Initial Site of Synthesis of Virus During Rescue of Simian Virus 40 from Heterokaryons of Simian Virus 40-Transformed and Susceptible Cells

GRACE H. WEVER, SAUL KIT, AND D. R. DUBBS

Division of Biochemical Virology, Baylor College of Medicine, Houston, Texas 77025

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Simian virus 40 (SV40) can be rescued from certain SV40-transformed hamster cells by fusion with susceptible African green monkey kidney (CV-1) cells, in the presence of ultraviolet-irradiated Sendai virus. We have determined the sites in which SV40 is produced during rescue in these heterokaryons. To determine the sequence, nuclei were isolated from fused cells at various times after fusion, separated on sucrose-density gradients, and assayed for infectious center formation and virus content on CV-1 monolayers. Virus was first detected in the transformed nucleus (40 hr postfusion), and later associated with both transformed and susceptible nuclei (68 to 72 hr). Viral rescue apparently does not depend upon the transfer of SV40 deoxyribonucleic acid to a susceptible CV-1 nucleus, since the transformed nucleus is the primary site of virus production. The time course of certain cytological events in the rescue process and in productive infection was found to be similar.

Virus can be rescued from certain simian virus 40 (SV40)-transformed cell lines by co-cultivation or fusion with susceptible lines (5, 7, 8, 12, 14, 16, 17). Under light microscopy, both transformed and susceptible nuclear types appear to contain virus several days after fusion (17). However, the subcellular sites in which virus is produced have not been determined.

Several lines of evidence indicate that a transformed nucleus can support viral replication, since rescue can occur in the absence of susceptible cells. (i) Virus is occasionally produced spontaneously, or after X-ray radiation by an SV40-transformed tumor line (14); (ii) virus is produced by transformed hamster kidney cells after induction by mitomycin (2). Thus, it seems logical to predict that, after the fusion of transformed and susceptible lines, virus will be found first in the transformed nucleus and that, after complete particles are released into the cytoplasm, infection of other nuclei in the heterokaryon occurs as a secondary process. An alternative explanation is that the fusion process triggers the release and transfer of SV40 deoxyribonucleic acid (DNA) to a susceptible or permissive nucleus in the heterokaryon for virus replication. Re-infection of the transformed nucleus must then be postulated to explain the presence of virus-specific changes in that nucleus.

It should be possible to determine whether

either of these sequences is consistent with actual cellular events by determining the initial site of synthesis of SV40. We have attempted to do this, using the following experimental approach. Nuclei were isolated from heterokaryons and separated on sucrose-density gradients; the various nuclear bands were then assayed on African green monkey kidney (CV-1) monolayers to determine (i) the percentage of nuclei which contained SV40, and (ii) the total viral content per nucleus in any given band. The distribution of the two nuclear types in the gradient was determined by autoradiography. These experiments were performed at various times after fusion to determine the sequence of sites in which SV40 appeared.

MATERIALS AND METHODS

Cell propagation and fusion. The growth of CV-1 and SV40-transformed hamster (TSV-5) cells has been described (4, 10). Prior to fusion, TSV-5 cells were radioactively labeled with ^3H -thymidine ($1\text{ }\mu\text{C}/\text{ml}$) for 24 hr, a time period sufficient to label 95 to 98% of the nuclei. CV-1 and TSV-5 cells were fused at a ratio of 2:1, or 1:1, by adding ultraviolet-irradiated Sendai virus to mixtures of the two cell types, as previously described (4), with the exception that 2 ml of fusion mixture was used per fusion tube. Cells (6×10^6) were seeded in 8-oz (226 g) prescription bottles and incubated at 37 C. The percentage of cell fusion was determined by planting 0.6×10^6 cells in Leighton

tubes containing gelatin-coated slides, and fixing the slides for autoradiography at the same time the bottles were harvested for nuclear isolation. Cells were synchronized by the addition of 10 μ g of 1- β -D-arabinofuranosylcytosine (ara-C) per ml for 24 hr. The cells were then rinsed, and fresh medium was added.

Nuclear isolation: unfused cells. Cells were harvested with trypsin-ethylenediaminetetraacetate, and washed once with Hanks balanced salt solution. Cells were suspended at a concentration of 10×10^6 /ml in homogenizing medium at 4 C (0.25 M sucrose-0.001 M MgCl_2 -0.001 M KH_2PO_4 - K_2HPO_4 , pH 6.5), and transferred to a Dounce homogenizer (7.0 ml capacity, tight-fitting pestle B) containing Triton X-100 (Packard Instrument Co. Inc., Downers Grove, Ill., liquid scintillation grade), final concentration 0.05%. The cell suspension was homogenized gently until approximately 80% of the cells were broken. This usually required 20 to 25 strokes. Breakage was monitored by phase contrast microscopy. An equal volume of 1.5 M sucrose-0.0015 M MgCl_2 -0.001 M KH_2PO_4 - K_2HPO_4 , pH 6.5, was added. (This solution and all subsequently mentioned sucrose solutions contained the same MgCl_2 and phosphate buffer.) The suspension was mixed and then centrifuged in 15-ml polyallomer tubes in a Sorvall SS34 rotor at 3,750 rev/min at 5 C for 10 min. The pellicle and supernatant fraction were carefully removed and discarded, and the pellet was resuspended in 0.5 ml of suspending medium (0.25 M sucrose). The nuclear suspension was diluted with 3 ml of 1.8 M sucrose, centrifuged at 8,000 rev/min in an SW39L rotor of a model L-2 centrifuge (Spinco) for 15 min at 5 C. The pellet was resuspended in 0.1 ml of suspending medium, diluted with 0.35 ml of 2.6 M sucrose; 0.2 ml of this suspension was layered over a gradient formed 2 to 3 hr prior to use. The gradient consisted of the following: 1.4 ml of 2.6 M sucrose, 0.9 ml of 2.5 M sucrose, 0.9 ml of 2.4 M sucrose, and 0.9 ml of 2.3 M sucrose. The gradient was centrifuged at 25,000 rev/min for 30 to 45 min in the SW39L rotor. The tube was then sliced between the interfaces and above the pellet by using a Beckman tube slicer. The nuclear concentration of each sample was determined in a hemocytometer. Samples of these bands were fixed for autoradiography with 4% formaldehyde overnight at 4 C. The nuclei were washed three times with distilled water, suspended in a minimum volume of water, and spotted on gelatin-coated slides with capillary tubes. The slides were air-dried, Feulgen-stained, and dipped in Kodak NTB-2 liquid emulsion. After 7 to 12 days of exposure, the autoradiographs were developed in Dektol for 2 min, rinsed in water for 30 sec, and acid-fixed for 3 min. The percentage of labeled nuclei in each band was determined by light microscopy. Cytoplasmic contamination was determined by phase-contrast microscopy of freshly isolated and fixed, stained preparations of nuclei.

Nuclear isolation: fused cells. The procedure described above was modified as follows. After harvest, smaller unfused cells were selectively removed by two slow-speed centrifugations (40 sec at approximately $300 \times g$, setting "1" on a Clay-Adams Safeguard centrifuge). The final cell suspensions contained 85 to 95% fused cells, both homokaryons and heterokary-

ons. The cell pellet was resuspended in 5 volumes of homogenizing buffer (0.001 M KH_2PO_4 - K_2HPO_4 , pH 6.5, 0.0004 M MgCl_2) for 3 min at 4 C, diluted with an equal volume of 1.5 M sucrose, and centrifuged in 15-ml tubes at 3,750 rev/min for 10 min in a Sorvall SS34 rotor. Nuclear pellets were resuspended in 0.5 ml of 0.25 M sucrose, diluted with 3 ml of 1.8 M sucrose, and centrifuged for 15 min at 10,000 rev/min in 5-ml polyallomer tubes in the SW39L rotor. The final pellets were resuspended in 0.25 M sucrose, diluted with 2.4 ml of 2.3 M sucrose and layered over 2 ml of 2.45 M sucrose. After 20 min of centrifugation at 18,000 rev/min in the SW39L rotor, the tube was sliced above and below the interface. The top layer, interface, and pellet were individually relayered on a second set of gradients (Table 1) and centrifuged for 60 min at 35,000 rev/min in the SW39L rotor.

Assay for SV40. To determine the percentage of fused cells or nuclei which would give infectious centers, 0.1-ml portions of serially diluted samples were plated on CV-1 monolayers from which the fluid medium had been removed by aspiration. The plates were immediately overlaid with five drops of agar, allowed to cool, and then overlaid with 5 ml of agar. After 1 week of incubation, a second agar overlay was added (4). Plaques were enumerated 12 to 18 days after plating. The total virus yield per fused cell or per isolated nucleus was determined by plating samples of sonically treated material, serially-diluted, on CV-1 monolayers.

RESULTS

Nuclear isolation and separation. Preliminary studies were made with mixtures of unfused CV-1 and TSV-5 cells to determine the feasibility of nuclear separation. A clean preparation of CV-1 nuclei, with almost no cytoplasmic contamination, was obtained in the bottom gradient band (Table 2). TSV-5 nuclei were concentrated above

TABLE 1. *Composition of the second sucrose density gradients for the separation of nuclei from fused CV-1 and TSV-5 cells*

First gradient band relayered	Second gradient	
	Sucrose	Vol
	M	ml
Top	2.15	0.9
	2.30	0.9
	2.45	2.0
2.3/2.45 M Interface	2.30	1.40
	2.45	0.95
	2.50	0.90
	2.70	0.90
Pellet	2.45	0.90
	2.50	0.95
	2.55	0.95
	2.70	1.40

the 2.3 M layer. However, these were always 20 to 30% contaminated with CV-1 nuclei with cytoplasm. These CV-1 nuclei could not be removed by rebanding. Many of the nuclei at the top of the gradient were large CV-1 nuclei with considerable cytoplasmic contamination.

The nuclear isolation procedure used for fused cells was a compromise; fused cells were more difficult to rupture than unfused mixtures, yet the nuclei were more fragile and more frequently carried cytoplasmic fragments. Similar difficulties in the isolation of nuclei from cells productively infected by herpes simplex have also been described (15). The breakage rate was improved in our experiments by including hypotonic shock prior to homogenization, and by changing the Mg^{++} and Triton X-100 concentrations in the homogenizing medium. The difficulties encountered in cell rupture at 0 to 16 hr after fusion were possibly due to membrane changes incurred during cell fusion. The increased resistance of fused cells to breakage after 16 hr could be related to cytoplasmic changes associated with virus production.

Nuclei isolated from fused cells were also more difficult to separate on sucrose gradients, because of the increased cytoplasmic contamination. A clean band of CV-1 nuclei was again found at the bottom interface of the gradient (2.5/2.6 M sucrose). The upper bands contained varying proportions of CV-1 nuclei with considerable cytoplasm. The procedure was therefore modified to include two sequential gradients, which usually

produced an improvement in separation. Typical data are presented in Table 3.

The ratio of unlabeled to labeled nuclei recovered from the first gradient corresponded to the initial fusion ratio. This would indicate that little selection of either nuclear type occurred during nuclear isolation when fused nuclei did not form an appreciable portion of the population. Nuclear fusion has been observed in all of our experiments. At 24 hr, approximately 10% of the nuclei in heterokaryons appear fused; by 48 hr this has increased to 23%. However, the conditions of the isolation procedure select against fused nuclei. Owing to their larger diameter and apparently greater fragility, they are readily damaged during homogenization, and are re-

TABLE 2. Sucrose density gradient separation of 3H -thymidine-labeled TSV-5 and unlabeled CV-1 nuclei isolated from mixtures of unfused CV-1 and TSV-5 cells

Gradient region	Labeled nuclei (%)
<i>M</i>	
Pellicle	42
2.2 (Nuclear layer)	63
2.2/2.3 Interface	80
2.3	59
2.3/2.4 Interface	45
2.4	23
2.4/2.5 Interface	8
2.5	5
2.5/2.6 Interface	3

TABLE 3. Sucrose density separation of 3H -thymidine-labeled TSV-5 and CV-1 nuclei isolated from fused cells at 40 and 68 hr postfusion

Time of harvest (hr postfusion)	Gradient	Gradient band	Labeled nuclei (%)
40	First	Top	48
		Interface	42
		Pellet	13
	Second		
		Rebanded top	2.15 M
		Rebanded interface	2.3/2.45 M Interface
			2.45/2.5 M Interface
			2.5/2.7 M Interface
		Rebanded pellet	2.45/2.5 M Interface
			2.5/2.55 M Interface
			2.55/2.7 M Interface
68	First	Top	27
		Interface	74
		2.45 M	9
		Pellet	4
	Second		
		Rebanded interface	2.45/2.5 M Interface
			2.5/2.7 M Interface

moved during the subsequent centrifugation-wash steps which precede the gradient separation. No fused nuclei are found in the lowest gradient bands. Some fused nuclei may be present in the top band; these are difficult to score with certainty in autoradiographs, but probably do not account for more than a few per cent of the total.

Approximately 10 to 20% of the nuclei in the starting homogenate were layered onto the first gradient, and 80% of these were recovered in the gradient fractions sampled for infectious center formation. Most of the loss was due to mechanical problems of sampling small volumes; the remainder (no more than 10%) were in the unsampled region between the top band and the 2.3/2.45 interface.

SV40 production. Nuclei were assayed on monolayers by using the infectious center technique to determine the percentage of nuclei which contained SV40 and the total virus yield per nucleus. A time course of infectious center formation is shown in Table 4. Nuclei isolated from TSV-5 cells, or from fused cells up to 30 hr after fusion, did not form infectious centers. Gradient-separated nuclei from fused cells at 34, 40, and 68 hr postfusion are shown in Table 5.

Although somewhat better separation of the nuclei was obtained by using a second gradient (Table 3), the data for infectious centers were usually less reliable, since nuclei were now spread

over a larger volume. In the analysis described below, only the most reliable data from this table and from similar experiments have been used.

Our primary objective was to determine the site, or sequence of sites, of SV40 production after fusion of transformed and susceptible cell lines. A complete separation of TSV-5 and CV-1 nuclei would have been the best material for such an analysis, since SV40 production could have been correlated directly with either or both nuclear types at any given time. Instead, our experimental procedures gave virtually pure CV-1 preparations, but TSV-5 nuclei were always contaminated with CV-1 nuclei, even when 1:1 fusion ratios were used. We therefore employed simultaneous equations, using the percentage of each nuclear type in a given gradient band (A, B) and the percentage of nuclei in that band which formed infectious centers (C) to calculate the

TABLE 4. *Time course of infectious center formation by nuclei from fused CV-1 and TSV-5 cells*

Time of harvest (hr postfusion)	Infectious center formation/nucleus
24	0
32	3.3×10^{-6}
40	4.0×10^{-4}
72	3.1×10^{-3}

TABLE 5. *Infectious center formation by sucrose density gradient-separated nuclei isolated from fused CV-1 and TSV-5 cells at 34, 40, and 68 hr after fusion (separate experiments)*

Harvest time (hr)	Gradient	Gradient region (M)	Nuclei/ml	Infectious centers/ml	Infectious centers/nucleus
34	First	2.0	21.5×10^6	7×10^2	3.3×10^{-6}
		2.3/2.45	1.5×10^6	5×10^1	3.3×10^{-6}
		Pellet	1.5×10^6	6×10^1	4.0×10^{-5}
40	First	2.0	9.5×10^6	50×10^2	5.3×10^{-4}
		2.3/2.45	3.7×10^6	18×10^2	4.9×10^{-4}
		Pellet	2.9×10^6	35×10^1	1.2×10^{-4}
	Second	Rebanded 2.0	4.4×10^6	9×10^2	2.0×10^{-4}
		Rebanded 2.3/2.45	0.86×10^6	31×10^1	3.6×10^{-4}
		2.45/2.5	0.36×10^6	2×10^1	0.56×10^{-4}
		2.5/2.7	0.33×10^6	1×10^1	0.30×10^{-4}
		Rebanded pellet	0.21×10^6	6×10^1	2.9×10^{-4}
		2.5/2.55	0.23×10^6	3×10^1	1.3×10^{-4}
		2.55/2.7	0.38×10^6	1×10^1	0.27×10^{-4}
68	First	2.0	1.12×10^6	8.0×10^1	7.1×10^{-5}
		2.3/2.45	5.50×10^6	2.9×10^2	5.3×10^{-6}
		2.45	0.13×10^6	1.0×10^1	7.7×10^{-6}
		Pellet	1.25×10^6	1.5×10^1	1.2×10^{-5}
	Second	Rebanded 2.3/2.45	0.10×10^6		
		2.45/2.5	0.47×10^6	5.0×10^1	1.1×10^{-4}

contribution of each nuclear type to infectious center formation (X, Y). The data for these equations were drawn from bands which had relatively large differences in their relative contents of TSV-5 and CV-1 nuclei. For any two bands, the following equations were used:

$$\text{Band 1: } A_1X + B_1Y = C_1$$

$$\text{Band 2: } A_2X + B_2Y = C_2$$

The results of these analyses are presented in Table 6. The following statements summarize the data. (i) At 40 hr, 91 to 100% of the nuclei which contained SV40 were TSV-5 nuclei. (ii) By 68 to 72 hr, this percentage shifted to between 34 and 60%, i.e., CV-1 nuclei now contributed appreciably. The same results were obtained whether cells were unsynchronized or were synchronized by ara-C treatment.

Our use of this method of analysis is based on the assumption that nuclei of either type have not been stratified in the gradient according to their ability to form infectious centers. Otherwise, the values for X and Y would be different for each gradient band. We tested this assumption by

an indirect experimental approach. CV-1 cells were infected with SV40 and grown for 40 hr postinfection. Nuclei were then isolated and layered on sucrose density gradients. The data in Table 7 show that the percentage of CV-1 nuclei containing SV40 does not vary appreciably throughout the gradient. If this result can be extended to fused cells, our method of analysis is valid.

To test the possibility that virus was adsorbed to nuclei during isolation, SV40 was added in a control experiment to a homogenate of fused cells harvested at 16 hr after fusion, when no virus can be detected. The amount of virus added corresponded to the amount normally present at 40 hr postfusion. Unadsorbed virus was removed during the two centrifugation-wash steps which precede the gradient separation of the CV-1 nuclei. The data in Table 8 show that (i) most of the adsorbed virus is found in nuclear bands which have some cytoplasmic contamination, i.e., the top and interface band, and (ii) the amount of virus adsorbed by the nuclei corresponds to 10, 0.1, and less than 0.1% of the amount usually

TABLE 6. *Relative contributions of TSV-5 and CV-1 nuclei to infectious center formation at 40, 68, and 72 hr after fusion*

Cell treatment	Hr after fusion ^a	Gradient bands used for analysis	Labeled nuclei	Infectious centers/nucleus	Gradient bands compared	(X) Infectious centers/TSV-5 nucleus	(Y) Infectious centers/CV-1 nucleus	Infectious centers formed by TSV-5 nucleus
		<i>M</i>	%		<i>M</i>			%
Unsynchronized, fused 2:1	40	Top	48	5.3×10^{-4}	Top and 2.3/2.45	10.7×10^{-4}	0.70×10^{-4}	94
		2.3/2.45	42	4.9×10^{-4}	Top and pellet	11.9×10^{-4}	-0.41×10^{-4}	104
		Pellet	13	1.2×10^{-4}	2.3/2.45 and pellet	12.3×10^{-4}	-0.46×10^{-4}	104
Unsynchronized, fused 1:1	40	Top	29	4.8×10^{-5}	Top and 2.3/2.45	33.2×10^{-5}	-6.80×10^{-5}	126
		2.3/2.45	24	2.8×10^{-5}	Top and 2.15	18.1×10^{-5}	-0.63×10^{-5}	104
		2.15 ^b	37	6.3×10^{-5}	2.3/2.45 and 2.15	23.3×10^{-5}	-3.67×10^{-5}	119
Synchronized, fused 2:1	40	Top	6	0.7×10^{-5}	Top and 2.15/2.3	4.7×10^{-5}	0.44×10^{-5}	91
		2.3/2.45	6	0.6×10^{-5}	2.3/2.45 and 2.15/2.3	6.0×10^{-5}	0.26×10^{-5}	96
		2.15/2.3 ^b	13	1.0×10^{-5}				
Synchronized, fused 1:1	40	2.3/2.45 ^b	36	5.0×10^{-5}	2.3/2.45 and 2.45/2.5	1.2×10^{-4}	0.10×10^{-4}	92
		2.45/2.5 ^b	17	2.9×10^{-5}	2.3/2.45 and 2.5/2.7	1.3×10^{-4}	0.12×10^{-4}	92
		2.5/2.7 ^b	11	1.7×10^{-5}	2.45/2.5 and 2.5/2.7	2.0×10^{-4}	-0.05×10^{-4}	108
Unsynchronized, fused 2:1	68	Top	27	7.2×10^{-5}	Top and 2.3/2.45	4.3×10^{-5}	8.3×10^{-5}	34
		2.3/2.45	74	5.3×10^{-5}	Top and 2.45	5.1×10^{-5}	8.0×10^{-5}	39
		2.45	9	7.7×10^{-5}	2.3/2.45 and 2.45	4.3×10^{-5}	8.0×10^{-5}	35
Unsynchronized, fused 2:1	72	2.3/2.45	55	4.3×10^{-5}	2.3/2.45 and 2.45/2.7	5.6×10^{-5}	3.8×10^{-5}	60
		2.45/2.7	10	3.0×10^{-5}				
Synchronized, fused 2:1	68	Top	16	13.3×10^{-5}	Top and 2.3	9.1×10^{-5}	14.1×10^{-5}	39
		2.3 ^b	32	12.5×10^{-5}				

^a Or after release of synchronization by changing medium.

^b Second gradient.

TABLE 7. *Formation of infectious centers by gradient-separated CV-1 nuclei isolated from CV-1 cells 40 hr after infection with SV40^a*

Multiplicity of infection (PFU ^c /cell)	Nuclear sample	Infectious centers/ml	Nuclei/ml	Infectious centers/nucleus
0.02	Unbanded nuclei	75×10^4	21.3×10^8	35×10^{-3}
	2.05/2.3 M Interface	64×10^3	3.8×10^8	17×10^{-3}
	2.45/2.7 M Interface	54×10^3	1.9×10^8	28×10^{-3}
1	Unbanded nuclei	66×10^5	20×10^8	33×10^{-2}
	Top of gradient	50×10^4	1.6×10^8	31×10^{-2}
	Nuclear layer/2.05	66×10^4	1.5×10^8	44×10^{-2}
	2.45/2.7 M Interface	34×10^4	1.5×10^8	23×10^{-2}

^a Nuclei were washed as described in Materials and Methods prior to layering on sucrose density gradients.

^b Plaque-forming units.

TABLE 8. *Infectious center formation by gradient-separated nuclei from (i) fused cells, 16 hr postfusion; (ii) fused cells, 16 hr postfusion, to which virus was added during homogenization^a; and (iii) fused cells, 40 hr postfusion^b*

Time of harvest (hr postfusion)	Amt of virus added (PFU ^c /cell)	Infectious centers/nucleus		
		Top	2.3/2.45 M Interface	Pellet
16	0	0	0	0
16	3×10^{-4}	3.4×10^{-5}	2.2×10^{-7}	0
40	0	5.5×10^{-4}	4.9×10^{-4}	1.2×10^{-4}

^a Virus was added directly to the homogenate. Nuclei were washed as described in Materials and Methods prior to layering on sucrose density gradients.

^b Nuclei were not relayered on a second gradient for this experiment.

^c Plaque-forming units.

present in the same nuclear bands in 40-hr fusion samples. This error has been neglected in our calculations since it does not appreciably affect the results.

Virus pool size. The size of the virus pool in either CV-1 or TSV-5 nuclei in fused cells can be directly calculated from our data for several gradient samples. (i) At 40 hr, when TSV-5 nuclei contribute 91 to 100% of the infectious centers, the value of plaque-forming units per infectious center ranges from 2 to 5; and (ii) at 68 to 72 hr, for the lowest gradient band which contains 95 to 98% CV-1 nuclei, 18 plaque-forming units are found per infectious center. If this value can be used for CV-1 nuclei in other gradient bands, the pool size of TSV-5 nuclei can be calculated. The values obtained for TSV-5 nuclei in this manner are smaller than at 40 hr, or are negative numbers (range 0.4 to -0.3). One explanation for this is that the viral pool size for TSV-5 and/or CV-1 nuclei is not constant throughout the gradient. A second explanation is that, by 72 hr after fusion, the TSV-5 nuclei show appreciable membrane damage due to virus production. Calculations of infectious center

formation or viral pool size for TSV-5 nuclei at this time point would then be invalid. In any case, neither of these explanations changes the conclusions drawn from these experiments. At the earliest time points, when virus is associated almost exclusively with TSV-5 nuclei, gradient-separated nuclei do not show appreciable damage. Virus appears in CV-1 nuclei only at later time points, and these nuclei also appear morphologically intact under phase-contrast microscopy. Only the magnitude of the contribution of TSV-5 nuclei at later time points remains in question.

DISCUSSION

The results of these experiments show that, after fusion of TSV-5 and CV-1 cells, SV40 first appears in the transformed TSV-5 nucleus and later is associated with both nuclear types. Based on these results, we suggest the following time table of events. SV40 DNA synthesis is activated in the transformed TSV-5 nucleus, and a pool of virus particles is formed. Virus production is confined to the TSV-5 nucleus until at least 40 hr after cell fusion. Virus particles are subsequently released into the cytoplasm and secondar-

ily infect other nuclei in the heterokaryon. The ratio of CV-1 and TSV-5 nuclei containing virus at this time (approximately 68 to 72 hr) corresponded in two experiments to the initial cell fusion ratio (2:1), suggesting that all nuclei in a heterokaryon produce virus.

This schedule of events in the rescue process agrees quite well with cytological observations of productive infection by SV40. Mayor et al. (13) reported that virus particles were found at 48 hr after infection only in the nucleus, and at 60 hr in both the nucleus and cytoplasm. Granboulan et al. (9) found that, in the electron microscope, virus could be seen in the nuclei in rare instances as early as 10 to 12 hr postinfection, but usually appeared between 24 and 48 hr postinfection. Cytoplasmic virus usually appeared by 6 days postinfection. (The fact that virus formation was not synchronous in their study prompted us to synchronize our cells prior to or after fusion with ara-C, with the hope that the sequence of viral association with the two nuclear types would be more clearly seen. However, the results with synchronized and unsynchronized cells were essentially the same.) The parallels between these studies and ours suggest that the rescue process may be very similar to productive infection. Similarities between these two processes have also been extended to the molecular level. Infectious DNA synthesis begins at approximately the same time in both rescue and productive infection (11).

The ability of the transformed nucleus to serve as the initial replicating site for virus production indicates that the first step in the rescue process is *not* the transfer of SV40 DNA to a permissive or susceptible nucleus in the heterokaryon. Instead, a transformed nucleus itself is capable of supporting viral replication. The contribution of the permissive cell is still not understood, and is further obscured by the fact that some transformed lines produce virus spontaneously, or after treatment with X-rays or mitomycin (2, 14). Thus, virus production and transformation are not necessarily mutually exclusive responses of a given cell type to an oncogenic virus. Both responses can be exhibited at least by those lines which can be transformed.

The fact that SV40 is associated with both TSV-5 and CV-1 nuclei later in the rescue process raises the following questions. (i) Are CV-1 nuclei simply repositories for virus particles synthesized elsewhere? (ii) If CV-1 nuclei also synthesize SV40, is SV40 DNA or a completed virus particle transferred to the CV-1 nucleus? (iii) At what time during rescue does the transfer process occur?

The following answers are based on our results.

(i) The virus associated with CV-1 nuclei at 68 to

72 hr after fusion could represent a redistribution of the viral pool synthesized in the TSV-5 nucleus. We have no reason to believe, however, that CV-1 nuclei are incapable of replicating SV40 during rescue, since virus-specific changes appear in susceptible nuclei (17). The continued synthesis of viral DNA (11) and the increased virus content of the nuclei at 68 to 72 hr (Table 4) suggest that production of virus by CV-1 nuclei could account for part of these increases. (ii) Our experiments did not determine whether SV40 is transferred as a DNA molecule or a complete virion. Mechanisms for survival and transfer of SV40 DNA through the cytoplasm to the nucleus must exist, since certain cell lines can be transformed or productively infected by SV40 DNA alone (1, 3, 6). Infectious DNA survives for at least 50 hr after abortive infection of mouse kidney cells by SV40 DNA (11). If SV40 DNA is transferred to CV-1 nuclei, our first question can be answered: virus found in CV-1 nuclei at 68 to 72 hr is actually synthesized there. (iii) It is likely that the transfer of SV40 to the CV-1 nucleus does not occur at a very early time point. Otherwise, we would expect to find the same percentage of infectious center formation by both nuclear types at early time points. On the other hand, we do not exclude early transfer, since a time lag in the CV-1 nucleus (somewhat analogous to the eclipse observed in productive infection) may be required before virus can be replicated at this site.

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