and for estimating confidence intervals associated with these parameters. The formula of the Weibull cumulative distribution function is $F(x) = 1 - \exp(-(x/\alpha)^\beta)$, where x is the interinitiation distance, α is called the scale parameter, and β is the shape parameter. β can be considered as describing the shape of the frequency distribution curve for interinitiation distances. Differences in α indicate whether the entire curve is rescaled by a constant value. The mean of a Weibull distribution is $\alpha(\Gamma(\beta+1)/\beta)$, where $\Gamma(x)$ is the gamma function $\int_0^\infty t^{x-1} e^{-t} dt$. If $\beta = 1$, then the distribution

TABLE 3. Parameters for Weibull distribution on pooled interinitiation distances

Category	P^a	Shape parameter, β	Scale parameter, α	b	99% CI ^b	95% CI	U	99% CI	95% CI
Serum deprived	$\ll 0.005$	1.63	15.91	0.614	0.277	0.283	2.77	0.125	0.095
Virus infected	$\ll 0.005$	1.80	13.14	0.554	0.250	0.256	2.58	0.112	0.085
Serum enriched	$\ll 0.005$	1.71	15.35	0.586	0.266	0.272	2.73	0.110	0.084

^a P , Probability of finding the result observed, under exponentiality.

^b CI, Confidence interval (i.e., $b \pm CI$ or $U \pm CI$).

TABLE 4. Probability values for pooled Weibull b and U 99% confidence interval

Category	U	b
Virus infected vs serum deprived	$0.001 < P < 0.005$	$P > 0.5$
Serum deprived vs serum enriched	$P > 0.5$	$P > 0.5$
Virus infected vs serum enriched	$0.005 < P < 0.01$	$P > 0.5$

of interinitiation distances is exponential with a mean equal to α . If β is greater than 1, there are fewer long interinitiation distances than would be expected from a random model with the same α . If β is less than 1, there are more short interinitiation distances and more long ones than would be predicted by a random model with the same α .

To evaluate the differences between experimental variables for β and α values, it was necessary to determine the variances within each set. For the statistics b , where $b = 1/\beta$, and U , where $U = \ln \alpha$, it was possible to determine the 99% confidence intervals, since b and U can be approximated by a normal distribution. The confidence interval is considered to be equivalent to the parameter $\pm (2.57s)/n^{1/2}$ (where s = the standard deviation and n = the sample size), and it is thus possible to calculate variances for standard F and t testing. Virus infection in the Muntjac cells resulted in significant differences in the U statistic when compared with serum-deprived and serum-enriched cells (Table 4; $P < 0.01$). There was no significant difference between serum-deprived and serum-enriched cells in the U statistic ($P > 0.5$), and no significant differences were observed for any of the comparisons in Muntjac variables in the b statistic ($P > 0.5$). Although the shapes of the Muntjac curves as measured by b are not changing, SV40

infection did result in measurable alterations in the distribution of interinitiation distances reflected in a decrease in α .

The next step was to examine the organization in localized regions of the fiber. In the following series of tests, the strands are analyzed individually, and the probability (P) values are pooled. As previously described (7), we used three separate tests for deviation from exponentiality: the Keiding test, the Kolmogorov-Smirnov test with a fitted mean, and the Weibull test. Keiding (31) developed a test of exponentiality based upon the chi-square distribution and a minimum observation. When this observation is assumed to be 0, exponentiality is rejected in all of the cases. If the test minimum is fitted from the sample, exponentiality is not rejected for the serum-deprived Muntjac cells. Virus-infected Muntjac cells and Muntjac cells grown in the presence of serum are initiating nonrandomly even if the test is performed with a fitted test minimum.

The Kolmogorov-Smirnov test of exponentiality with a fitted mean (12) is not a sensitive test; yet, as Table 5 shows, it definitely rejected exponentiality for the serum-enriched and virus-infected conditions in Muntjac cells. Rejection of exponentiality for the serum-deprived condition is dependent on the P level chosen ($0.01 < P < 0.025$). The specific test of exponentiality against a Weibull alternative (15) overwhelmingly rejected exponentiality in favor of a Weibull distribution for all three conditions (Table 5). The variation in α and β in individual strands was too great to allow analyses of significance as were carried out for pooled strands.

To summarize, in the entire population, initiation sites were separated by shorter distances after virus infection. This was not, however, associated with demonstrable changes in the spatial organization within localized regions of the fiber.

DISCUSSION

Initiation of DNA synthesis in Muntjac cells exhibits demonstrable synchrony in that there is

TABLE 5. Two-tailed tests of exponentiality performed on individual strands

Category	Durbin Kolmogorov-Smirnov test, P^a	Keiding test P		Weibull test, shape parameter β			Weibull test, scale parameter α			Smith-Finch V test exponential vs Weibull
		$\alpha = 0$	α estimated	Maximum	Minimum	Median	Maximum	Minimum	Median	
Serum deprived	0.011	< 0.005	0.1–0.2	2.78	0.90	1.48	22.10	9.93	17.59	< 0.005
Virus infected	0.005	< 0.005	0.005–0.01	3.32	0.97	1.78	19.96	10.64	13.08	< 0.005
Serum enriched	0.009	< 0.005	< 0.005	4.33	0.65	1.49	23.25	11.13	16.52	< 0.005

^a P , Probability of finding the result observed, under exponentiality.

a high degree of clustering of initiation times around the median. This control over the timing of initiation over localized regions of the fiber is disturbed when the cells are incubated in medium without FBS. There was a significant increase in the frequency of "outliers" in serum-deprived cells. Infection with SV40 resulted in an induction of cellular DNA synthesis (i.e., an increase in the percentage of cells in S phase) and a distribution of initiation times which was closer to the median. Initiations along the fiber occurred more closely together in time in virus-infected cells than in serum-deprived cells, but the clustering did not reach the level seen in serum-enriched cells. Potential physical sites may become available for DNA replication even in serum-deprived cells, but apparently cannot initiate at the proper times because of the low level of initiation factors. SV40 infection may partially remove this barrier. Even if new sites are activated after SV40 infection, they are governed by the temporal control over initiation events operating in normal cells. The only way that there can be an increase in the clustering around the median is if initiation at "new" sites occurs concurrently with initiations at the majority of preexisting sites.

To relate our findings to the demonstration that the length of the S phase does not change in Muntjac cells after SV40 infection (17), it is necessary to consider the concept of control mechanisms governing the temporal ordering of replication in entire segments of the genome (for a review, see references 2, 13, 20). Particular chromosomal regions are replicated at specific times within the S phase. A decrease in the length of time needed to replicate one or more segments because of a virus-induced increase in synchrony or a decrease in interinitiation distance need not necessarily affect the time of onset of replication in the next segment. Thus, the overall length of the S phase may be much more stable than would be suspected from the events occurring in localized regions of the fiber.

At 24 h after the infection of serum-deprived Muntjac cells with SV40, initiations appear to be physically closer together. This decrease is reflected in a change in the scale parameter α of the Weibull distribution of the pooled data. A significant decrease in α means that, on the average, all of the interinitiation distances are shortened proportionally, with the longer interinitiation distances being shortened to a greater extent. In SV40-infected Muntjac cells, if an initiation site is located at a point along a strand, the probability of finding a second site at any distance away from it along the strand is higher than it would be in the uninfected control cell.

There was a suggestion from the Kolmogorov-Smirnov test and the Keiding test that initiations

in serum-deprived cells occur in a more random (exponential) manner than in serum-enriched cells, but the differences were not statistically significant. There were no profound shifts in the organization of DNA replication within individual strands after SV40 infection, unlike changes observed after the exposure of cells to prolonged treatments with FUDR (7) or infection with reovirus (29). For this reason, we do not believe that entire clusters of replication units are being induced or repressed by SV40 infection in an order different from that operating in normal genome replication.

It is difficult to compare our data meaningfully with those of Martin and Oppenheim (37) and Oppenheim and Martin (39), who found the geometric mean interinitiation distance to be lower in SV40-transformed relative to nontransformed Chinese hamster lung and BALB/c 3T3 cells in complete medium, but to be higher in isoleucine-deprived medium. They were dealing with cellular DNA replication in established transformed lines, rather than events occurring early after infection and used a different method of deprivation. The labeling procedures also differed. Martin and Oppenheim employed a brief pulse-label and measured distances between track centers. Our procedure of following a short pulse of high-specific-activity label with a long period of labeling at low specific activity gave us greater confidence in identifying origins in prepulse and postpulse figures and correctly recognizing replication units that were on the same strand. We also believe that in our system, the nonparametric statistical analyses utilized made the fewest possible assumptions and provided precise and accurate representations of the data.

It is noteworthy that large T antigen has specific affinity for sequences at the origin of viral DNA replication (43, 48) and also binds to cellular DNA (17, 42). We consider that the additional sites used in SV40-infected Muntjac cells are sites normally employed by uninfected cells, but are being recognized by T antigen or a T-antigen-containing replication complex in the infected cells. T antigen may facilitate the formation of DNA replication complexes or promote the efficiency of recognition of existing origins in such a way that when multiple sites become available for replication in a segment of DNA, initiations can occur more closely together in time.

ACKNOWLEDGMENTS

This work was supported by Research Grant CA-18608 and by Program Project Grant CA-18213 awarded by the National Cancer Institute. B.R.J. is a postdoctoral fellow under the Institutional National Research Award CA-09256 from the National Cancer Institute.

We thank Robert Memoli for his assistance in scoring the autoradiograms. We also gratefully acknowledge the assistance of Terry Woodfield in the utilization of the TWOSAM program and thank Emanuel Parzen for helpful discussions.

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