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Inhibition of Herpes Simplex Virus Type 1 Replication in Temperature-Sensitive Cell Cycle Mutants

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Herpes simplex virus type 1 DNA synthesis and infectious progeny production were studied in five different conditional hamster (BHK-21) cell cycle mutants. At the nonpermissive temperature (39.5°C), both events were strongly inhibited in four of these cell lines. The degree of inhibition was a reproducible characteristic of each cell mutant and in two cases was dependent upon the multiplicity of infection. Experiments involving shifts to the nonpermissive temperature at least 3 h postinfection at 33.5°C suggested that the defects in viral replication were not due to faulty adsorption, penetration, or uncoating, whereas experiments involving shifts of infected cells from the nonpermissive temperature to 33.5°C revealed the reversible nature of the inhibition.

The self-explanatory terms "permissive," "semipermissive," and "nonpermissive" are used to describe the commonly occurring interactions between viruses and cells. Variations in the degree of permissiveness can presumably be due to defects in all phases of the viral reproductive cycle, ranging from adsorption to maturation and release, and the genetic determinant of this variation can reside in either the virus or the cell. In the case of oncogenic DNA-containing animal viruses, in which semi- and nonpermissive interactions may lead to viral transformation, the inhibition of replication often resides in defects in specific viral genes and/or host functions necessary for the late phase of virus reproduction. For example, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) transform cells in nonpermissive systems, and the cause of the nonpermissiveness can reside in either the virus (9, 12, 15, 17) or the cell (8).

The importance of the study of semi- and nonpermissive interactions in understanding the roles of specific host functions in viral replication and transformation prompted our investigation of HSV-1 replication in various hamster cell lines containing temperature-sensitive (*ts*) mutations affecting cell cycle progression (1, 10, 17a; see below). Since some of these mutants had been shown previously to be conditionally defective in supporting adenovirus type 2 replication (13, 14), it was hoped that similar results would be obtained with HSV-1. In this communication we report the conditional inhibition of HSV-1 DNA synthesis and infectious progeny production in four different *ts* hamster cell cycle mutants. The degree of inhibition was a reproducible characteristic of each cell mutant and in some cases was dependent upon the multiplicity of infection (MOI).

MATERIALS AND METHODS

Virus infection. HSV-1 strain MacIntyre (American Type Culture Collection), previously propagated at a low MOI (0.01 PFU/cell) to avoid the appearance of defective particles, was used to infect various cell types (see below) at about 25% confluence in 35-mm plastic dishes at either 33.5, 37, or 39.5°C. After a 1-h adsorption period (0.1 ml), infected cells were washed three times with 3 ml of temperature-equilibrated Dulbecco-modified Eagle medium (GIBCO) containing 10% calf serum and incubated in 2 ml of this medium under an atmosphere containing 5% CO_2 for the times indicated in the text.

Cell cultures to be assayed for infectious virus production were frozen and thawed three times and titrated on African green monkey kidney (BSC-1) cells at 37°C; after virus adsorption, BSC-1 monolayers were overlaid with 4 ml of modified Eagle medium containing 2% methylcellulose-5% calf serum, and plaques were counted after 4 days.

Cells. *ts* cell cycle mutants, as well as the hamster kidney line (BHK-21) from which they are derived, were provided by Claudio Basilico. Mutants *ts* AF-8 (1, 10) and *ts*-13 (17a) are arrested in the G-1 phase of the cell cycle at the nonpermissive temperature (39.5°C); *ts* HJ-4 is probably blocked very late in G-1 (17a), whereas *ts* BTN-1 and *ts* BN-2 seem to become arrested in late G-1 or early S (T. Nishimoto and C. Basilico, manuscript in preparation). All mutants exhibit greatly reduced DNA synthesis after appropriate incubation times at the restrictive temperature (see Table 1), but only *ts* BTN-1 expresses extensive inhibition (80%) of protein synthesis; inhibition of RNA synthesis at the nonpermissive temperature is about 70% in *ts* AF-8 (1) and 10% in both *ts* HJ-4 and *ts*-13 (17a). In addition, survival rates determined from plating efficiencies of the various cell mutants after incubations at 39.5°C for the time periods corresponding

to experiments described below are about 75% for *ts* AF-8 after 68 h (1), 40% for *ts*-13 after 38 h, 90% for *ts* HJ-4 after 68 h (17a), and 30% for both *ts* BTN-1 and *ts* BN-2 after 28 h. A cell line containing a *ts* mutation affecting 28S rRNA production (*ts*-422E) was also provided by C. Basilico (and derived from BHK-21 [18]).

Measurement of viral DNA synthesis. Infected cells (in 35-mm dishes containing 2 ml of medium) were labeled with 2.5 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear) per ml for the times indicated in the text. In most experiments (see Fig. 2 and 3), cells resuspended in their own culture medium were adjusted to a final volume of 6 ml by the addition of a solution containing 1.2% sodium lauryl sulfate–10 mM EDTA–10 mM Tris-hydrochloride (pH 7.9) and then incubated for 1 h at 37°C. The resulting lysate contained ^{3}H-labeled HSV DNA originally present in both cells (cell associated) and medium (non-cell associated). In some experiments (see Fig. 1), cell monolayers were washed free from their own culture medium and directly resuspended in 2 ml of the 1.2% sodium lauryl sulfate solution, in which case the resulting lysate contained only cell-associated viral DNA. In general, by 20 h postinfection (p.i.) approximately 50% of the ^{3}H-labeled viral DNA was present in the culture medium.

The amount of viral DNA in these lysates was determined by centrifuging portions of them to equilibirum in 7-ml CsCl density gradients (input density, 1.70 g/ml) in a Beckman 50Ti rotor at 30,000 rpm and 25°C for 48 h. Collected fractions were precipitated onto nitrocellulose filters with equal volumes of cold 10% trichloroacetic acid–0.04 M sodium pyrophosphate and, after three washes with cold 5% trichloroacetic acid–0.04 M sodium pyrophosphate, were counted in a toluene-based scintillation fluid.

Measurement of host DNA synthesis. Host DNA synthesis was measured from the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material in sodium lauryl sulfate cell lysates prepared from uninfected nonconfluent cells. Percent inhibitions of host DNA synthesis in various cell types were calculated by comparing the total radioactivity incorporated after 20 h at either 33.5 or 39.5°C. Precipitates were dissolved in NCS tissue solubilizer (Amersham/Searle) before counting.

RESULTS

Properties of uninfected *ts* mutants. The six *ts* growth mutants used in this work were derived from BHK-21 cells. They exhibit low reversion frequencies (from 10^{-4} to 10^{-6}) as well as low leakiness, and in all cases growth is arrested at 12 to 24 h after the shift to the nonpermissive temperature. Moreover, these mutants are all affected in different functions, as shown by genetic and biochemical analyses.

ts AF-8 is a cell cycle mutant that becomes arrested in the G-1 phase at 39.5°C. Synchronization-shift-up experiments place the critical thermosensitive step early in G-1, between the blocks induced by serum starvation and isoleucine deprivation (1). These cells remain viable at 39.5°C for very long times, thus mimicking a G-0 state. *ts*-13 is another cell cycle G-1 mutant, but the thermosensitive step appears to be located at a distal point in G-1 with respect to *ts* AF-8. *ts*-13 cells at 39.5°C are arrested in G-1, but lose viability (as measured by plating efficiency at 33°C) quite rapidly (17a). *ts* HJ-4 also becomes arrested in G-1 at the nonpermissive temperature, but its critical period appears to be quite late in G-1, probably near the onset of the S phase (17a).

ts BTN-1 and *ts* BN-2 are cell cycle mutants with defects that may directly affect the process of DNA replication. In the case of *ts* BTN-1, a decrease in the rate of DNA synthesis per cell can be observed shortly after exposure to the nonpermissive temperature. This mutant also exhibits a considerable inhibition of protein synthesis (although less drastic than that of DNA synthesis), and it is not yet clear which event is the primary defect (Nishimoto and Basilico, manuscript in preparation).

ts BN-2 is probably defective in the initiation of DNA synthesis, since cells accumulate in G-1 and S after exposure to the nonpermissive temperature. If synchronized at the G-1/S boundary at 33°C and then shifted to 39.5°C, they perform a limited round of DNA synthesis and arrest during the S phase, as would be expected for cells that could complete initiated replicons, but could not initiate new ones. Both *ts* BTN-1 and *ts* BN-2 lose viability at 39.5°C.

ts-422E has a defect in the processing of 28S rRNA precursors at 39.5°C and stops dividing after about one cell doubling at this temperature (18). For convenience, Table 1 summarizes those properties of the six *ts* mutants that are most relevant to the studies described in the next sections.

Kinetics of HSV-1 DNA synthesis in wild-type BHK-21 cells as a function of MOI and temperature. Since all of the cell mutants examined in this study were originally derived from the same BHK-21 line, control experiments determining the effects of MOI and temperature on HSV-1 DNA replication in these "parental" cells were performed (experiments regarding infectious virus production are presented in a following section). Extents of viral DNA synthesis were monitored from incorporations of [^{3}H]thymidine into cell-associated DNA banding at the viral density (1.725 g/ml) in equilibrium CsCl gradients, as described above, and the results of one such study are shown in Fig. 1. The general conclusion from this study is that the higher the MOI, the earlier the onset and the greater the amount of viral DNA synthesis. In addition, viral DNA synthesis is about 50% inhibited at the higher temperature. It should be noted, how-

ever, that such inhibition is not a reproducible finding, and in some experiments no inhibition was observed; although a well-documented explanation of this fluctuation has not been obtained, a careful comparison of experimental conditions has suggested that small variations in incubation temperatures (such as 39.7°C compared with 39.5°C) are the likely cause. In any event, we consider twofold variations in viral DNA synthesis to be potentially within the realm of experimental error; the inhibitions described in the next section are well beyond this range. It should also be noted that a direct relationship between the incorporation of [^{3}H]-thymidine into viral DNA and the actual amount of viral DNA synthesized can be complicated by the effect of viral thymidine kinase activity on increasing the endogenous pool of thymidine nucleotides. Thus, the general conclusion that the higher the MOI, the earlier the onset and the greater the amount of viral DNA synthesis would probably be accentuated by a direct measurement of viral DNA, since high MOIs should have increased the thymidine nucleotide pool and reduced the incorporation of exogenous [^{3}H]thymidine shown in Fig. 1. In the data presented in the next sections, the incorporation of [^{3}H]thymidine into viral DNA is used as a measure of viral DNA synthesis since the results are generally analyzed by comparing radioactivity incorporations after uniform labeling at a constant MOI and varying temperature.

Conditional inhibition of HSV-1 DNA synthesis in *ts* cell cycle mutants. The effects of MOI and temperature on HSV-1 DNA replication in five *ts* cell cycle mutants, as monitored from incorporations of [^{3}H]thymidine into total viral DNA (both cell associated and non-cell associated), are shown in Fig. 2 and 3 and summarized in Table 2. At the nonpermissive temperature (39.5°C) and low MOI (0.5 PFU/cell), viral DNA synthesis was essentially completely inhibited in four of the mutants (*ts* AF-8, *ts*-13, *ts* BTN-1, and *ts* BN-2), whereas at the nonpermissive temperature and high MOI (50 PFU/cell), viral DNA synthesis was completely inhibited in only two of them (*ts* BTN-1 and *ts*

TABLE 1. *Properties of ts cell mutants*[a]

Cell type	Defect at nonpermissive temp (39.5°C)	Plating efficiency at 33.5°C after incubation at 39.5°C for various times
ts AF-8	G-1 progression	75% after 68 h at 39.5°C
ts-13	G-1 progression	40% after 38 h at 39.5°C
ts BTN-1	S progression	30% after 28 h at 39.5°C
ts BN-2	S progression	30% after 28 h at 39.5°C
ts HJ-4	Progression into S	90% after 68 h at 39.5°C
ts-422E	Processing of 28S rRNA precursors	60% after 24 h at 39.5°C

[a] See the text for details.

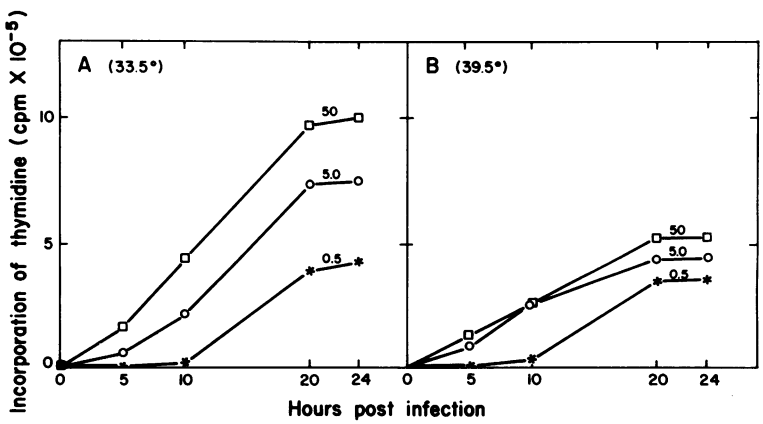

FIG. 1. *Kinetics of HSV-1 DNA synthesis in wild-type BHK-21 cells as a function of MOI and temperature. (A) Total incorporation of [^{3}H]thymidine into cell-associated (10^6 cells) viral DNA as a function of time at the indicated MOIs of 50, 5, and 0.5 PFU/cell and at 33.5°C; (B) total incorporation of [^{3}H]thymidine into cell-associated (10^6 cells) viral DNA as a function of time at the indicated MOIs of 50, 5, and 0.5 PFU/cell and at 39.5°C. Cells were incubated for 48 h prior to infection. In both (A) and (B), total incorporation was obtained by summing the results of pulse-labelings performed from 0 to 5, 5 to 10, 10 to 20, and 20 to 24 h, respectively.*

BN-2). Moreover, one of the cell lines (*ts* HJ-4) supported HSV-1 DNA synthesis at 39.5°C regardless of MOI, although low-multiplicity infections of this line yielded abnormally small amounts of viral DNA at both permissive and nonpermissive temperatures.

Indeed, the data presented in Fig. 3C and D suggest that 33.5°C infections may actually be

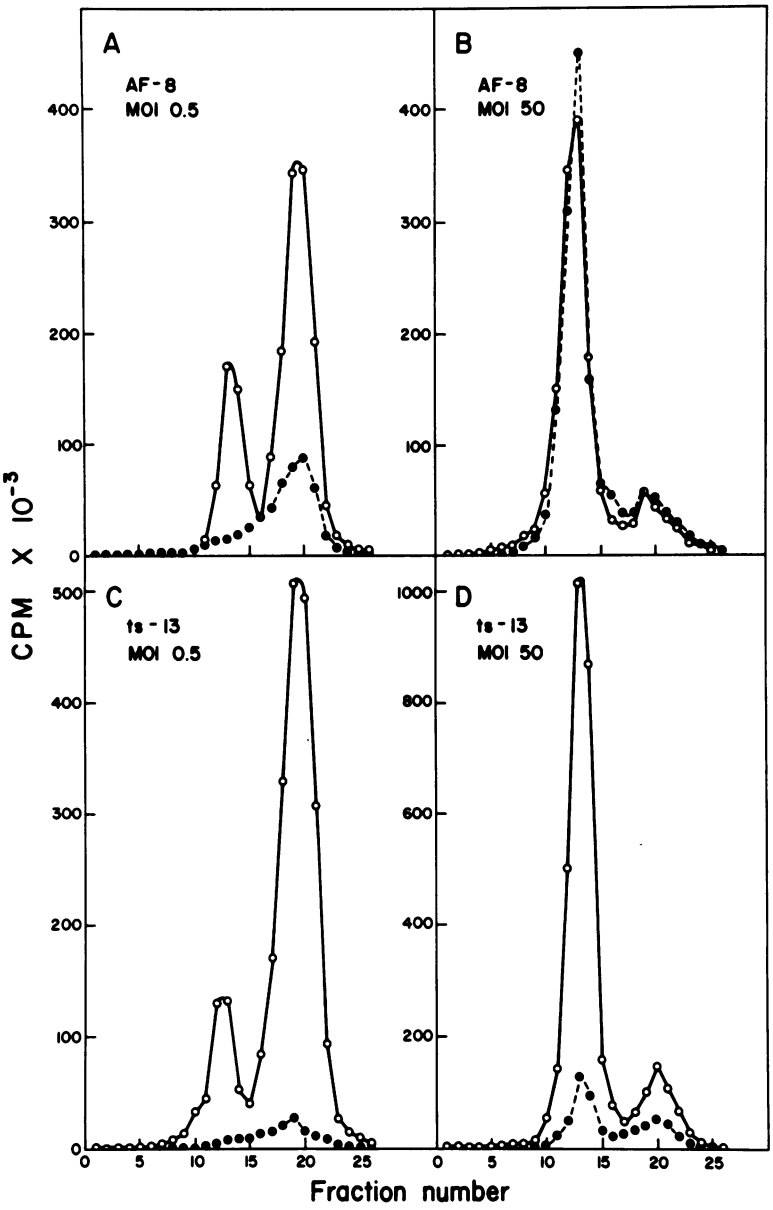

FIG. 2. *Radioactivity profiles of HSV-1 and host cell DNAs isolated from infected cell mutants ts AF-8 and ts-13 after CsCl equilibrium density gradient centrifugation. Cells were uniformly labeled with [³H]-thymidine for 20 h p.i., and the total DNA (both cell associated and non-cell associated) was centrifuged to equilibrium in CsCl as described in the text. Symbols: ○, labeled DNA isolated from 10⁶ cells infected at 33.5°C; ●, labeled DNA isolated from 10⁶ cells infected at 39.5°C. The MOIs and cell types are indicated in each panel; HSV DNA peaks are to the left of host cell DNA peaks. The percent inhibition of HSV DNA synthesis was calculated from the ratio of viral DNA radioactivity present at 39.5°C to viral DNA radioactivity present at 33.5°C; when no distinct peak was evident at 39.5°C as in (A) and (C), inhibition was considered to be 100%. Note the decreased levels of labeled host cell DNA at 39.5°C and/or a high MOI.*

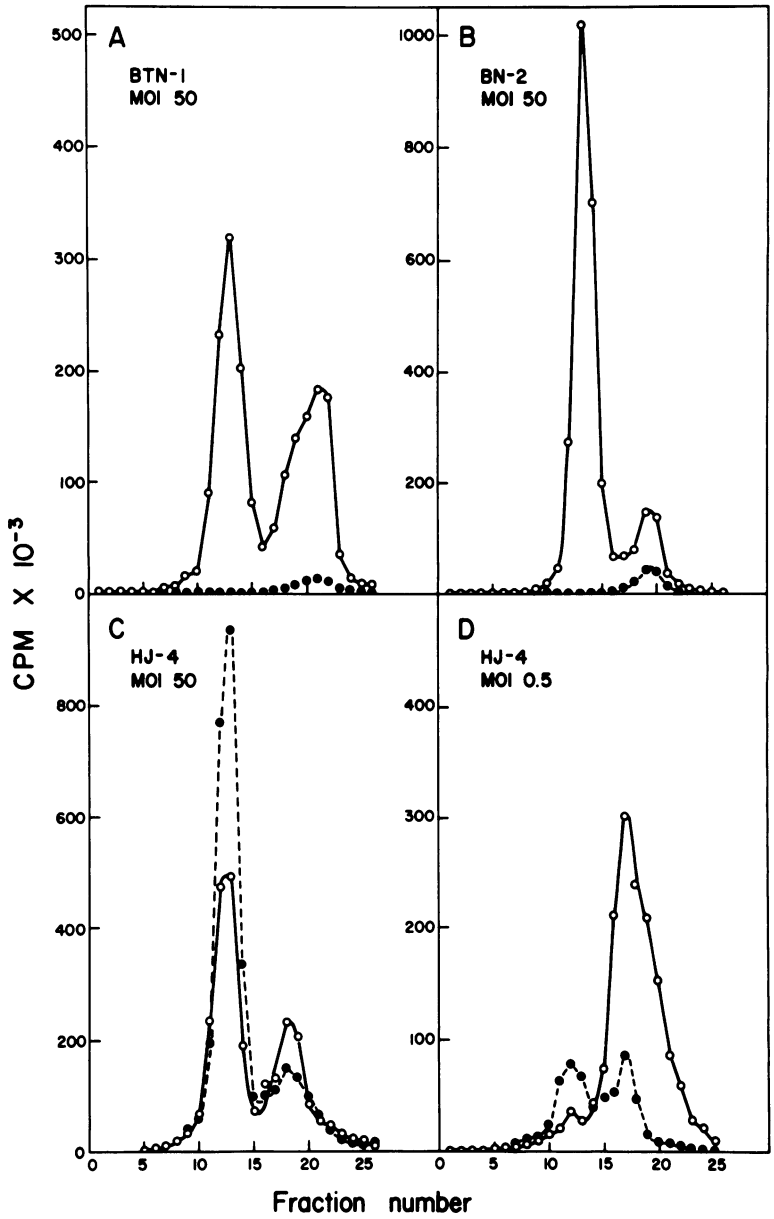

FIG. 3. *Radioactivity profiles of HSV-1 and host cell DNAs isolated from infected cell mutants ts BTN-1, ts BN-2, and ts HJ-4 after CsCl equilibrium density gradient centrifugation. Cells were uniformly labeled with [³H]thymidine for 20 h p.i., and the total DNA (both cell associated and non-cell associated) was centrifuged to equilibrium in CsCl as described in the text. Symbols: ○, labeled DNA isolated from 10⁶ cells infected at 33.5°C; ●, labeled DNA isolated from 10⁶ cells infected at 39.5°C. The MOIs and cell types are indicated in each panel; HSV DNA peaks are to the left of host cell DNA peaks.*

less efficient than those at 39.5°C; this supposition has been confirmed from studies of infectious virus production (see below).

The data in Table 2 also indicated that mutant *ts*-422E cells (18) supported HSV-1 DNA replication to the same extent as did the parental BHK-21 line. In fact, the CsCl equilibrium density gradient patterns of viral DNA isolated from infected *ts*-422E cells at both high and low MOIs and temperatures were indistinguishable from those obtained from wild-type cells (data not shown). This result was consistent with the known defect of *ts*-422E, since a failure to correctly process an rRNA precursor at 39.5°C

would not be expected to interfere with HSV replication; it should be noted that the production of adenovirus type 2 in this mutant was also normal at the nonpermissive temperature (14).

In addition to the experiments reported in Fig. 2 and 3, shift-up and shift-down studies were performed to clarify the nature of conditional viral DNA synthesis inhibition. For example, infected (MOI, 0.5 PFU/cell) *ts* AF-8 cells grown at 33.5°C were shifted to 39.5°C at either 4.5 or 10 h p.i. and subsequently labeled for 3 h with [^{3}H]thymidine beginning at 14 h p.i. When the amounts of labeled viral DNA synthesized during this period were compared with the amounts synthesized during this same period in the original batch of infected cells maintained for the full 17 h at 33.5°C (about 10^5 cpm/10^6 cells), inhibitions of 75% (4.5-h shift-up and 70% (10-h shift-up) were observed. Similar results were obtained from shift-up experiments involving mutants *ts*-13, *ts* BTN-1, and *ts* BN-2, and in the case of *ts* BTN-1 approximately 95% inhibition was observed when shift-ups were performed 3 h p.i. at an MOI of 50.

TABLE 2. *Comparison of HSV-1 DNA synthesis in various BHK-21 cell mutants at permissive (33.5°C) and nonpermissive (39.5°C) temperatures*[a]

Cell type	Preinfection incubation time at 39.5°C (h)	Inhibition (%) of host cell DNA synthesis at 39.5°C[b]	MOI (PFU/cell)	Inhibition (%) of viral DNA synthesis at 39.5°C[c]
ts AF-8	48	97	0.5	100
			50	0
ts-13	18	92	0.5	100
			50	90
ts BTN-1	8	95	0.5	100
			50	100
			200	100
ts BN-2	8	89	0.5	100
			50	100
			200	100
ts HJ-4	48	94	0.5	0
			50	0
ts-422E	24	0	0.5	0
			50	0
Wild type	48	0[b]	0.5	0
			50	0

[a] The various cell types were incubated at 39.5°C for the times indicated and then infected with virus at the MOIs shown. Percent inhibitions of viral DNA synthesis were calculated by comparing the total radioactivity incorporated into HSV-1 DNA in 20 h at 33.5°C and 39.5°C, as described in the legend to Fig. 2 and the text.

[b] See text. Wild-type cells have a higher rate of host cell DNA synthesis at 39.5°C in the absence of virus infection.

[c] One hundred percent inhibition indicates that no distinct peak of viral DNA was found in CsCl gradients containing DNA isolated from infected cells at 39.5°C. Zero percent inhibition indicates that the size of the peak of viral DNA found in CsCl gradients containing DNA isolated from infected cells at 39.5°C was within experimental variability (twofold), the same as that of the peak of viral DNA found at 33.5°C.

The reversibility of the inhibition, at least in *ts* BTN-1 cells, was demonstrated by shifting down infected cells (MOI, 50 PFU/cell) from 39.5 to 33.5°C at either 10, 15, or 20 h p.i. and then exposing the cultures to [^{3}H]thymidine for an additional 20 h at this permissive temperature. In all cases, significant amounts of viral DNA (125,000, 42,000, and 6,000 cpm/10^6 cells, respectively) were synthesized.

The results of these and other shift-up experiments strongly suggest that viral adsorption and/or penetration are not responsible for the observed inhibitions of viral DNA replication at the nonpermissive temperature. Also, studies of low-multiplicity infection in serum-starved, early G-1-arrested *ts* AF-8 cells at 33.5°C revealed that the inhibitions of viral DNA synthesis and progeny virus production at 39.5°C were not due to a more or less nonspecific cell cycle effect (a conclusion supported by the results of shift-up experiments, in which inhibition of HSV DNA synthesis took place before the cell cycle block characteristic of each mutant was reached). Cells synchronized in early G-1 at the permissive temperature supported HSV DNA replication and infectious virus production with the same kinetics as did unsynchronized ones (data not shown), a result in agreement with published studies of herpes replication in S-phase synchronized KB cells (2).

Conditional inhibition of HSV-1 infectious progeny production in *ts* cell cycle mutants. The kinetics of HSV-1 infectious progeny synthesis in wild-type BHK-21 and mutant *ts* AF-8 cells as a function of MOI and temperature are shown in Fig. 4, and the yields of infectious virus produced 24 h p.i. by all of the cell cycle mutants are presented in Table 3. In general, the results of these studies were in good agreement with those obtained from examinations of DNA synthesis, even though faulty maturation and virus inactivation probably occurred to some extent in all cell types at 39.5°C (4–6). In the case of high-multiplicity and high-temperature infection of wild-type cells, the net increase of infectious virus was partially obscured by the input; however, comparisons to similar, but nonproductive, infections of *ts* BTN-1, *ts* BN-2, or *ts*-13 cells (Table 3) indicate that the 24-h titer in wild-type cells essentially represents newly synthesized virus. Figure 4B shows that a small amount of virus production occurred in low-multiplicity infections in *ts* AF-8 at 39.5°C, but this quantity would not be expected to yield a readily measurable level of viral DNA in CsCl gradients (Fig. 2A). The possible decreased virus production during high-multiplicity (39.5°C) infections of *ts* AF-8 as compared with wild-type cells (Fig. 4)

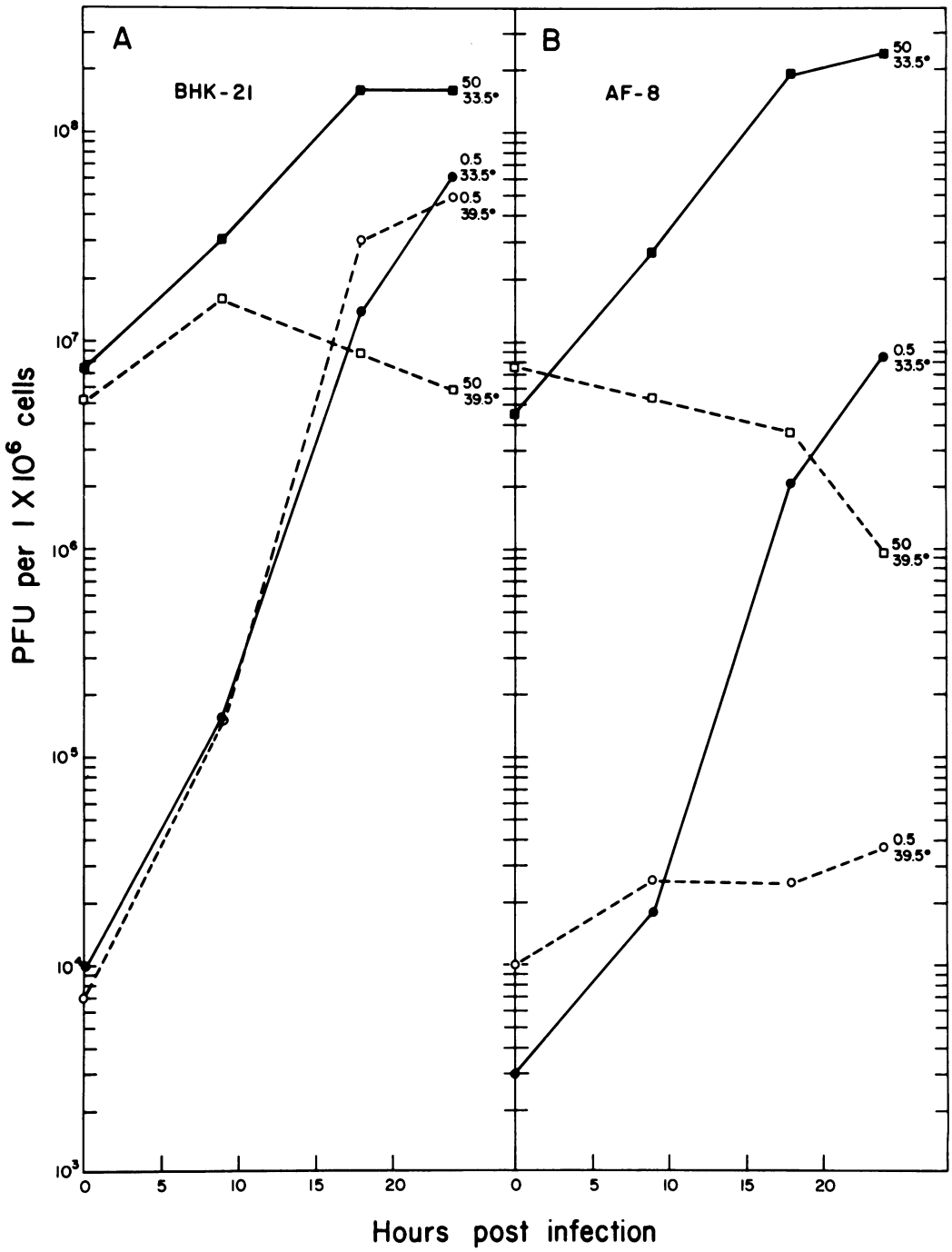

FIG. 4. *Infectious virus production in wild-type BHK-21 and mutant ts AF-8 cells. Cells were infected at the indicated MOIs and temperatures and assayed for infectious virus at various times p.i. as described in the text. Titers are expressed as PFU per 10^6 cells assayed. (A) Virus production in BHK-21 cells. The reduction of virus production in wild-type cells infected at a high temperature and MOI (4–6) was expected. The apparently normal production of virus in these cells infected at a high temperature and low MOI was not. It is possible that these observations may be in some way correlated with the early (10 h) onset of cytopathogenicity at a high MOI. (B) Virus production in ts AF-8. Both wild-type and mutant cells were preincubated at 39.5°C for 48 h prior to infection. Zero time assays were performed after a 1-h adsorption period and three washings with medium.*

was a reproducible observation, but could be within the range of experimental error. It should also be noted that the production of infectious virus in *ts*-422E cells (defective in rRNA processing) at both high and low MOIs and temperatures was indistinguishable from that observed for the parental BHK-21 line (data not shown); this result was consistent with the data obtained for viral DNA synthesis (Table 2) in these two cell types.

The behavior of *ts* HJ-4 concerning the production of infectious virus is extremely peculiar and seems to be unrelated to the growth cycle conditional defect in this cell mutant. As noted above, low-multiplicity infections of this line yielded abnormally small amounts of viral DNA at either permissive or nonpermissive temperatures, although both high- and low-multiplicity infections resulted in lower amounts of viral DNA at the permissive temperature. The results of virus production studies (Table 3) followed a similar, but more highly accentuated pattern, since no infectious virions were produced during 33.5°C infections, regardless of MOI. A conceivable explanation for the anomalous behavior of *ts* HJ-4 in supporting HSV-1 replication could be the presence of an additional, independent mutation, a possibility that will eventually be examined by studying viral reproduction in spontaneous phenotypic revertants of this cell line. Indeed, an examination of viral production in spontaneous revertants of all of our *ts* growth-arrested cell lines will be necessary to conclusively prove that one defect is responsible for the inhibition of both cell cycle progression and HSV-1 replication.

TABLE 3. *Infectious virus production in various BHK-21 cell mutants at permissive (33.5°C) and nonpermissive (39.5°C) temperatures*[a]

Cell type	MOI (PFU/cell)	Temp (°C)	PFU/10^6 cells after adsorption	PFU/10^6 cells at 24 h p.i.
ts AF-8	0.5	33.5	2.9×10^3	8.6×10^6
		39.5	1.0×10^4	3.7×10^4
	50	33.5	4.5×10^6	2.4×10^8
		39.5	7.7×10^6	9.6×10^5
ts-13	0.5	33.5	2×10^4	7.7×10^5
		39.5	2×10^4	1.1×10^2
	50	33.5	2.2×10^6	2.0×10^7
		39.5	2.0×10^6	8.8×10^3
ts BTN-1	50	33.5	3.3×10^6	1.6×10^8
		39.5	1.5×10^6	6.8×10^4
ts BN-2	50	33.5	2.6×10^6	1.1×10^8
		39.5	2.2×10^6	4.4×10^4
ts HJ-4	0.5	33.5	2.4×10^3	<400
		39.5	5.8×10^3	7.3×10^3
	50	33.5	4.0×10^5	3.2×10^3
		39.5	1.2×10^5	5.0×10^6
Wild type (BHK-21)	0.5	33.5	1.05×10^4	6.2×10^7
		39.5	6.9×10^3	4.9×10^7
	50	33.5	7.4×10^6	1.6×10^8
		39.5	5.1×10^6	5.9×10^6

[a] Preinfection incubation times at 39.5°C were the same as those presented in Table 2. Cells infected at the indicated MOIs were assayed for infectious virus as described in the text. The titer obtained after a 1-h adsorption period (zero time p.i.) has not been subtracted from the titer obtained after 24 h of infection. The data presented in this table are typical of those obtained by us from many other examinations of infectious virus production.

DISCUSSION

The conditional inhibition of HSV-1 DNA synthesis and infectious progeny production in four *ts* cell cycle mutants has been described. Since these recessive mutations are each in different complementation groups, as defined by cell hybridization studies (17a; Nishimoto and Basilico, manuscript in preparation), it was not surprising to find that the precise characteristics of the inhibition often differed (Table 2). For example, the extent of viral DNA synthesis at 39.5°C was strongly dependent upon MOI in *ts* AF-8, slightly dependent upon MOI in *ts*-13, and apparently independent of MOI in both *ts* BTN-1 and *ts* BN-2. Moreover, the results of shift-up experiments indicated that the inhibition of viral DNA synthesis was due to the high-temperature inactivation of a cellular component required for HSV-1 replication following the stages of adsorption, penetration, and uncoating and suggested that this component is directly involved in DNA replication. Future investigations of virus-specific RNA and protein syntheses in both uniformly incubated (39.5°C) and shifted-up (33.5 to 39.5°C) infected cells should help clarify this suggestion, as should an examination of the temperature sensitivity of α, β, and γ DNA-polymerizing activities in uninfected cells.

It is hoped that the late G-1- or early S-phase cell cycle mutants *ts* BTN-1 and *ts* BN-2 will be particularly well suited for such studies, since relatively short preinfection incubation times at 39.5°C are required for total viral DNA synthesis inhibition, even at high MOIs (Table 2).

Elucidation of the mechanism of multiplicity-dependent HSV-1 replication inhibition in the G-1-arrested cell cycle mutants *ts* AF-8 and *ts*-13 also warrants further investigation.

Infection of temperature-arrested *ts* AF-8 cells (48 h, 39.5°C) with HSV-1 previously passaged under permissive conditions in *ts* AF-8 resulted in the same multiplicity-dependent inhibition of viral DNA synthesis and infectious virus production as did virus passaged in wild-type BHK (K. Yanagi, unpublished data); thus, it does not appear that the overcoming of viral DNA synthesis inhibition at 39.5°C by high-multiplicity

infection is due to the incorporation of a cellular component (the presumed *ts* constituent of *ts* AF-8) into progeny virions. One possible explanation regarding multiplicity-dependent viral DNA synthesis could be that a balance of both cellular and viral enzymes is normally used, or is required, for accurate HSV genome replication. In *ts* BTN-1 and *ts* BN-2 cells, the presence of a mutated host enzyme, normally required for viral DNA synthesis irrespective of the level of herpes-specified enzyme activities, could completely inhibit viral DNA replication at all MOIs, whereas in *ts* AF-8 and *ts*-13 cells different mutated host factors normally used, but not absolutely essential, for viral DNA synthesis might reduce such synthesis to very low levels only in the absence of extensive amounts of herpes-coded enzymes. The reported in vitro insensitivity of the HSV-1 DNA polymerase to cytosine arabinoside (triphosphate) inhibition (11), coupled with the well-known in vivo inhibitory effect of this drug on HSV-1 DNA synthesis (3, 7), is an observation consistent with such a hypothesis; also of interest is the reported resistance of DNA synthesis in HSV-1-transformed hamster cells to cytosine arabinoside inhibition (16). It should be noted that infection of *ts* AF-8 cells at the nonpermissive temperature and high MOI (50 PFU/cell) in the presence of cytosine arabinoside (40 μg/ml) resulted in a complete inhibition of viral DNA synthesis (Yanagi, unpublished data), suggesting that the viral enzyme was still not acting independently. This result is consistent with the supposition that more than one host factor participates in viral DNA synthesis.

In any event, the existence of cell cycle mutants capable of conditionally inhibiting HSV-1 replication should be of great value in studying both viral transformation and replication, as well as the nature of specific cellular gene products.

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