VIRUS GENES 2:3, 253-267, 1988

Transcriptional Activation is not Responsible for Increased Levels of Autonomously Expressed Simian Virus 40 T-Antigen in Herpes Simplex Virus-Infected Cells

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Received October 31, 1988
Accepted November 24, 1988

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Key words: herpes simplex virus, *trans*-activation, SV40 T-antigen, metallothionein

Abstract

Herpes simples virus type 1 (HSV-1) superinfection of CV-1 cells weakly *trans*-activated a plasmid-borne metallothionein 1 (MT-1) promoter, but activated the expression of a marker gene controlled by an authentic HSV-1 promoter to a high level. In contrast, CMT-3 cells, which are CV-1 cells stably transformed with the simian virus 40 (SV40) large T-antigen (T-Ag) gene controlled by the MT-1 promoter, contained high levels of T-Ag following HSV-1 superinfection, but only if cells were preincubated in the presence of heavy-metal ions. This T-Ag was functional in that it could mediate the increase in copy number of a marker plasmid containing the SV40 origin of DNA replication. Pulse and continuous labeling of preinduced CMT-3 cells showed that T-Ag expression was not induced by HSV-1; but rather, HSV-1 superinfection resulted in the stabilization of pre-existing protein.

Introduction

Activation of heterologous cellular and viral genes by herpes simplex virus type 1 (HSV-1) infection remains a poorly understood phenomenon, although there is considerable interest in the problem, particularly with respect to the etiology of acquired immune deficiency syndrome (AIDS) (1-4). Indeed, activation of the ex-

pression of one or more critical cellular genes is one possible mechanism for herpesvirus-induced cellular transformation (5). Several groups have used subtractive cDNA cloning to identify cellular genes that can be activated upon infection by HSV-1 (6, 7). However, only the heat/stress responsive genes have been specifically identified as members of this group (8, 9). These stress proteins can be thought of as gene products whose transcriptional control elements may be readily accessible for transcription. This and the high levels of transcriptional activity from a variety of plasmid-borne indicator genes in cells infected with herpes viruses (10-12) suggested to us that the physical accessibility of the heterologous promoter in question may be one determinant of the response to HSV infection. Thus, we decided to investigate the ability of HSV gene products to *trans*activate the metallothionein (MT-1) promoter as a function of whether this promoter had been preinduced with heavy metals, in that treatment of cells with metal ions might alter the promoter from inaccessible to accessible states (13). Although we found that, irrespective of preinduction with heavy-metal ions, HSV-1 was unable to strongly activate either a plasmid-borne MT-1 promoter or a chromosomal MT-1 promoter controlling the expression of the SV40 T-antigen (T-Ag) gene, we did find that HSV-1 superinfection stabilized preexisting T-Ag. This finding has important implications concerning mechanisms by which HSV may affect the activities of proteins known to alter the host cell phenotype.

Methods

Drugs and inhibitors

Thymidine-1-β-arabinofuranoside (Ara-T, Sigma) was used at a final concentration of 50 μg/ml. CMT-3 cells were induced with $ZnCl_2$ and $CdSO_4$ at final concentrations of 100 μM and 1 μM, respectively.

Cells, virus, DNA transfections, and CAT assays

These methods were used as described previously (14, 15). The CMT-3 cell line derived from the simian kidney cell line, CV-1, both provided by Y. Gluzman (16), contains the gene for the SV40 large T-Ag linked to the mouse MT-1 promoter. Treatment of CMT-3 cells with heavy-metal ions (Zn^{2+}/Cd^{2+}) results in MT-1-induced expression of T-Ag and consequently mediates replication of transfected constructs carrying the SV40 *ori*. Cultures of rabbit skin (RS) cells, CV-1, or CMT-3 cells (10^6/60 mm^2 culture dish) were transfected by calcium phosphate coprecipitation with 5 μg of $CsCl_2$ gradient purified-form I plasmid DNA. Transfected cells were then either mock-infected or superinfected with HSV-1 in the absence or presence of 50 μg/ml Ara-T at multiplicities and for time periods detailed in the relevant figure legends. CAT assays were performed when required, as described

previously (14, 15). One unit of CAT activity catalyzes the acetylation of one nanomole of chloramphenicol per minute at 37°C, pH 7.8 (Pharmacia). Using 0.25 μCi of [^{14}C]chloramphenicol (35-45 mCi/mmol; Amersham) in a 90 min reaction at 37°C, a 50% conversion is equivalent to 0.0334 units of CAT enzyme. Therefore, conversion of X% acetylation to U/mg is given by the equation:

$$\frac{X \times 0.0334}{50 \times \text{protein in mg}} = \text{U/mg}.$$

The protein content of extracts was determined using the BioRad protein assay kit with bovine serum albumin as the standard.

Recombinant plasmids

Construction of pSVOd-CAT (*Xba*I) has been detailed previously (14). This plasmid contains an unique *Xba*I site, into which was cloned, by addition of *Xba*I linkers (Collaborative Research), either the HSV *ori*$_s$ or the SV40 *ori*. The HSV *ori*$_s$ from the terminal short repeat region of the viral genome (TR$_s$) was cloned as a 231-bp *Sma*I fragment corresponding to base pairs 550-781 of the sequence of McGeoch et al. (17). The SV40 *ori* was cloned as a 210-bp *Sph*I-*Hin*dIII piece (SV40 coordinates 5172-133) containing the SV40 "core" origin and GC boxes essential for replication (18). Eighteen hours after transfection with SV40 *ori*$^+$ constructs, CMT-3 cells were incubated in medium containing Zn^{2+}/C^{2+} to induce T-Ag-mediated replication of these plasmids. Cells were then deinduced by incubation in normal medium.

The plasmid rMT-1/Δ222CAT contains the chloramphenicol acetyl transferase (CAT) gene controlled by the rat MT-1 promoter (13) and was kindly provided by R. Andersen. The plasmid pSVOd CAT VSP (−280/+154) contains the CAT gene controlled by the promoter/leader sequences of the HSV virion stimulatory protein (VSP) gene and has been described previously (15).

Measurement of plasmid copy number

Transfected cells were harvested at postinfection times detailed in the figure legends and then were analyzed for template copy number by Southern blotting of total DNA using a CAT-specific probe prepared by random-primed cDNA synthesis (19). DNA was digested either with *Hin*dIII to linearize plasmid DNA or with *Hin*dIII and *Dpn*I. Since *Dpn*I requires methylated adenine residues for cleavage, it digests only input plasmid DNA and leaves undigested SV40 *ori*$^+$−mediated replicated plasmids. Densitometric analysis of nonreplicating (*ori*$^-$) constructs provided a control for efficiency of transfection, and analysis of replicating (*ori*$^+$ and SV40 *ori*$^+$) constructs allowed determination of the increase in template copy number.

Immunofluorescence

Monolayers of 10^4 CMT-3 cells grown on 10-well immunofluorescence coverslips were induced with Cd^{2+}/Zn^{2+} and infected as detailed in the legend to Fig. 2. Coverslips were then fixed with methanol at −20°C and were permeabilized with 1% Triton X-100 (Sigma). Cells were then incubated at 37°C for 1 hr with primary antibodies against T-Ag and HSV-1 polypeptides. The monoclonal antibodies against T-Ag, pAb416, and pAb419 (20) were kindly provided by E. Harlow as tissue culture supernatants and were mixed 1:1 in phosphate-buffered saline (PBS) containing 1% BSA. One microliter of rabbit antiserum raised against HSV-infected cells (AL3L, provided by G. Cohen) was added to the diluted T-Ag antibodies, and this cocktail was reacted with the monolayers for 1 hr at 37°C. Cells were then washed three times with PBS/1% BSA and bound antibodies were detected by treatment with secondary antibodies as follows: Goat anti-mouse IgG conjugated to rhodamine and goat anti-rabbit IgG conjugated to fluorescein were mixed 1:1, diluted 1:50 in PBS/1% BSA, and reacted with the monolayers. The coverslips were then washed sequentially through 50%, 60%, 80%, and 95%, ethanol, dried in air, and mounted using 90% glycerol/10 mM Tris, pH 7.8, between slides, ready for viewing using a Leitz photomicroscope with an X63 objective lens. Anti-T-Ag staining was visualized by excitation of rhodamine at 540 nm, giving red fluorescence, and anti-HSV staining was detected as green fluorescence following excitation of fluorescein at 480 nm. Fields were photographed using Kodak X-pan black-and-white film with 2 min regulated exposure times.

Immunoprecipitation

Cultures of 10^6 CMT-3 cells were induced with Cd^{2+}/Zn^{2+} and infected with HSV-1 for the time periods described in the legends to Figs. 4 and 5. These legends also detail the interval of radiolabeling of polypeptides. This was performed by overlaying cells with growth medium containing 10 μCi/ml of [^{35}S]methionine (1000 Ci/mmol, Amersham). Cells were washed three times in PBS then resuspended in 200 μl of extraction buffer (150 mM NaCl; 50 mM Tris HCl, pH 7.8; 0.1 mM EDTA; 0.5% Tween 20 [Sigma]) and sonicated for 10 sec on ice. The cell lysate was cleared by microcentrifiguration for 10 min at 4°C, and the supernatant was collected. The protein content, determined using the BioRad assay kit, was generally at 2 mg/ml. Aliquots containing 200 μg of soluble protein were reacted with antibodies against T-Ag and HSV-VP5 (pAb416/pAb419, 1:1 [20] and NC-1 [21]). The resulting immune complexes were then precipitated by incubation with 50 μl of a 10% solution of *S. aureus* protein A sepharose (Pharmacia) at 4°C for 2 hr. This was followed by two washes each in extraction buffer and in disruption buffer containing 2 M urea, and then one wash each in disruption buffer containing 1% β-mercaptoethanol then 0.1% $NaDodSO_4$. The protein-A complex was resuspended in 40 μl PAGE disruption buffer (0.5 M Tris HCl, pH 7.0; 2% $NaDodSO_4$; 5%

β-mercaptoethanol; 5% glycerol; 0.005% bromophenol blue) and heated at 95°C for 10 min, and then the soluble protein faction was subjected to electrophoresis through 10% $NaDodSO_4$-polyacrylamide gels (22). After staining with Coomassie brilliant blue and drying, gels were exposed to x-ray film for 2-3 days.

Results

Pretreatment with heavy metals did not render the metallothionein promoter responsive to HSV-1 activation

A number of experiments were carried out to determine whether the rat metallothionein (rat MT-1) promoter could be activated by HSV-1 superinfection and whether preinduction of promoter activity had any effect on viral activation. As described in the methods section, cultures of 10^6 cells were transfected with appropriate supercoiled plasmid DNA, treated with Zn^{2+}/Cd^{2+} and then were infected or mock-infected with HSV-1. Control cultures were "mock-induced" by incubation in fresh medium lacking heavy-metal ions and hereafter will be referred to as *uninduced.* A typical experiment is shown in Fig. 1, and the quantitation of two similar experiments is presented in Table 1.

Heavy-metal induction of the rat MT-1 promoter resulted in a greater than 10-fold increase in CAT activity compared to uninduced cells (1.10–1.02 vs. 0.06–0.09 U CAT/mg protein); however, HSV-1 superinfection of induced cells had only a slight further stimulatory effect upon CAT levels in induced cells (1.16–1.5 vs. 1.10–1.02 U CAT/mg protein). A slightly greater stimulation of CAT

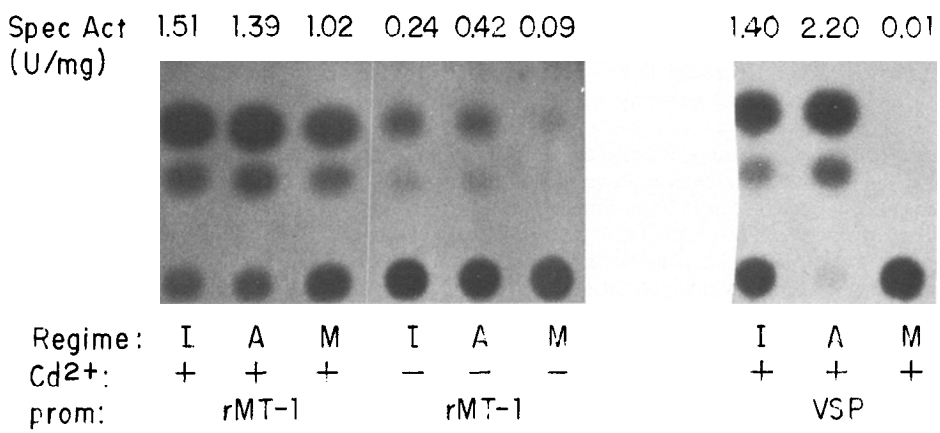

Fig. 1. CAT expression from MT-1 or VSP promoters at 11 hr postinfection. Cells were mock-infected (M) or were infected in the presence (A) or absence (I) of 50 μg/ml Ara-T after pretreatment (+) or mock-treatment (−) with medium containing zinc and cadmium ions.

Table 1. Effect of HSV-1 superinfection on CAT expression controlled by the rat MT-1 or HSV-1 VSP promoter

Plasmid[a]	Zn^{2+}/Cd^{2+} induced[b]	HSV inf[c]	+ Ara-T[d]	Time of harvest (hr postinf)[e]	CAT activity (U/mg)[f]
Rat MT-1 222	+	+	−	12	1.51
Rat MT-1 222	+	+	+	12	1.39
Rat MT-1 222	+	−	−	12	1.02
Rat MT-1 222	−	+	−	12	0.24
Rat MT-1 222	−	+	+	12	0.42
Rat MT-1 222	−	−	−	12	0.09
Rat MT-1 222	+	+	−	18	1.16
Rat MT-1 222	+	+	+	18	1.24
Rat MT-1 222	+	−	−	18	1.01
Rat MT-1 222	−	+	−	18	0.16
Rat MT-1 222	−	+	+	18	0.35
Rat MT-1 222	−	−	−	18	0.06
HSV-1 VSP (+154)	+	+	−	12	1.4
HSV-1 VSP (+154)	+	+	+	12	2.2
HSV-1 VSP (+154)	+	−	−	12	0.01
HSV-1 VSP (+154)	+	+	−	18	1.88
HSV-1 VSP (+154)	+	+	+	18	2.8
HSV-1 VSP (+154)	+	−	−	18	0.01

[a]Approximately 10^6 CV-1 cells were transfected with 5 μg of a plasmid containing the chloramphenicol acetyl transferase gene linked to the rat MT-1 or HSV-1 VSP promoter (Col 1).

[b]Transfected cells were treated 18 hr later with medium containing or lacking Zn^{2+}/Cd^{2+} ions for 6 hr, washed twice with warm saline, and incubated for a further 6 hr in medium free of heavy-metal ions.

[c]Cells were mock-infected or infected at 1-2 PFU/cell with HSV-1 (KOS).

[d]Infection was in the presence or absence of 50 μg/ml thymidine 1-β-D arabinofuranoside.

[e]Cells were harvested 12 or 18 hr postinfection; extracts were prepared and assayed for CAT activity, as described previously (14,15). The protein content of extracts was determined with a BioRad protein assay kit using bovine serum albumin as the standard.

[f]One unit of CAT activity catalyzes the acetylation of one nonomole of chloramphenicol per minute at 37°C, pH 7.8 (Pharmacia). Using 0.25 μCi Amersham [^{14}C]chloramphenicol (35-45 mCi/mmol) in a 90 min reaction at 37°C gives a 50% conversion equivalent to ~0.0334 units CAT enzyme. Therefore, X% conversion is given by (X × 0.0334)/(50 × protein in mg) = U/mg.

activity controlled by the rat MT-1 promoter was observed in uninduced cells superinfected with HSV-1. Here 3-5 times higher CAT activity was recovered from the virus-treated uninduced cells compared to uninfected controls (Table 1). Rat MT−1 controlled CAT enzyme levels were higher in superinfected cells in which viral DNA replication was inhibited with Ara-T. A similar effect has been observed with assays of HSV promoter driven CAT activity (14, 23), and can also be seen in Fig. 1 and Table 1 when CAT activity was controlled by the HSV-1 virion stimulatory protein (VSP or α-TIF), an authentic and active viral promoter (15, 24, 25).

Although total levels of CAT activity in cells containing the rat MT-1 promoter-controlled construct approached those seen in cells containing the HSV-1 VSP promoter (Table 1), the proportional increase of activity due to heavy-metal ion induction was significantly less due to the high basal activity in uninduced, mock-infected cultures. Such levels of basal activity controlled by the rat MT-1 promoter in the absence of Zn^{2+}/Cd^{2+} induction have been reported previously (13).

We conclude from these experiments that HSV-1 superinfection can activate the rat MT-1 promoter, but is significantly less efficient than the activation observed for the HSV-1 virion stimulatory protein promoter. It is also clear that preinduction of the MT-1 promoter with heavy metals does not materially increase this promoter's ability to be activated by *trans*-acting viral factors.

Higher levels of autonomously expressed T-Ag are found in HSV-1-infected cells

An apparently different situation was observed with an integrated metallothionein promoter in CMT-3 cells. These cells are derived from CV-1 cells and are stably transformed with the SV40 T-Ag gene controlled by the mouse MT-1 promoter. Thus transcription of the gene and subsequent expression of functional T-Ag protein is readily induced by treatment of CMT-3 cells with medium containing heavy-metal ions (16). Cultures of 10^4 CMT-3 cells on immunofluorescence coverslips were preinduced for 6 hr with medium containing Zn^{2++} and Cd^{2+}, rinsed twice in saline, and incubated for a further 6 hr in normal growth medium. Cultures were then infected with 0.1, 1, or 10 PFU/cell of HSV-1 or were mock-infected. Fig. 2 depicts the results obtained using an m.o.i. of 1. The presence of T-Ag and HSV-1-specific polypeptides was detected by reaction with a mixture of the appropriate antibodies, followed by immunofluorescence, as detailed in Methods. Each monolayer was analyzed concurrently for T-Ag levels and the presence of HSV polypeptides by excitation of individual fields at appropriate wavelengths. Fig. 2A shows that a significant amount of T-Ag was present in cells preinduced and infected. All cells showing the presence of T-Ag were also positive for HSV-1 antigens (Fig. 2B). Similarly, cells infected at 0.1 PFU/cell after preinduction also showed increased levels of T-Ag, but only in cells expressing HSV polypeptides (data not shown). In contrast, infection of uninduced cells or mock-infection of preinduced cells did not show this high level of T-Ag (Figs. 2C-2F).

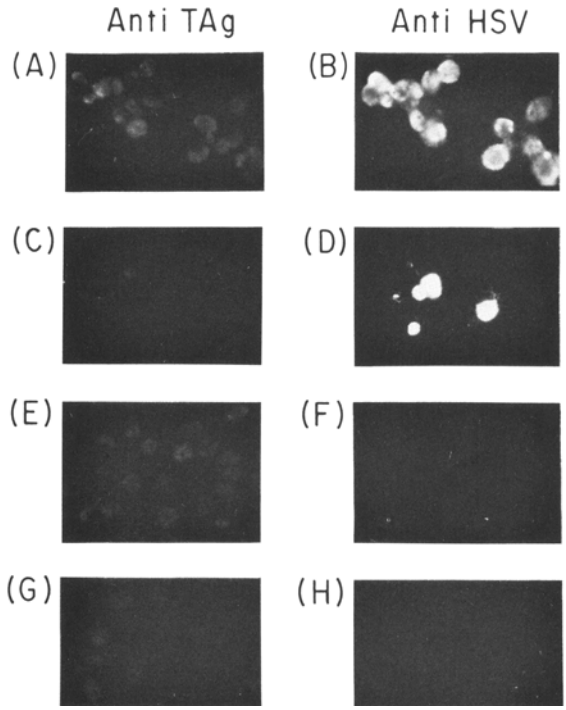

Fig. 2. Immunofluorescence staining of CMT-3 cells for the presence of SV40 T-Ag and HSV-1 polypeptides. T-Ag was detected using murine monoclonal antibodies and goat antimouse conjugate (left-hand panels A, C, E, and G), and HSV-1 antigens were detected using a general rabbit antiserum and goat antirabbit conjugate (right-hand panels B, D, F, and H). Panels A & B: induced and infected at 1 PFU/cell; panels C & D: not induced but infected; panels E & F: induced but not infected; panels G & H: not induced and not infected.

Extra T-Ag functions to support additional SV40 ori*-mediated DNA replication*

The T-Ag present in the HSV-1-infected, preinduced cells was shown to be functional by its ability to mediate a further increase in copy number of a plasmid containing the SV40 origin of DNA replication (SV40 *ori*). A typical experiment is shown in Fig. 3A and is described in the figure legend. CMT-3 cells were transfected with a plasmid, pSVOd-CAT, lacking "poison" sequences (26) and containing no origin (*ori*$^-$), the SV40 *ori,* or the HSV-1 IRs/TRs origin of DNA replication (HSV *ori*$_s$) (27; see Methods for details of constructs).

Eighteen hours after transfection with 5 μg of supercoiled plasmid DNA, cultures of 10^6 CMT-3 cells were treated with Zn^{2+}/Cd^{2+} to induce T-Ag synthesis and then were rinsed and allowed to recover in growth medium for 6 hr. Cultures were then infected at 1-2 PFU/cell with HSV-1 or were mock-infected. At 6 or 18

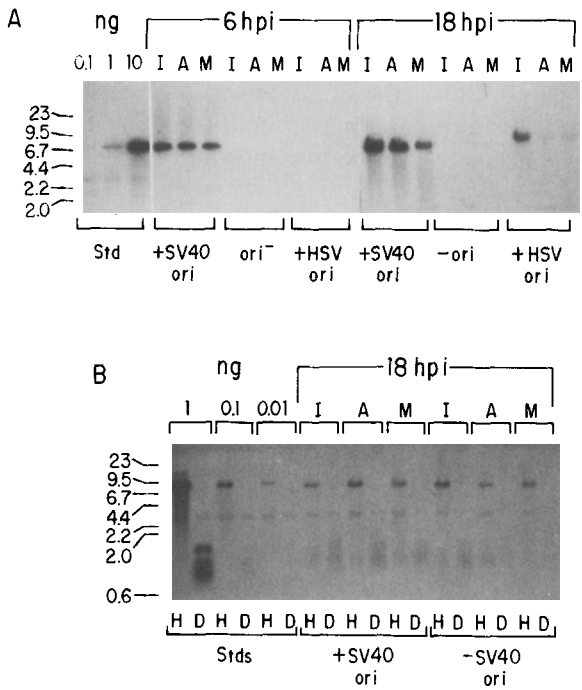

Fig. 3. Amplification of plasmids containing the SV40 origin of DNA replication in infected and uninfected CMT-3 or CV-1 cells. Triplicate cultures of 10^6 CMT-3 cells (A) or CV-1 cells (B) were transfected (14,15) with 5 μg of supercoiled pSVOd-CAT (*Xba*I) containing the SV40 origin of DNA replication (+SV40 *ori*), the HSV-1 origin of DNA replication from the terminal short-repeat region (TR_S) of the viral genome (+HSV ori_S), or containing no eucaryotic replication origin (ori^-), as detailed in Methods. Eighteen hours after transfection, cells were incubated with Zn^{2+}/Cd^{2+}-containing medium for 12 hr. This treatment induced T-Ag synthesis and replication of SV40 *ori* plasmids in CMT-3 cells (A, lanes +SV40 *ori* vs. ori^-). Cells were deinduced in normal growth medium for 6 hr, and were then mock-infected (M) or infected at 1-2 PFU/cell with HSV-1 (KOS) in the presence (A) or absence (I) of 50 μg/ml thymidine 1-β-D-arabinofuranoside (Ara-T). Total DNA was isolated (at postinfection times, indicated above lanes, digested) with *Hin*dIII alone (Figs. 3A and 3B, lanes marked *H*) or with *Hin*dIII and *Dpn*I (Fig. 3B, lanes marked *D*) and electrophoresed through a 0.8% agarose gel. Standards (Stds) containing 5 μg of cellular DNA and 0.1 ng, 1 ng, or 10 ng of pSVOd-CAT (*Xba*I) (Fig. 3A); or 5 μg cellular DNA and 1 ng, 0.1 ng, or 0.01 ng (Fig. 3B) were also digested and electrophoresed in parallel. Gels were blotted and hybridized with a random-primed probe of the CAT gene.

hr postinfection, total DNA was extracted from the cultures, digested with *Hin*dIII to generate linear plasmid, and the levels of DNA were assayed by densitometric analysis of Southern blot hybridizations. Copy numbers of plasmids bearing the SV40 *ori* (Fig. 3A, lanes marked +SV40 *ori*) were the same in infected cells as in mock-infected cells at 6 hrs postinfection (Fig. 3A); but by 18 hrs postinfection, the DNA levels of the SV40 *ori* plasmid in infected cells had increased a further three- to fourfold. Unlike the situation with replication of the plasmid containing the HSV-1 ori_s, additional replication of the SV40 *ori*-containing plasmid in infected cells was insensitive to the presence of 50 μg/ml Ara-T (Fig. 3A, compare lanes headed *I* and *A* for constructs +SV40 *ori* and +HSV *ori*). Therefore, this additional plasmid replication in infected cells was not mediated by the Ara-T-sensitive HSV-1 DNA polymerase, but rather was catalyzed by host-cell DNA polymerases, such as Pol α, which complexes with T-Ag (28).

To further show that replication of SV40 *ori* plasmids in HSV-infected cells was not directly catalyzed by HSV-1 polypeptides, we performed the control experiment shown in Fig. 3B. Here, plasmids containing or lacking the SV40 *ori* were transfected into CV-1 cells that do not contain the T-Ag gene. The cells were treated as described above for CMT-3 cells (namely, incubated with Zn^{2+}/Cd^{2+} medium and infected at 1 PFU/cell), and HSV-1 induced replication of the SV40 *ori*-containing plasmid was assayed by testing recovered DNA for its sensitivity to cleavage by *Dpn*I. This restriction enzyme requires methylated adenine residues for cleavage and consequently digests only input plasmid DNA grown in dam^+ bacteria, but will not cleave DNA replicated in mammalian cells. The complete sensitivity of plasmid DNA, recovered from CV-1 cells 18 hr postinfection, to *Dpn*I digestion (Fig. 3B, lanes marked D) demonstrated the lack of any measurable plasmid replication in HSV-1-infected cells in the absence of T-Ag.

De-novo synthesis is not responsible for higher T-antigen levels in infected cells

In light of the weak activation of CAT gene expression from the MT-1 promoter in response to HSV superinfection, the higher levels of functional T-Ag present in HSV-1-infected, preinduced CMT-3 cells compared to mock-infected, preinduced cells was surprising. For this reason, we used immunoprecipitation of radioactively labeled polypeptides to measure the synthesis of T-Ag in CMT-3 cells under conditions of preinduction and subsequent HSV-1 superinfection. Our results indicated that T-Ag present in the infected, preinduced CMT-3 cells was not synthesized during the HSV-1 infection period. The specific experimental protocol is outlined in the legends to Figs. 4 and 5.

In the first experiment (Fig. 4), T-Ag was isolated at 18 hr postinfection from extracts of induced or uninduced cells by reaction with the monoclonal antibodies pAb416 and pAb419 (20), and precipitation of the resulting immune complex with *S. aureus* protein A-sepharose. A monospecific polyclonal rabbit antiserum against the HSV-1 major capsid protein (VP5) (21) was included as a control for

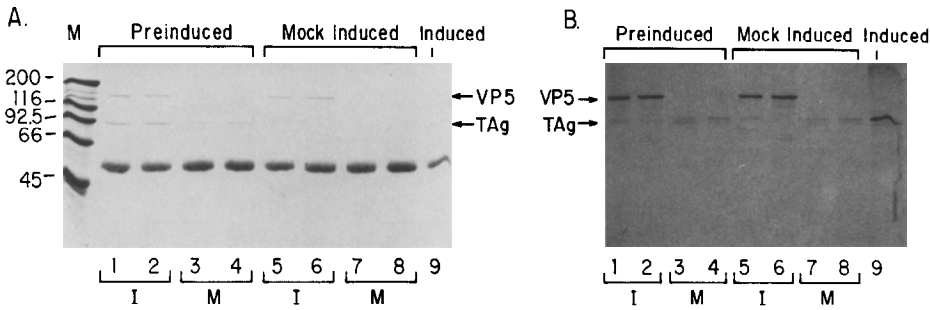

Fig. 4. Greater quantities of T-Ag are recovered from induced, infected CMT-3 cells. A. Coomassie stained gel. B. Autoradiogram of the same gel. Paired cultures of CMT-3 cells were induced, deinduced, and infected (I) or mock-infected (M) in the presence of [^{35}S]methionine. T-Ag and VP5 were coprecipitated with a mixture of anti-T-Ag and anti-VP5 antibodies. Parallel cultures were mock-induced, deinduced, and infected or mock-infected in the presence of [^{35}S]methionine (lanes 5-8). One further culture was labeled at the time of induction with [^{35}S]methionine but was not infected (lane 9). Migration of VP5, T-Ag, and molecular-weight markers are indicated.

productive viral infection. Immunoprecipitated polypeptides were then separated by $NaDodSO_4$ polyacrylamide gel electrophoresis (22). Fig. 4A (lanes 1 and 2) shows that polypeptides migrating with apparent molecular weights corresponding to those expected for T-Ag and VP5 were readily detectable in the preinduced, infected cells using Coomassie brilliant blue staining. However, only the VP5 protein incorporated a significant amount of radiolabel during the period of viral infection (Fig. 4B, lanes 1 and 2). Uninduced, infected cells contained significant amounts of VP5 protein, detectable both by protein staining and autoradiography (Figs. 4A and 4B, lanes 5 and 6), but there was no readily detectable band of radioactivity corresponding to T-Ag, indicating the absence of T-Ag synthesis during the period of labeling and superinfection. As a control, the presence of heavy metals during the entire labeling period resulted in the expression of enough T-Ag to be readily detectable by protein staining (Fig. 4A, track 9); and as seen in the same track 9 of Fig. B, there was significant radioactivity incorporated into this material.

A second experimental protocol was used to show that all the T-Ag recovered from infected cells was indeed synthesized prior to infection. Cultures were incubated with [^{35}S]methionine-containing medium, according to the regime detailed in the legend to Fig. 5, to facilitate identification of polypeptides synthesized de novo during each state of induction, deinduction, and HSV superinfection. Uninduced cultures were radiolabeled in parallel. T-Ag was synthesized during the induction period and, to a lesser extent, during the period of deinduction (Fig. 5, lanes 1 and 5), but little T-Ag synthesis was evident in the uninduced cultures (Fig. 5, lanes 2 and 6). There was little or no T-Ag synthesis during the time of virus infection, although the [^{35}S]-methionine medium was utilized in that VP5 was radiolabeled in these cultures (Fig. 5, regime 6, lanes 11 and 12). T-Ag im-

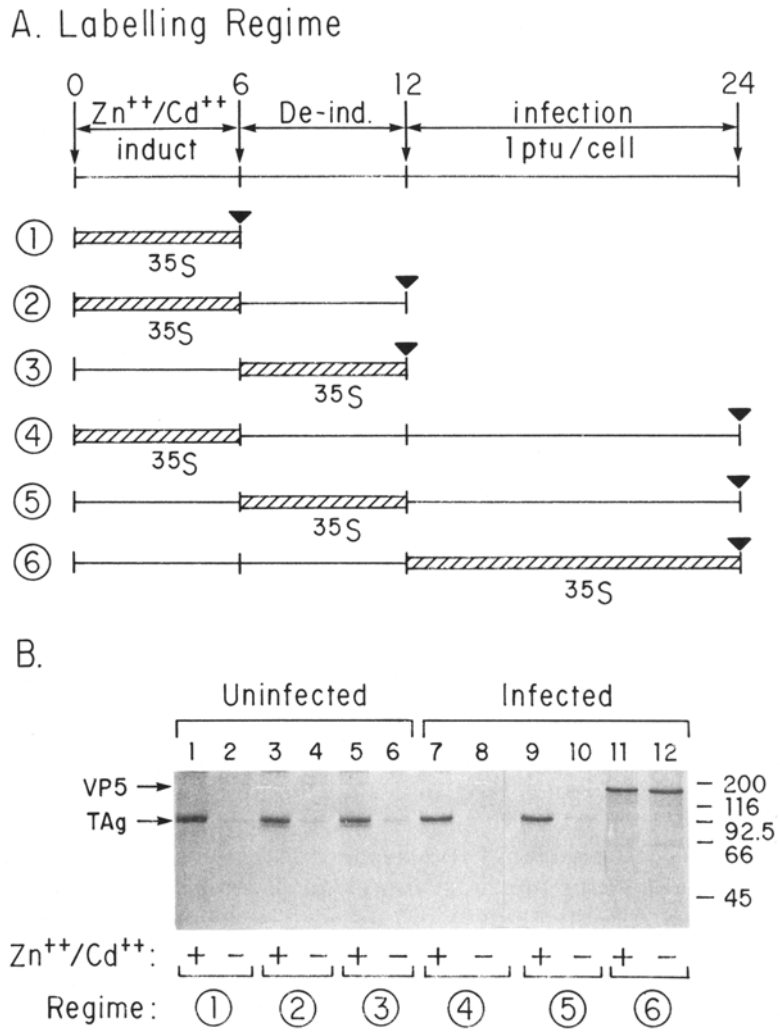

Fig. 5. T-Ag is maintained but not synthesized in infected cells. A. Diagram of [^{35}S]methionine labeling regimes 1 through 6 during induction, deinduction, or infection. Cross-hatching indicates labeling period, and filled arrowhead indicates time of harvesting. B. Autoradiogram of immunoprecipitation of T-Ag and VP5. Cells were treated with Zn^{2+}/Cd^{2+} medium (+) or were not treated (−), and [^{35}S]methionine medium was added at either the time of induction, time of deinduction, or time of infection, according to regimes 1-6. Tracks 1-6: uninfected; tracks 7-12: infected. The position of VP5 and T-Ag is indicated, with the position of molecular-weight markers indicated.

munoprecipitated from infected cells prelabeled at the time of induction or during the deinduction period was radiolabeled (Fig. 5, lanes 7 to 10). Therefore, the higher levels of T-Ag found in infected cells, typically as shown in Fig. 4A, but also true for the Coomassie brilliant blue stained gel of Fig. 5 (not shown), were derived from T-Ag synthesized during or immediately after the time of induction, which was then turned over less rapidly in HSV-1-infected cells, rather than as a result of de-novo T-Ag synthesis following HSV superinfection. The presence of a fainter, slightly lower molecular weight band in samples harvested following the 6 hr period of deinduction (regimes 2 and 3), rather than immediately after the Zn^{2+}/Cd^{2+} induction period (regime 1), suggested that some type of turnover or post-translational modification may occur in uninfected cells. This second species was not found in HSV-infected cells, where the single form detected was the same species found at the time of colabeling and induction (regime 1).

Discussion

The experiments described here indicated that the MT-1 promoter is only weakly responsible for activation by HSV superinfection. However, HSV superinfection is able to mediate posttranslational stabilization of functional SV40 T-Ag in CMT-3 cells.

Since the functions of SV40 T-Ag in infected and transformed cells are both diverse and complex (29), and the number of gene products induced by HSV-1 infection is large (30), we can only speculate as to the nature of this stabilization. Covalent modification of T-Ag, such as phosphorylation and/or ADP-ribosylation, may alter the stability as well as the specific activity of the protein (31, 32). Levels of protein phosphorylation and ADP ribosylation have both been reported to increase in HSV-infected cells (30, 33). T-Ag appears to associate with at least three cellular proteins, AP-2, p53, and DNA polymerase (28, 34, 35), and the interaction between p53 and T-Ag stabilizes the former protein (34, 36). Further, c-*myc* has been reported to increase T-Ag-mediated DNA replication from the SV40 *ori* in the absence of de-novo T-Ag synthesis (37). Alterations in the levels of T-Ag binding proteins as a result of HSV-1 infection could increase the stability of T-Ag. A direct interaction of HSV-1 gene products with T-Ag, thus giving greater stability, is another possibility. Given that the higher levels of T-Ag found in infected cells were also found in cells infected in the presence of Ara-T, then the viral gene products that bring about this effect appear to be structural components of the infecting virions or members of the immediate-early or early class of viral polypeptides.

Although mechanistically unclear, the observations described above have important implications. Firstly, they emphasize that an alteration in the level or activity of a gene product does not necessarily represent a change in the level of transcription from the corresponding gene. Secondly, and more specifically, her-

pes simplex virus infection may lead to alterations in the phenotype of an infected cell by modulating the levels or activities of heterologous polypeptides.

Acknowledgments

This work was supported by funds provided by grants Ca11861 of the National Institutes of Health and MV372 from the American Cancer Society. Further support was provided by the UCI Focused Research Program in the Molecular Biology of Eucaryotic Viruses. We thank C. Blair and M. Rice for technical assistance, R. Wrightsman and B. Hamkalo for help with immunofluorescence, J. Foreman for describing the immunoprecipitation method, and M. Flanagan for helpful discussions. We also thank R. Andersen, Y. Gluzman, E. Harlow, G. Cohen, J. Manning, and M. Cumsky for generously providing plasmids, cells, antibodies, and *S. aureus* protein A sepharose used in this study.

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