JOURNAL OF VIROLOGY, Dec. 1974, p. 1530-1546

Vol. 14, No. 6
Printed in U.S.A.

Simian Virus 40-Host Cell Interactions

II. Cytoplasmic and Nucleolar Accumulation of Simian Virus 40 Virion Protein

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Received for publication 21 June 1974

We have used immunofluorescence in parallel with transmission and scanning electron microscopy to characterize the unusual cytoplasmic and nucleolar accumulation of Simian virus 40 (SV40) virion protein (C antigen) at restrictive temperatures (39 to 41 C) in monkey cells infected with a temperature-sensitive mutant of SV40 defective in virion assembly, *tsB11*. Cytoplasmic and nucleolar accumulation of C antigen did not occur in wild-type-infected cells at any temperature. Wild-type- and *tsBll*-infected cells were not distinguishable at 33 C by immunofluorescence or electron microscopy. Temperature-shift experiments using metabolic inhibitors of DNA (cytosine arabinonucleoside, 20 μg/ml), RNA (actinomycin D, 5 μg/ml), and protein synthesis (cycloheximide, 2×10^{-4} to 10×10^{-4} M) were used to investigate the requirements for ongoing DNA, RNA, and protein synthesis in the distribution of virion protein between the nucleus, nucleolus, and cytoplasm. The transport of C antigen from the nucleolus and cytoplasm into the nucleus was complete after a temperature shift-down (41 and 39 to 33 C). Limited virus particle formation occurred after the shift-down in the presence of actinomycin D and cycloheximide, indicating some of the 39 to 41 C synthesized virion protein could be used for capsid assembly at 33 C in the absence of further virion protein synthesis. Nucleolar and cytoplasmic accumulations of C antigen occurred in the absence of drugs after a shift-up (33 to 39 C and 41 C) indicating a continuous requirement for the *tsB11* mutant function. Furthermore, the virion protein synthesized at 33 C remained confined to the nucleus when the cells were shifted to 39 and 41 C in the presence of actinomycin D or cycloheximide. In the presence of cytosine arabinonucleoside, however, the virion protein accumulated in large aggregates in the nucleus and nucleolus after the shift-up, but did not migrate into the cytoplasm as it did in drug-free *tsB11*-infected control cells. Colchicine (10^{-3} M) had no effect on the abnormal accumulation of C antigen during shift-up or shift-down experiments suggesting that microtubular transport plays little if any role in the abnormal transport of *tsB11* virion protein from cytoplasm to nucleus. Although virus particles were never observed by electron microscopy and V antigen was not detected by immunofluorescence at 39 or 41 C in *tsB11*-infected cells, dense amorphous accumulations were formed in the nucleoli and cytoplasm. We suggest that the *tsB11* function is continuously required for the normal transport of SV40 virion protein between the cytoplasm, nucleolus, and nucleus and for the assembly of capsids and virions. Several possible mechanisms for the altered *tsB11* function or protein are discussed. One of the virion proteins may also be involved in some presently undetermined nucleolar function during SV40 productive infection.

The molecular mechanisms underlying the cytoplasmic-nuclear transport of proteins in mammalian cells are poorly understood (7). Similar mechanisms are probably used to transport the cytoplasmically synthesized, virus-specific proteins that are involved in the viral functions and nuclear assembly of several DNA-containing animal viruses (e.g., Simian virus 40 [SV40], polyoma, adenovirus, herpesvirus). These transported virus-specific proteins include the tumor (T) antigens, which are early viral functions not requiring viral DNA synthesis, as well as virion structural proteins, which are late functions requiring viral DNA synthesis

(2). The investigation of mutant viruses that are altered in the normal cytoplasmic-nuclear transport of virus-specific proteins should provide some insight into the underlying cellular transport mechanisms and possibly into the oncogenic mechanisms possessed by these viruses. Such mutant viruses have been isolated for T antigen transport in SV40 (3) and virion protein transport in polyoma (1) and adenovirus (10).

We have begun to characterize the defective transport of virion protein in productive monkey cells infected by a temperature-sensitive mutant of SV40, *tsB11*, that was shown to be defective in virion assembly by Tegtmeyer and Ozer (21). Immunofluorescence has been used in parallel with transmission and scanning electron microscopy to characterize the abnormal cytoplasmic and nucleolar accumulation of the virion protein during *tsB11* infection using temperature-shift experiments and metabolic inhibitors of DNA, RNA, and protein synthesis. Tegtmeyer et al. have recently characterized the mutant for viral DNA synthesis, virion protein synthesis, and nucleoprotein complex assembly (22).

MATERIALS AND METHODS

Cell line and medium. The TC7 subline (17) of African green monkey kidney CV-1 cells was grown in powdered Dulbecco-Vogt modified Eagle medium (Flow Laboratories, Rockville, Md.) supplemented with 9% fetal bovine serum (FBS, Flow Laboratories) and buffered with 60 mM N-Tris-glycine (Tricine, Sigma Chemical Co., St. Louis, Mo.) and 1.0 g of $NaHCO_3$ per liter at pH 7.7.

Virus stocks. Wild-type virus (SV-S [20]) and *tsB11* (21) were grown in TC7 cells at 33 C with an MOI of 0.01 and were prepared for cell infection as previously described (18). In all experiments, TC7 cells were mixed in suspension with virus at an MOI of 5 and were incubated for 30 min at 25 C before plating in 32- or 52-mm plastic petri dishes (Falcon). After plating, the infected cultures were incubated for various periods at 33, 39, or 41 C in medium supplemented with 5% FBS. Mock infections were carried out by using TC7 cell lysates without virus.

Serological assays. The infected monolayers in 32-mm petri dishes were fixed and stained by the indirect immunofluorescent technique as previously described (16). Individual dishes were simultaneously stained for all three antigens (T, C, and V) by dividing each dish into thirds with vacuum grease. Antigen-positive cells were observed using a ×40 water immersion objective that provided a final magnification of ×500. The preparation, characterization, and dilution of the anti-T, anti-C, and anti-V sera have been previously described (19), but can be summarized as follows. The anti-T serum does not react with U antigen. The anti-C serum reacts with the 45,000 (VP1), 35,000 (VP2), and 25,000 (VP3) dalton virion proteins and intact capsids, but not with capsomeres (15). The anti-V serum reacts only with intact capsids.

Transmission electron microscopy. Mock- and viral-infected cells were gently removed from 52-mm plastic petri dishes with 0.05% doubly crystallized trypsin-0.06% ethyleneglycol-bis-(beta-amino-ethyl ether) N,N'-tetraacetic acid (EGTA, Sigma Chemical Co.), resuspended in Dulbecco-Vogt medium with 5% FBS, and centrifuged at 5000 rpm for 5 min in-a Sorvall GCL centrifuge at 25 C. After rinsing once with phosphate buffered saline (PBS), the pellet was fixed with 2.5% glutaraldehyde in PBS (pH 7.4) for 20 min at 4 C, post-fixed with 1.0% osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in Araldite (6). The sections were cut with an LKB ultramicrotome and stained with uranyl acetate and lead citrate for examination with a Siemens Elmiskop 101.

Scanning electron microscopy. Mock- and virus-infected cells were grown on glass cover slips in 32-mm plastic petri dishes. The monolayers were washed twice with isotonic PBS, fixed for 15 min with 0.1% glutaraldehyde in isotonic PBS at 4 C, and then fixed in 1.0% glutaraldehyde for 60 min at 4 C. After two rinses with isotonic PBS, the monolayers were dehydrated with increasing concentrations of ethanol and finally heated with freon using the critical point drying method (4). The monolayers were then shadowed with gold and examined with an Autoscan scanning electron microscope.

Light microscope autoradiography. TC7 cells were infected in suspension with WT and *tsB11* at an MOI of 5, plated into 32-mm petri dishes, and incubated at 33, 39, and 41 C. Newly synthesized DNA was labeled with [*methyl*-^{3}H] thymidine (3 μCi/ml, 15 Ci/mM) for various times. The cells were then rinsed twice with serum-free medium (SFM) and incubated 15 min to 1 h in medium containing 5% FBS, but no additional thymidine. After the chase, the monolayers were rinsed five times with PBS and then fixed in absolute ethanol:glacial acetic acid (3:1) for 15 min at room temperature. After air drying, 0.5 ml of Eastman Kodak NTB3 emulsion was placed in each petri dish and the excess was poured off. The dishes were developed after 7 days at 4 C and the cells were stained with Giemsa.

RESULTS

Time course of infection. Previous studies have shown that *tsB11*-infected monkey cells synthesize VP1 at normal rates at 41 C, but are inhibited in the accumulation of VP1 in the nucleus and completely blocked in the subsequent assembly of capsids (V antigen) (21, 22). Because of this lack of capsid formation, the free capsid proteins VP1, VP2, and VP3 could be detected by anti-C serum in *tsB11*-infected cells at 39 and 41 C. Mono-specific antisera for VP1, VP2, and VP3 are not presently available. Although T antigen was confined solely to the nuclei excluding the nucleoli at 39 to 41 C as

previously described (19), the anti-C serum staining occurred in the cytoplasm and nucleoli as well as in the nuclei.

To investigate the time course of the abnormal C antigen accumulation at 39 to 41 C, the following experiment was performed using transmission and scanning electron microscopy as well as monitoring the T, C, and V antigens. TC7 cells were infected with *tsB11* virus at an MOI of 5 and were incubated at 39 and 41 C. At various times, the infected cells were fixed for immunofluorescent staining or processed for electron microscopy. The immunofluorescent data are given in Table 1. *T antigen*: Appearance of T preceded C, but the final number of T- and C-positive cells was equal at 48 HAI. The cytoplasm and nucleoli were always negative for T. *C antigen*: At 18 h after infection (HAI), about 5% of the total cells had bright-C positive nuclei with equally bright nucleoli and negative cytoplasm at both 39 and 41 C. By 24 HAI, the nuclear staining at 41 C was fainter, producing a marked contrast to the increasingly bright nucleoli. At 39 C, some cytoplasmic fluorescence had appeared in a few percent of the cells along with negative nuclei and brightly positive nucleoli. By 30 HAI, the nuclear staining at 41 C had decreased and was completely absent in about 10% of the cells with the nucleoli remaining very bright (Fig. 1C). At 39 C, a similar percentage of cells had lost their nuclear staining leaving most of the fluorescence in the cytoplasm with some residual fluorescence in nucleoli. Late in the infection (48 HAI), the majority of infected cells at 41 C exhibited bright nucleoli without nuclear fluorescence and many cells had faint cytoplasmic staining (Fig. 1D). In most of the infected cells at 39 C, nucleolar staining was decreased, nuclear staining was absent, and cytoplasmic staining was bright (Fig. 1B). Figure 1A demonstrates the reversibility of the abnormal accumulation following a shift to 33 C. *V antigen*: Detectable V-positive fluorescence was not observed at any time during the infections at either 39 or 41 C. Wild-type virus-infected cells at 33, 39, and 41 C and *tsB11*-infected cells at 33 C were not distinguishable, having T, C, and V antigen staining confined to the nuclei excluding nucleoli at all times (data not shown, see ref. 20).

Transmission electron microscopy. In parallel with the above immunofluorescent monitoring, replicate-infected monolayers were prepared for transmission electron microscopy. Mock- and wild-type-infected monolayers were included as controls. No differences between the mock- or viral-infected cultures were seen until 18 HAI at 41 C when the nucleoli in both the *tsB11* and wild-type-infected cells began to enlarge with some condensation of the nucleolonema. By 24 HAI at 41 C in *tsB11*-infected cells, small dense accumulations of amorphous material were within the nucleoli (Fig. 3A) and many nucleoli were separating into pieces. The wild-type-infected cells had numerous vacuoles, a few separating nucleoli, and some virus particles confined to nuclei excluding the nucleoli, but no amorphous accumulations (Fig. 2B). By 36 and 48 HAI at 41 C in *tsB11*-infected cells, large dense accumulations were present in and around nucleoli and occasionally in the cytoplasm (Fig. 3B, 4A and B). Virus particles and vacuolation were never observed. In the wild-type-infected cells at 39 and 41 C, virus particles continued to accumulate in the nuclei excluding the nucleoli with an occasional cell having cytoplasmic particles associated with

TABLE 1. Time course of T, C, and V antigen appearance in *tsB11*-infected TC7 cells at restrictive temperatures

Time (HAI)	Total cells displaying the monitored antigen in the nucleus (Nu), Nucleolus (Nl), or cytoplasm (Cy) (%)											
	T antigen 39 and 41C			C antigen[a] 39C			C antigen[a] 41C			V antigen 39 and 41C		
	Nu	Nl	Cy	Nu	Nl	Cy	Nu	Nl	Cy	Nu	Nl	Cy
12	1	0	0	0	0	0	0	0	0	0	0	0
18	12	0	0	5	0	0	4	0	0	0	0	0
24	34	0	0	25	1[f]	3	45	20	0	0	0	0
30	43	0	0	39	8[f]	12	45	33	0	0	0	0
36	59	0	0	10	41[f]	52	22	40	10[f]	0	0	0
48	57	0	0	5	30[f]	55	20	44	20[f]	0	0	0

[a] [f], Faint.

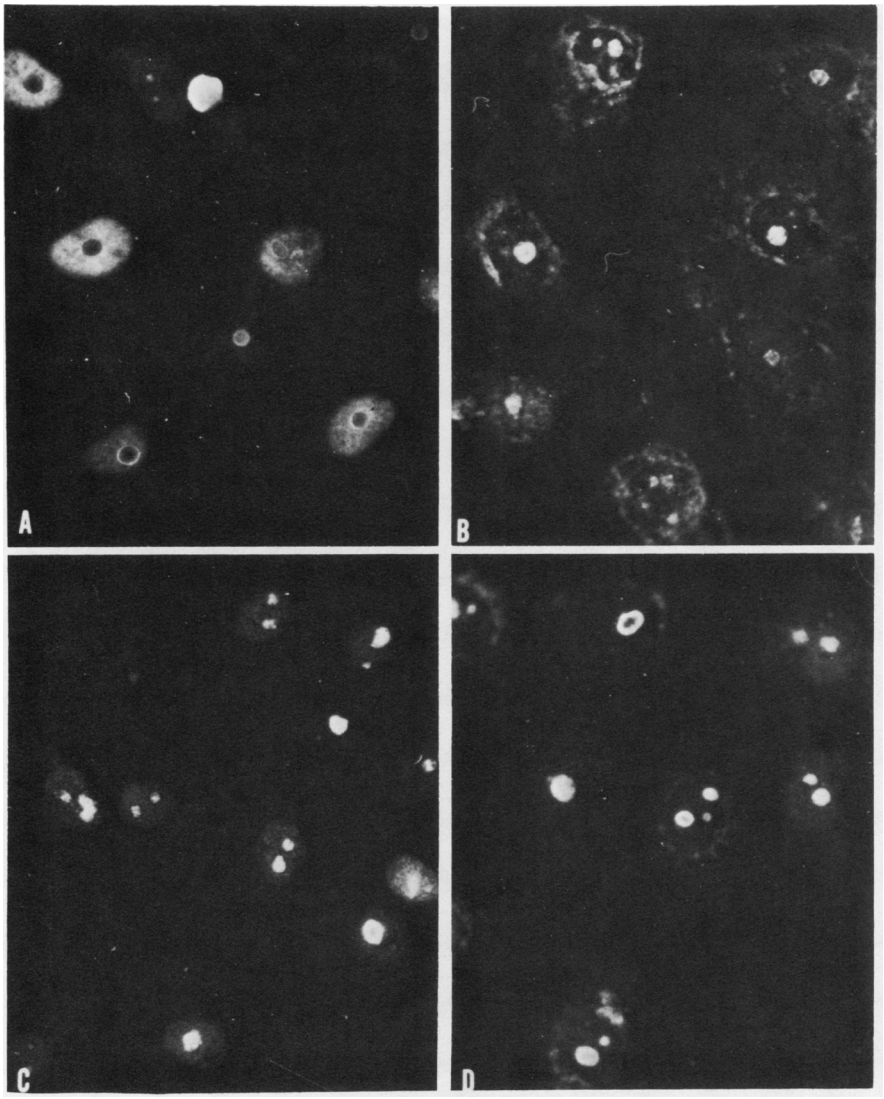

FIG. 1. *Location of C antigen in tsB11-infected TC7 cells by immunofluorescence using SV40 anti-C serum: (A) Cells incubated at 41 C for 48 h and then shifted to 33 C for an additional 24 h; (B) incubation for 48 h at 39 C; (C) incubation at 41 C for 30 h; and (D) incubation at 41 C for 48 h. Original magnification ×650.*

the outer nuclear membrane and some membranes of the endoplasmic reticulum. At 39 C in the *tsB11*-infected cells, some accumulations similar to the accumulations at 41 C were present in the nucleoli, whereas extensive accumulations appeared in the cytoplasm often associated with microtubules (Fig. 4A and B). However, virus particles and vacuolation were never observed. The *tsB11*-infected cells at 33 C were not distinguishable from wild-type-infected cells at 33, 39, or 41 C. Mock-infected cells had no accumulations, virus particles, separating nucleoli, or significant vacuolation at any time or temperature (Fig. 2A).

Scanning electron microscopy. Scanning electron microscopy was done in parallel with the immunofluorescent and transmission electron microscopy experiments above using mock-, wild-type-, and *tsB11*-infected cells. The results are presented in Fig. 5, 6, and 7. The wild-type virus infections at all three temperatures produced swollen nuclei with slightly enlarged nucleoli and little cytoplasmic accumulation of new material. However, there was a

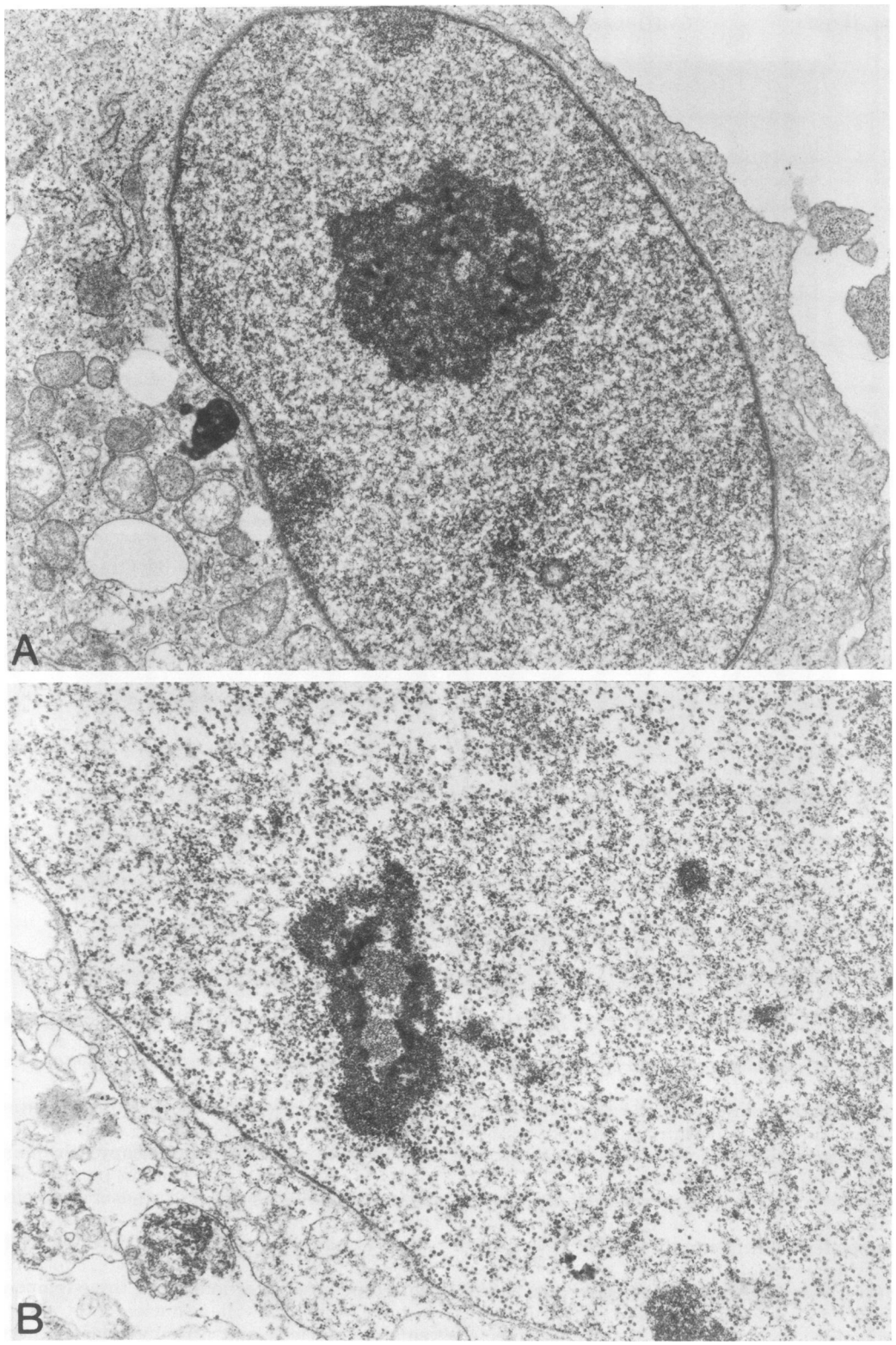

FIG. 2. *Transmission electron micrographs of: (A) uninfected TC7 cell at 39 C for 48 h (×11,000) and (B) wild-type-infected TC7 cell at 39 C for 48 h (×19,500).*

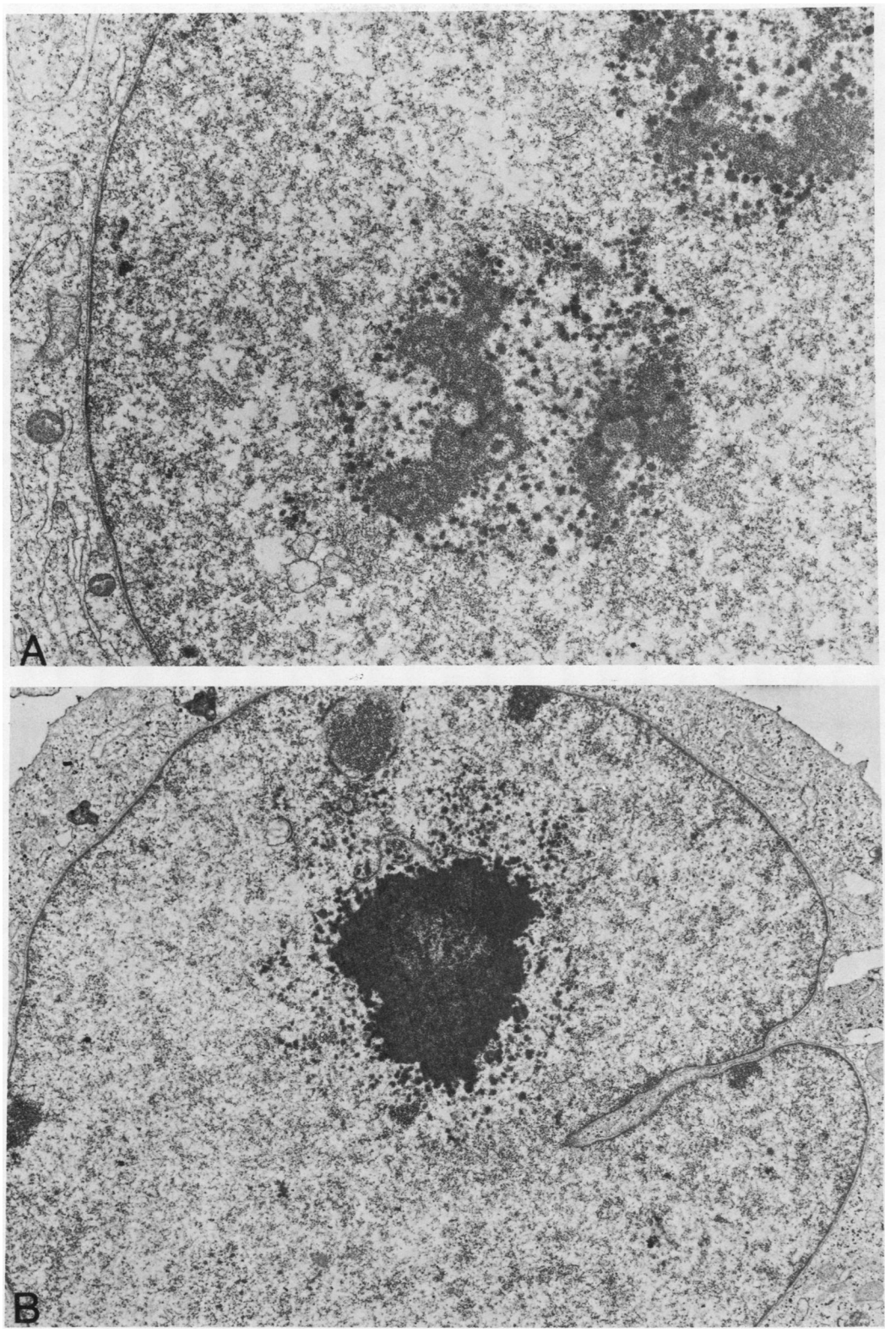

FIG. 3. *Nucleolar accumulations in transmission electron micrographs of tsB11 infected cells at: (A) 41 C for 24 h (×14,000) and (B) 41 C for 48 h (×11,000).*

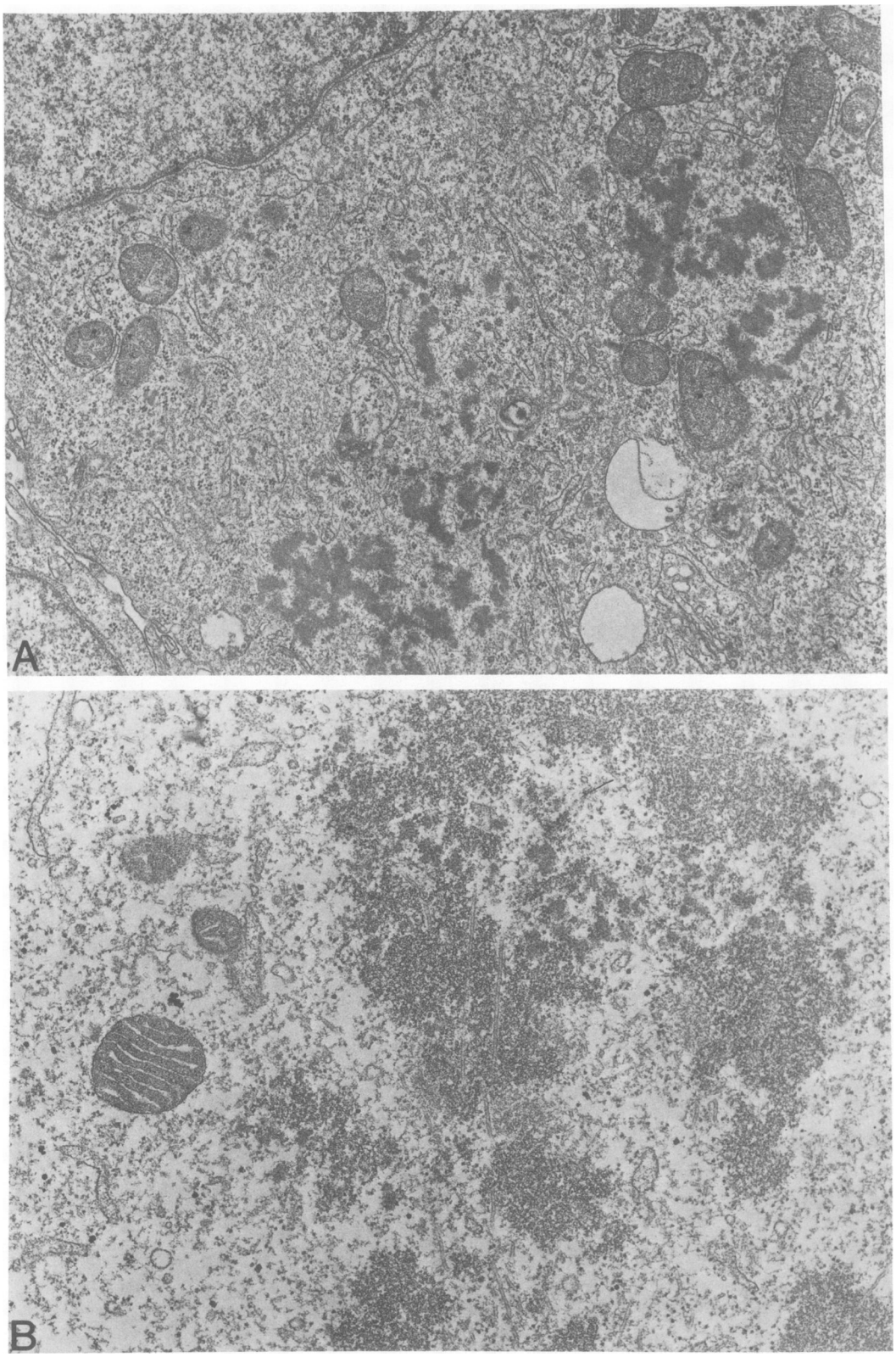

FIG. 4. *Cytoplasmic accumulations in transmission electron micrographs of tsB11-infected TC7 cells at: (A) 39 C for 48 h (×18,000) and (B) 39 C for 48 h (×27,000).*

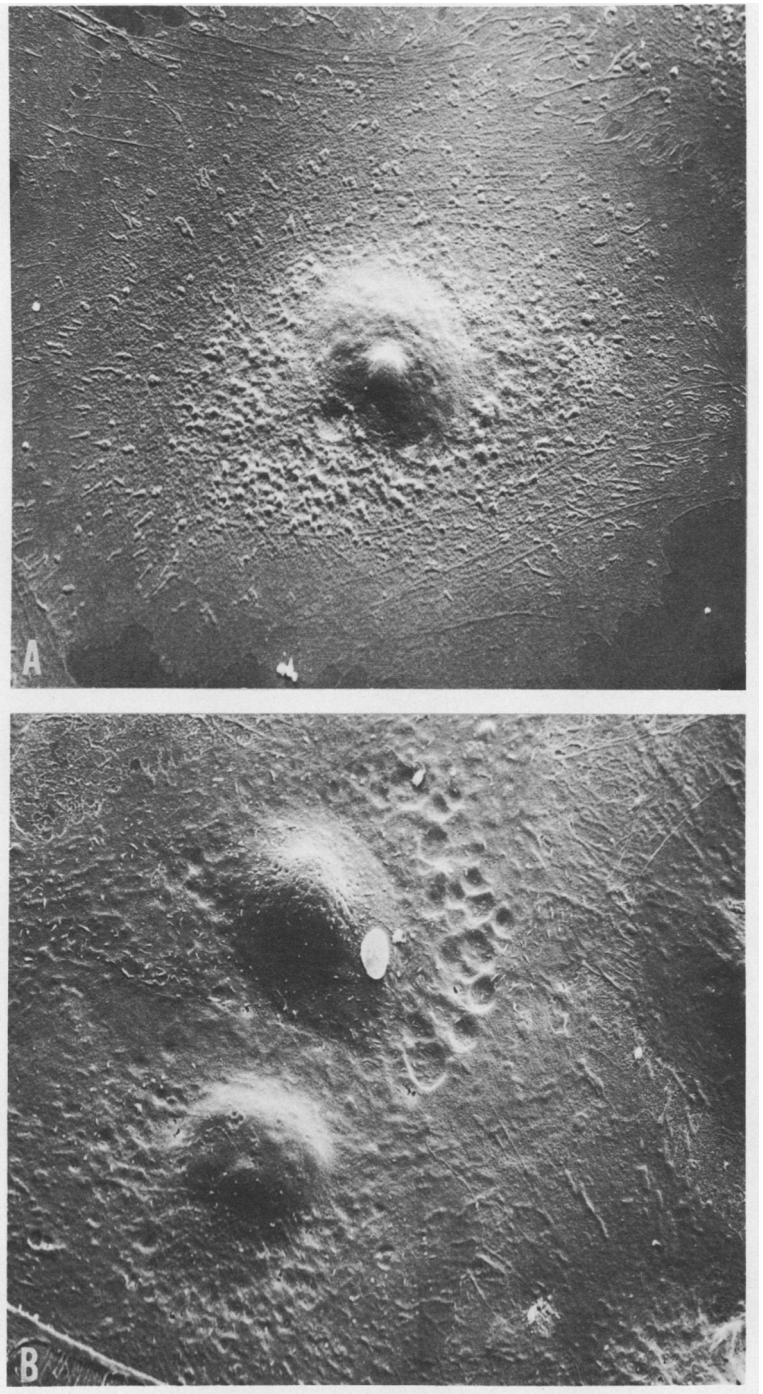

FIG. 5. *Scanning electron micrographs of: (A) uninfected TC7 cell at 39 C for 48 h (×1,030) and (B) wild-type-infected TC7 cells with nuclear swelling and cytoplasmic vacuolation at 39 C for 48 h (×1,020).*

preferential accumulation of microbodies and mitochondria on one side of the nucleus which did not occur in the mock-infected cells. Late in infection, cytoplasmic vacuoles accumulated with their initial appearance corresponding to the perinuclear site of microbody accumulation (Fig. 5B), although the reason for the origin of the vacuoles could not be determined. At this

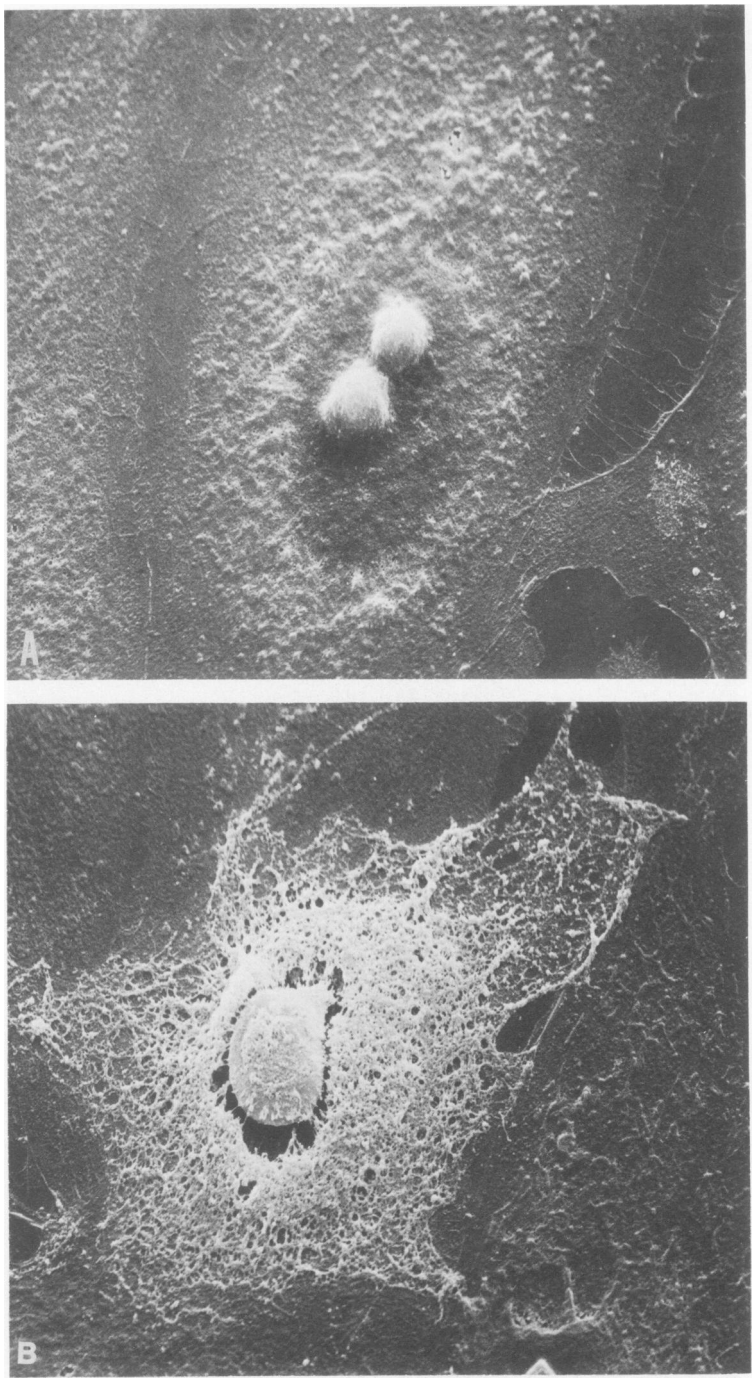

FIG. 6. *Scanning electron micrographs of tsB11-infected TC7 cells: (A) nucleolar accumulations at 41 C for 48 h (×1,650) and (B) late stage of infection at 41 C (×1,530).*

stage of infection, the swollen nuclei became partially separated from the cytoplasm, an event that corresponded to the breakdown of the outer nuclear membrane observed with transmission EM. Mock-infected cells had non-swollen nuclei, occasional single cytoplasmic vacuoles, normal sized nucleoli, and a fairly random distribution of microbodies and mitochondria (Fig. 5A).

The *tsB11*-infected cells at 33 C were not

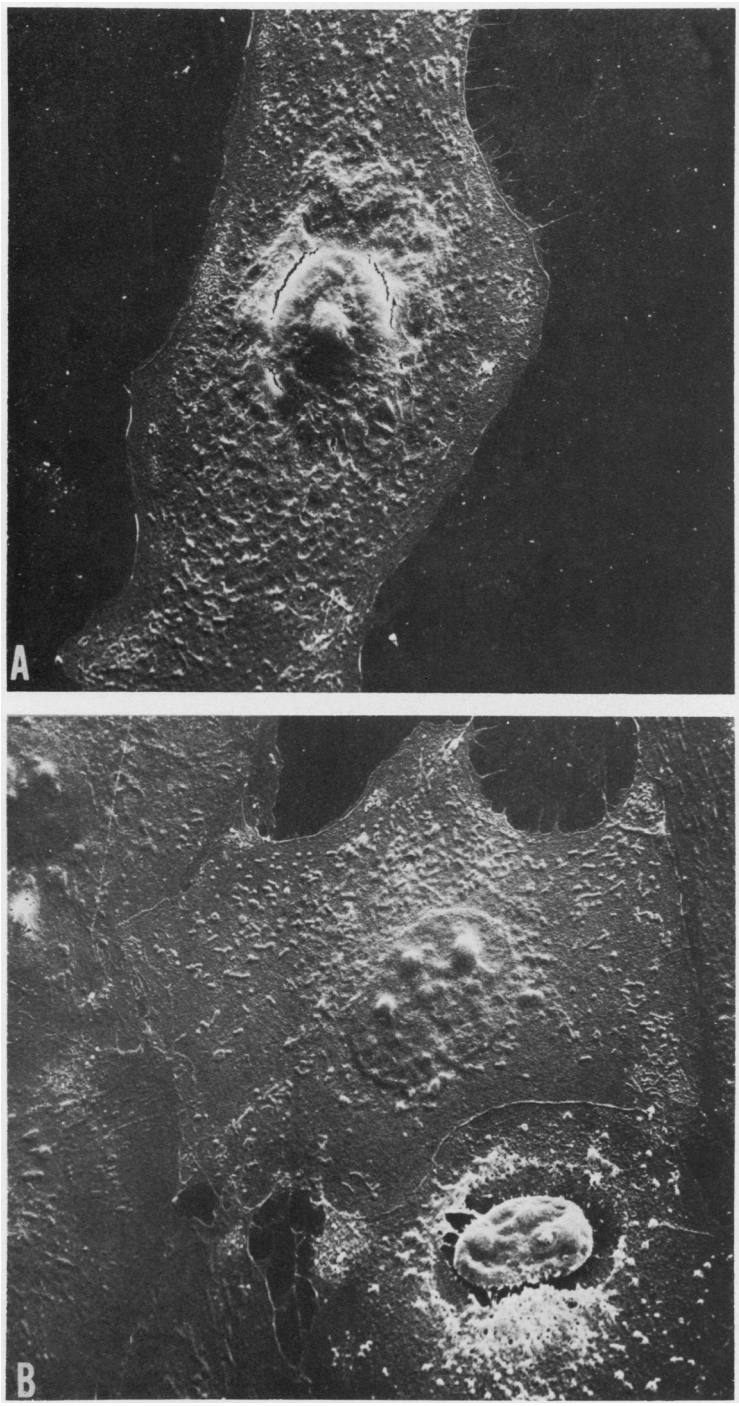

FIG. 7. *Scanning electron micrographs of tsB11 infected cells at: (A) 39 C for 48 h showing cytoplasmic accumulation, slight nucleolar enlargement, and early nuclear extrusion (×1,040) and (B) at 39 C for 48 h showing cell lysis (×1,030). Note uninfected cell in B.*

distinguishable from wild-type-infected cells at 33, 39, or 41 C. However, at 41 C the nonswollen nuclei in *tsB11*-infected cells contained large spherical bodies (Fig. 6A) that correspond to the nucleolar accumulations of C antigen seen in the immunofluorescent assay (Fig. 1D) and

transmission EM (Fig. 3B). The partial extrusion of infected nuclei and disrupted plasma membrane were similar to wild-type-infected cells although no cytoplasmic vacuoles occurred. The only differences between the 39 and 41 C *tsB11*-infected cells were the only slightly enlarged nucleoli and the increased amount of eccentrically located perinuclear material at 39 C (Fig. 7A). The *tsB11*-infected cells were killed as efficiently at 39 and 41 C as were the wild-type-infected cells (Fig. 6B, and 7B).

Light microscope autoradiography. Viral DNA synthesis in *tsB11*-infected TC7 cells at 39 and 41 C is normal. We therefore wanted to know if any of the newly synthesized viral DNA was present in the cytoplasmic accumulations of C antigen at 39 and 41 C. Mock-, wild-type-, and *tsB11*-infected TC7 cells were pulse labeled with [^{3}H] thymidine for 1, 6, and 12 h at 24, 36, and 48 HAI at 39 and 41 C and at 42 and 60 HAI at 33 C. Cells labeled for 1 h received a 15-min chase. Cells labeled for 6 or 12 h received a 1-h chase prior to fixation. Grains were present in large amounts in the nuclei, but were absent from the cytoplasm and nucleoli at anytime, regardless of the length or time of the pulse, except for an occasional wild-type-infected cell at 48 HAI at 39 and 41 C and at 60 HAI at 33 C. These cells correspond to dying cells in which the nuclear membrane is disrupted and the virus (wild type at 33, 39, and 41 C and *tsB11* at 33 C) is released into the cytoplasm.

Since viral DNA synthesis comprises only about 10% of the nuclear DNA synthesis (P. Tegtmeyer, unpublished data), the major problem in interpreting these data is estimating the lower limit of sensitivity in detecting viral DNA in the cytoplasm. We do not know this lower limit, but tentatively conclude that the majority of the newly synthesized *tsB11* DNA remains within the nucleus excluding the nucleolus and does not accumulate with C antigen in the cytoplasm at restrictive temperatures. We cannot rule out the possibility that some viral DNA is present within the cytoplasmic accumulations, possibly as nucleoprotein complexes.

Temperature shift-down experiment with metabolic inhibitors. The following shift-down experiment was performed in order to investigate the reversibility of the cytoplasmic and nucleolar accumulation of C antigen in *tsB11*-infected TC7 cells at restrictive temperatures. TC7 cells were infected and incubated at 39 and 41 C for 24 or 48 h and then were shifted to 33 C for a subsequent 24- or 48-h incubation after which they were stained for C and V antigens or prepared for electron microscopy. At 24 h after the downshift to 33 C, the cytoplasm and most nucleoli were negative for C antigen, while the nuclei were bright regardless of how long the cells were left at either 39 or 41 C (Fig. 1A). By 48 h after the downshift, all nucleoli were negative. The unusual distribution of C antigen at restrictive temperatures is therefore completely reversible.

The reversibility of the abnormal C antigen distribution after a downshift was not inhibited by metabolic inhibitors of DNA synthesis (cytosine arabinonucleoside, Ara-C, 20 μg/ml), RNA synthesis (actinomycin D, 5 μg/ml), or protein synthesis (cycloheximide, 2×10^{-4} to 10×10^{-4} mM) when the drugs were added individually to the cultures one hour prior to the downshift (Table 2). The concentrations of all three drugs are sufficient, when added at the time of infection, to inhibit 99.9% of the C and V antigen-positive cells after infection by wild-type virus at an MOI of 30 at 33, 39, and 41 C and of *tsB11* at 33 C (and C antigen at 39 and 41 C). T antigen was also equally inhibited at all three temperatures in wild-type- and *tsB11*-infected cells by actinomycin D and cycloheximide, but was not inhibited by Ara-C. The appearance of V antigen (capsid formation) after a downshift was variable in three different experiments. By 24 h after the downshift from either the 24- or 48-h 39 or 41 C incubation, V antigen was present in the nuclei excluding the nucleoli, but was faint regardless of the presence or absence of drugs, in contrast to the C antigen which was bright. By 48 h after the downshift, V antigen was brighter in the cultures without drugs or with Ara-C, but was still faint in the cultures containing actinomycin D or cycloheximide. We tentatively conclude that at least some of the virion proteins synthesized at 39 and 41 C can be assembled into virus after a downshift to 33 C without ongoing RNA or protein synthesis. As judged by the intensity of V antigen staining, the amount of virus assembled in the presence of actinomycin D or cycloheximide is decreased in comparison to the amount that can be made in the absence of drugs or in the presence of Ara-C. The appearance of virus particles estimated by transmission electron microscopy correlated very well with the brightness of V antigen. This observation confirmed that assembly of virus particles in the cells downshifted in the presence of actinomycin D or cycloheximide occurred in decreased amounts when compared to downshifted cells without drugs or with Ara-C at 48 h after the downshift. The number of infectious units produced after the downshift was reduced 90 to 99% in agreement with the V antigen data.

Temperature shift-up experiment with

TABLE 2. *Temperature shift-down experiments from 39 or 41 C to 33 C at 24 and 48 h after tsB11 infection at 39 or 41 C using metabolic inhibitors*

Time		Total cells displaying the monitored antigen only in the nucleus (Nu), nucleolus (Nl), or cytoplasm (Cy) (%)				Transmission electron microscopy			
		C antigen		V antigen					
		39 C	41 C	39 C	41 C	39 C		41 C	
						Par[a]	Acc[b]	Par	Acc
24 h	No drug (no shift)	Nu 11[d]	10[d]	0	0	0	0	0	0
		Nl 0	0	0	0	0	+	0	+
		Cy 0	0	0	0	0	0	0	0
24 h[c]	No drug	Nu 28[d]	28[d]	18[e]	8[e]	+	0	+	0
		Nl 0	0	0	0	0	0	0	0
		Cy 0	0	0	0	0	0	0	0
	Actino-D	Nu 20[d]	13[d]	13[e]	10[e]	±	0	±	0
		Nl 0	0	0	0	0	0	0	0
		Cy 0	0	0	0	0	0	0	0
	Cyclohex	Nu 12[d]	12[d]	7[e]	9[e]	±	0	±	0
		Nl 0	0	0	0	0	0	0	0
		Cy 0	0	0	0	0	0	0	0
	Ara-C	Nu 23[d]	28[d]	15[e]	5[e]	+	0	+	0
		Nl 0	0	0	0	0	0	0	0
		Cy 0	0	0	0	0	0	0	0
48 h	No drug (no shift)	Nu 1[d]	21[d]	0	0	0	±	0	±
		Nl 0	44	0	0	0	+	0	+++
		Cy 30	0	0	0	0	++	0	+
48 h[f]	No drug	Nu 34[d]	34[d]	27	9	++	0	++	0
		Nl 0	0	0	0	0	0	0	0
		Cy 1	4	0	0	0	0	0	0
	Actino D	Nu 30[d]	16[d]	6[e]	7[e]	±	0	±	0
		Nl 0	0	0	0	0	0	0	0
		Cy 0	0	0	0	0	0	0	0
	Cyclohex	Nu 30[d]	27[d]	5[e]	8[e]	±	0	±	0
		Nl 0	0	0	0	0	0	0	0
		Cy 4	12	0	0	0	0	0	0
	Ara-C	Nu 28[d]	42[d]	6[e]	4[e]	+	0	+	0
		Nl 0	0	0	0	0	0	0	0
		Cy 0	0	0	0	0	0	0	0

[a] Par = Mature viral particles, no virus crystals were observed at 24 h after the shift to 33 C.

[b] Acc = Amorphous accumulations, see Fig. 3 and 4.

[c] Infected cells shifted to 33 C at 24 h after infection at 39 and 41 C. Drugs added at 39 or 41 C 1 h prior to shift and left in during the subsequent 24 h at 33 C; actinomycin D 5 μg/ml; cycloheximide—10^{-3} mM; and cytosine arabinonucleoside, Ara-C, 20 μg/ml.

[d] Nucleoli also positive.

[e] Immunofluorescent staining present, but very faint.

[f] The infected cells were shifted to 33 C for a subsequent 24 h incubation after 48 h at 39 C or 41 C, drug concentrations same as in C above.

metabolic inhibitors. TC7 cells infected with *tsB11* were incubated at 33 C for 24 or 48 h and then shifted to 39 and 41 C for a subsequent 24-h incubation after which they were either stained for C and V antigens or were prepared for electron microscopy. Control *tsB11*-infected cultures were stained at 24 and 48 HAI at 33 C. The data are given in Table 3. At 24 HAI at 33

TABLE 3. *Temperature shift-up experiments from 33 to 39 C and 41 C at 24 and 48 h after tsB11 infection using metabolic inhibitors*

Time	Total cells displaying the monitored antigen only in the nucleus (Nu), nucleolus (Nl), or cytoplasm (Cy). (%)				Transmission electron microscopy			
	C antigen		V antigen		39 C		41 C	
	39 C	41 C	39 C	41 C	Par	Acc	Par	Acc
24 h[a] No drug	Nu 13	28	0	0	0	±	0	±
	Nl 0	28	0	0	0	+	0	+
	Cy 40	0	0	0	0	+	0	+
Actino-D	Nu 0	0	0	0	0	0	0	0
	Nl 0	0	0	0	0	0	0	0
	Cy 0	0	0	0	0	0	0	0
Cyclohex	Nu 0	0	0	0	0	0	0	0
	Nl 0	0	0	0	0	0	0	0
	Cy 0	0	0	0	0	0	0	0
Ara-C	Nu 0	1[d]	0	0	0	0	0	0
	Nl 0	0	0	0	0	0	0	0
	Cy 0	0	0	0	0	0	0	0
48 h[c] No drug	Nu 20[d]	12[d]	6[b]	1[b]	0	+	0	+
	Nl 15[b]	37	0	0	0	+	0	++
	Cy 48	5[b]	0	0	0	++	0	+
Actino-D	Nu 13[d]	9[d]	3[b]	2[b]	Rare	0	Rare	0
	Nl 0	0	0	0	0	0	0	0
	Cy 0	0	0	0	0	0	0	0
Cyclohex	Nu 12[d]	11[d]	3[b]	7[b]	Rare	0	Rare	0
	Nl 0	0	0	0	0	0	0	0
	Cy 0	0	0	0	0	0	0	0
Ara-C	Nu 22[d]	23[d]	0	1[b]	Rare	++	Rare	++
	Nl 0	0	0	0	0	+	0	+
	Cy 2[b]	1[b]	0	0	0	±	0	±

[a] *tsB11* infected cells shifted from 33 to 39 C or 41 C at 24 h after infection. Drugs added at 33 C 1 h prior to shift and left in during the subsequent 24 h at 39 or 41 C; actinomycin-D 5 μg/ml, cycloheximide—4×10^{-4} mM; and cytosine arabinonucleoside (Ara-C) 20 μg/ml.

[b] Immunofluorescent staining present, but very faint.

[c] The *tsB11* infected cells were shifted from 33 to 39 C or 41 C for a subsequent 24-h incubation at 48 h after infection, drug concentrations same as in a above.

[d] Nucleoli also positive.

C, no antigen was detectable. By 48 HAI at 33 C, about 20% of the cells had C-positive nuclei with negative cytoplasm and nucleoli, although V was barely detectable in nuclei at this time. Infected cells shifted at 24 and 48 HAI to 39 C had C-positive nuclei and most cells also had C-positive nucleoli and cytoplasm. The cells shifted to 41 C had bright C-positive nuclei with even brighter nucleoli although the cytoplasm in only a few cells was faintly C positive. The intensity of V antigen in the nuclei excluding nucleoli remained barely detectable as it was at 48 HAI at 33 C in the cells shifted to 39 and 41 C, indicating a marked inhibition of capsid assembly after the shift-up. This finding is in agreement with previous data (21) that a 48-h preincubation at 33 C is not sufficient to completely overcome the *tsB11* defect.

The following results were obtained when the metabolic inhibitors of DNA, RNA, and protein synthesis were added individually 1 h prior to the shift-up in the concentrations given in the

shift-down experiment described above. The cells preincubated for 24 h at 33 C and shifted to 39 or 41 C had no detectable C or V antigen in the presence of any of the inhibitors except for a few C-positive nuclei and nucleoli in the presence of Ara-C. The cells incubated for 48 h at 33 C and shifted to 39 or 41 C with actinomycin D and cycloheximide exhibited the same number of C positive nuclei with negative nucleoli and cytoplasm as was present at 48 HAI at 33 C indicating complete inhibition of further antigen formation by these two drugs. However, after 48 h at 33 C and the shift to 39 or 41 C, in the presence of Ara-C, the number of infected cells as judged by C antigen increased to the level found in the cultures without drugs and had C-positive nuclei and nucleoli. Furthermore, the cytoplasm remained negative even at 39 C where the cytoplasmic accumulation of C antigen is predominant over nucleolar accumulation in cultures without drugs. The nuclear accumulation of C antigen in the Ara-C-treated cultures did not have its usual homogeneity, but was clumped in the nuclei and the perinucleolar regions (Fig. 8). The V antigen remained barely detectable and confined to the nuclei excluding nucleoli in all of the drug-treated cultures.

We conclude that the C antigen made at 33 C retains its normal nuclear-nonnucleolar distribution at restrictive temperature when the altered *tsB11* function is inhibited by decreased RNA and protein synthesis. Only the C antigen made at restrictive temperatures is abnormally accumulated and/or transported. The fact that C antigen accumulates in an abnormal clumped fashion within nuclei at restrictive temperatures in the presence of Ara-C and does not accumulate in the cytoplasm suggests that DNA synthesis (presumably viral) is involved in the abnormal transport and accumulation of virion protein. Large amounts of newly synthesized viral DNA, however, could not be demonstrated in the cytoplasmic accumulations by

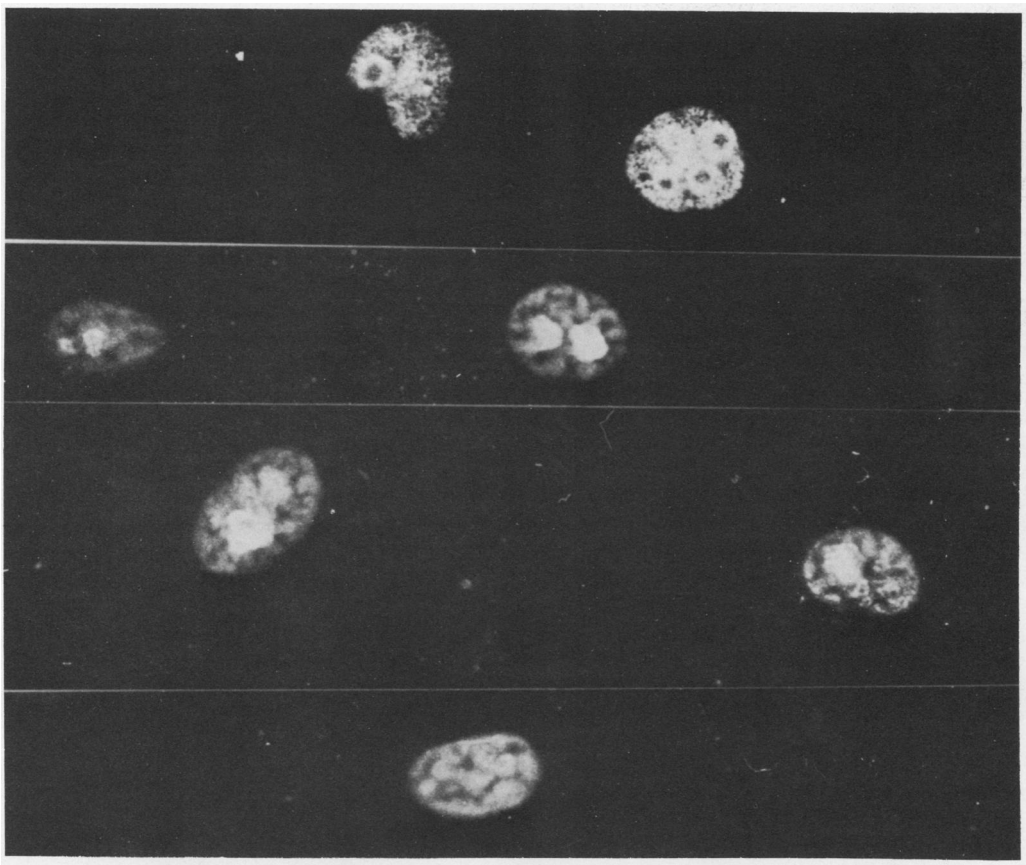

FIG. 8. *Location of C antigen by immunofluorescence using SV40 anti-C serum in tsB11-infected TC7 cells incubated for 48 h at 33 C and then shifted to 41 C for 24 h in the presence of Ara-C. Note abnormal nuclear and perinucleolar accumulations of C antigen* (×1,300).

light microscope autoradiography. Transmission electron microscopy demonstrated a small number of virus particles in the infected cells at 48 HAI at 33 C and after the shift-up regardless of drug treatment in agreement with the barely detectable V antigen.

Microtubule disruption experiment. The cytoplasmic-nuclear transport of virion protein in *tsB11*-infected TC7 cells is abnormal at restrictive temperatures producing accumulations in the nucleoli and cytoplasm instead of the normal accumulation confined to the nucleus. The role of microtubular transport in this viral protein accumulation was investigated by treating wild-type and *tsB11*-infected cells with Colcemid (10^{-5} M and 2×10^{-6} M) or colchicine (10^{-3}, 10^{-4}, and 2×10^{-5} M) at the time of infection, 12 HAI, 22 HAI, or 1 h prior to a shift-up or shift-down in temperature-shift experiments similar to those described above. Under all conditions there were no differences between nontreated and drug-treated cells. No effect was observed in the wild type infections. Since the 10^{-3} and 10^{-4} M concentrations of colchicine are known to disrupt existing microtubules as well as to prevent assembly of new microtubules (14), we conclude that microtubular transport plays little if any role in the abnormal cytoplasmic-nuclear transport of virion protein observed in the *tsB11* mutant phenotype.

DISCUSSION

During infection by *tsB11* or WT virus in productive monkey cells at restrictive temperature, viral DNA synthesis, assembly of the triton-extractable nucleoprotein complex, and the rate of synthesis of the VP1, VP2, and VP3 virion proteins are similar (22) although capsids and virions are not assembled in the *tsB11*-infected cells (21). There is an abnormal cytoplasmic-nuclear distribution of VP1 in that it accumulates in the cytoplasm and nucleoli at restrictive temperature, but probably only transiently in the nucleus. This abnormal accumulation is completely reversible after a shift to permissive temperature. The cytoplasmic-nuclear distribution of VP2 and VP3 cannot be determined accurately because of their reduced amounts compared to VP1. In this paper we have used immunofluorescence in parallel with transmission and scanning electron microscopy during temperature-shift experiments with and without metabolic inhibitors to further delineate the phases of SV40 productive infection that involve the *tsB11* function. We suggest that the *tsB11* protein and/or function is either directly or indirectly involved with the following SV40 productive infection functions: (i) accumulation of immunologically reactive U antigen; (ii) cytoplasmic-nuclear distribution of VP1; (iii) the stability of VP1; (iv) assembly of capsids and virions; (v) a presently undefined nucleolar function; and (vi) the SV40-specific cytoplasmic vacuolation.

There are several possible explanations for the *tsB11* defect. All of these possibilities must be considered in the context that we do not know the individual location and extent of abnormal accumulation of the three virion proteins (VP1, VP2, and VP3) because the anti-C antigen serum used in our experiments reacts with all three of these proteins. Monospecific sera will have to be developed.

The first possible mechanism of the *tsB11* defect is that an early function (e.g., U antigen formation [11]) is defective. This early function could be required for the normal SV40-nucleolar interaction (see below) and/or for the processing and assembly of virion proteins. The accumulation of immunologically reactive U antigen is markedly inhibited in *tsB11*-infected TC7 cells at 39 and 41 C (19). This is in contrast to the synthesis of normal T antigen, another early function. R. S. Kauffman and H. S. Ginsberg (75th Abstr. Annu. Meet. Amer. Soc. Microbiol., 1974) recently reported the cytoplasmic accumulation of hexon protein in adenovirus-infected cells. This accumulation is due to a mutation in a gene separate from the hexon structural gene. It is presently not known whether the decreased amounts of U antigen are due to the decreased synthesis of a virus-coded U antigen, a decreased induction of a cell-coded U antigen, or to a modification of the immunospecificity of U antigen by the *tsB11* function. It is unlikely that *tsB11* is a double mutant because five similar mutants have been isolated in three different laboratories using three different wild-type parents (19; J. Robb, unpublished data), and the mutation maps exclusively in the Hin G fragment of the SV40 genome (10a).

The second possibility is that the mutation is present in one of the virion proteins (VP1) so that, although synthesis and cleavage of the virion proteins seems to be normal at restrictive temperature, the assembly of capsids and virions is inhibited leading to an accumulation of virion protein in the nucleolus and cytoplasm. The increased rate of degradation of VP1 (22) may be responsible for the inhibited assembly of capsids. Ozer (15) has suggested that capsids are rapidly assembled from the newly synthesized virion proteins. If the altered protein

prevents capsid assembly, then the nonassembled virion protein might be free to accumulate in the nucleolus and cytoplasm. This suggestion fits the observation that the C antigen synthesized at 33 C remains confined to the nucleus after a shift-up in the presence of inhibited RNA and protein synthesis. Also compatible is the observation that virions are assembled at 33 C in the presence of inhibited RNA and protein synthesis after a shift-down.

The accumulation of SV40 antigen (i.e., T, U, C, or V) in the nucleolus has not been previously reported by other investigators nor does it occur with wild type or any other SV40 temperature-sensitive mutant group (19). We suggest that there is some role that one or more virion proteins play in the nucleolar region during normal productive infection. During *tsB11* infection, the mutation produces an accumulation of this virion protein in the nucleolus where it evidently cannot be normally utilized or processed. Although the hypothesized function is not known, several speculative possibilities exist. Granboulan and Tournier (8) found that the first site of DNA synthesis in SV40-infected monkey cells occurs in the nucleolar region with subsequent spreading into the nucleus. Deak, Sidebottom, and Harris (5) have suggested that all mammalian RNA has to be processed by the nucleolus before it is transported to the cytoplasm. Recently, Hashimoto et al. (9) have suggested that SV40 supplies a function allowing certain adenovirus mRNA to be translated when monkey cells are co-infected (SV40-adenovirus helper function). Cellular RNA synthesis is stimulated after SV40 infection of monkey cells (13) and the SV40-nucleolar interaction might be important in this stimulation.

SV40 was originally named "Vacuolating Agent" thus producing the "va" of papova (12). The mechanism of the production of the vacuolation, the contents of the vacuoles, and their role in productive SV40 infection are unknown. We have found that although *tsB11* kills TC7 cells as efficiently at 39 to 41 C as wild-type infection, vacuolation does not occur although an excentrically located perinuclear accumulation of microbodies does occur. The *tsB11* function is therefore involved in an unknown manner with the SV40-induced vacuolation during productive infection.

ACKNOWLEDGMENTS

We thank Walter Eckhart, Harvey Ozer, and Peter Tegtmeyer for helpful discussions and critical review of the manuscript, Peter Tegtmeyer for the gift of *tsB11* virus, Harvey Ozer for the gift of SV40 anti-C serum. The assistance of Mike Wagner with scanning electron microscopy and Bob Garrett with transmission electron microscopy is gratefully appreciated.

This investigation was supported in part by Public Health Service research grant CA-12472 from the National Cancer Institute, Cancer Research Funds of the University of California, The Anna Fuller Fund, American Cancer Society Grant VC-72, and American Cancer Society Institutional Grant AcrS IN-93. J. R. is the recipient of Research Career Development Award CA-70567 from the National Cancer Institute.

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