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A Physical Domain of Herpes Simplex Virus ICP8 Is Expressed and Active in *Escherichia coli*

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In this report, we describe a series of procedures to assay the function of fusion genes in *Escherichia coli* and the specific application to the carboxy-terminal third of the herpes simplex virus type 1 (HSV-1) DNA-binding protein ICP8. *E. coli* cells containing the cloned HSV-1 *Bam*HI G fragment with the HSV-1 *Bam*HI-G-V site, map unit 0.388, nearest the *tet* promoter in pBR322 synthesized an active product containing a portion of ICP8. The new product induced phenotypic alterations in recipient hosts that were measurable and stable yet limited to the stability of the plasmid. The corresponding cloned DNA from the characterized HSV-1 DNA-binding protein mutant *ts*HA1 exhibited a predictable temperature-sensitive phenotype. Screening procedures based on the loss of induction of the parental plasmid-induced phenotype in *E. coli* cells allowed us to select additional mutations. One of these, which conferred a phenotype different from that of *ts*HA1, was transferred to the viral genome by marker transfer techniques. We suggest that any mutant could be isolated in any sequence, provided that the wild-type coding sequences induce alterations in *E. coli* cells. The observed alterations should have relevance in determining the mode of action of the protein in its normal environment.

The herpes simplex viruses type 1 (HSV-1) and 2 (HSV-2) specify approximately 50 proteins in infected cells (8). Genetic and biochemical analyses of a variety of mutants isolated by either classical techniques or mutagenic treatment of cloned HSV DNA have yielded some information on the total number of potential regulatory genes necessary for the early events of viral expression and DNA replication. One aid to further identification would be a rapid assay of in vitro-constructed mutants for regions of the genome whose transcripts and products are expressed early during infection. In this paper, we report on the development of an assay and its application to analyzing some of the sequences that encode the HSV-1 major DNA-binding protein.

Bayliss et al. (1) showed that a high-molecular-weight, HSV-specified protein has strong binding affinity for DNA. The gene for this protein, which was identified as ICP8 (M_r 129,000), has been mapped to approximately 0.40 map units on the HSV genome (3, 10, 11). A 4.2- to 4.5-kilobase β-class transcript has also been mapped to this position (7, 17); this transcript is transcribed in the leftward direction in the prototype arrangement (7). ICP8 exhibits pleiotropic regulatory properties in infected cells. Functional ICP8 is required for DNA synthesis and the synthesis of γ polypeptides (3). In the absence of functional ICP8, proper temporal termination of β protein synthesis is reduced (3) and functional transcripts for at least αICP4 are retained (5). In infected cells, active ICP8 is compartmentalized to the cell nucleus and is physically associated with progeny DNA, whereas nonfunctional ICP8 associated with the nuclear framework (9). In vitro analysis shows that ICP8 can aid in the dissociation of synthetic polydeoxyadenylic acid-polydeoxythymidylic acid (14). ICP8 affinity is greater for single-stranded DNA than for double-stranded DNA (1, 15), and such binding exhibits a high degree of protein clustering; thus, the single-stranded DNA is held in an extended configuration (18).

All of these properties suggested that ICP8 would be an excellent candidate for expression in *Escherichia coli* and for examining the effects of its expression on bacterial replicative processes. The results presented in this paper show that *E. coli* replication is inhibited by ICP8 expression and that the described procedures may be widely applicable to the study of gene or gene fragment function outside the normal environment, the assay of mutagenized gene-coding sequences, and the selection of conditional-lethal mutations and may aid in selecting the most appropriate in vitro-generated mutants for either further study or transfer back to the original genome.

MATERIALS AND METHODS

Viruses, plasmids, DNA, and bacteria. The properties of HSV-1(mP) and HSV-1(mP) *ts*HA1 have been described previously (3, 6). Intact viral DNA was purified by sodium iodide density gradient centrifugation (21) of infected Vero cell cytoplasmic nucleocapsids or whole-cell lysates. The HSV-1(mP) *Bam*HI G fragment was obtained from an HSV-1(mP) *Bam*HI fragment library as described previously (13). The *Bgl*II-*Eco*RI IF fragment from *ts*HA1 was originally cloned in a *Bgl*II-*Eco*RI vector that contained pBR322 sequences and the HSV-1 *Bgl*II-*Bam*HI FF fragment. *Bam*HI-G was excised from this cloned fragment and cloned in pBR322 by previously described methods (13). The cloned *Bam*HI G fragment from HSV-1(F) pRB102 (13) was obtained from B. Roizman, University of Chicago. Plasmids were routinely carried in *E. coli* HB101. Plasmids were purified by the cleared lysate technique and banded in CsCl density gradients as described previously (2). Other *E. coli* strains used were JM103, DH1, and N99 (K Str^r *galK*). Plasmid transfers to the *E. coli* K strain were performed after one passage through *E. coli* DH1 cells. Salicin indicator plates were made with M9 agar media as described previously (4).

Enzymes, restriction endonucleases, and other reagents. Restriction endonucleases were purchased from New England Biolabs, Inc., Beverly, Mass., and Bethesda Research Laboratories, Gaithersburg, Md. DNA ligase was purchased from New England Biolabs. [^{35}S]methionine (1,055 Ci/mmol) and [^{14}C]leucine, [^{14}C]isoleucine, and [^{14}C]valine (each >250

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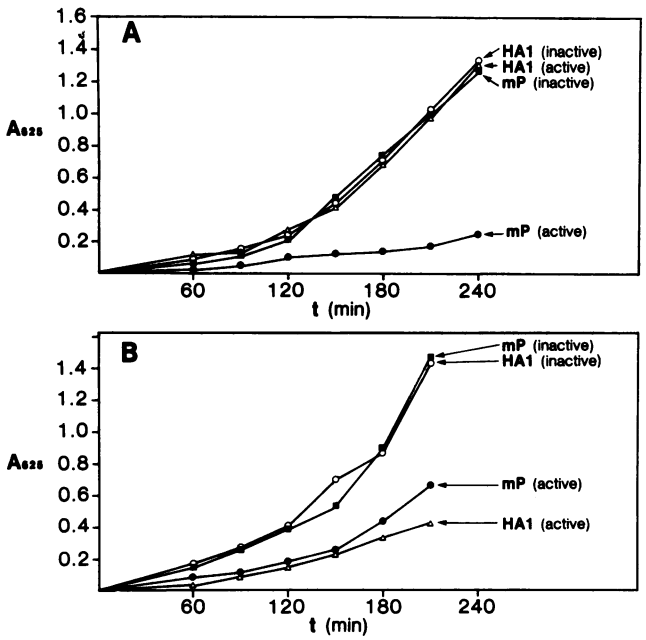

FIG. 1. Growth inhibition tests at 42°C (A) and 37°C (B) of *E. coli* bearing *Bam*HI-G-containing plasmids. mP, Clones of *Bam*HI-G from HSV-1(mP). HA1, Clones of *Bam*HI-G from HSV-1(mP) *ts*HA1. Active, *Bam*HI-G-V site is nearer the *tet* promoter; inactive, *Bam*HI-G-V site is further from the *tet* promoter. A_{625}, Absorbance measured at 625 nm.

mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Salicin was purchased from Sigma Chemical Co., St. Louis, Mo. Anti-ICP8 monoclonal serum (HSV-1 and HSV-2), designation 10-E3, was graciously provided by M. K. Shriver, Genetic Systems Inc., Seattle, Wash.

Plasmid-encoded proteins and gel electrophoresis. Plasmid-encoded proteins labeled with [^{35}S]methionine were prepared by the maxicell method of Sancar et al. (19). The processing of ^{14}C-amino acid-labeled infected-cell and maxicell lysates and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the labeled proteins have been described previously (11, 12).

Marker transfer. Marker transfer was performed by transfecting rabbit skin cells with intact HSV-1(mP) DNA and selected HSV-1 *Bam*HI-G mutants. Viral mutant selection and titration were performed on Vero cells (3). Marker rescue was performed in the same manner with mutant viral DNA and cloned HSV-1 DNA fragments.

RESULTS

Growth inhibition by *Bam*HI-G-containing plasmids. The growth of *E. coli* HB101 cells containing *Bam*HI-G from HSV-1(mP) in pBR322 was examined. Two plasmids containing the HSV-1 insert in opposite orientations were used. When the *Bam*HI-G-V site at HSV-1 map coordinate 0.388 was nearest to the *tet* promoter, extensive inhibition of bacterial growth was observed (Fig. 1). Neither the oppositely oriented insert nor pBR322 alone (data not shown) affected growth. This activity is encoded in other HSV-1 strains, since *Bam*HI-G from HSV-1(F) demonstrated the same activity when cloned in pBR322 (data not shown). We designated this growth-inhibiting property of *Bam*HI-G-containing plasmids and any active derivative plasmids ΔICP8 activity. We then determined the ΔICP8 activity in the previously described mutant HSV-1(mP) *ts*HA1 (3) by comparing it with that in HSV-1(mP) *Bam*HI-G-containing plasmids at two temperatures (Fig. 1). At 37°C, plasmids containing active-orientation inserts inhibited the growth of *E. coli* HB101. The opposite orientation had no effect. At 42°C, only the active-orientation insert from HSV-1(mP) inhibited the growth of *E. coli* HB101, whereas the plasmid from HSV-1(mP) *ts*HA1 did not. In agreement with previously reported studies, these analyses indicated that the mutation in *ts*HA1 was located in *Bam*HI-G (3) and that this protocol could easily assay such mutants.

We have previously reported that HSV-1 containing plasmids that confer resistance to coumermycin A1 could not transform *E. coli* JM103 (12). In standard transformation protocols, ΔICP8-active plasmids also did not transform strain JM103. Plasmids containing *Bam*HI-G from *ts*HA1 did transform this strain at 42 but not at 37°C, indicating that strain JM103 transformation correlated with plasmid-directed ΔICP8 activity and could serve as an indicator for the presence of a mutation.

Identification of the protein encoded in the active plasmid. Since mapping studies with HSV-1 × HSV-2 recombinants and in vitro translation experiments indicated that ICP8-coding sequences were contained in the right end (in the prototype arrangement) of *Bam*HI-G (3, 11) the following experiment was performed to confirm that part of the ICP8 protein is synthesized in bacterial cells containing the active plasmid. Labeled maxicell preparations containing either the active or inverted (inactive) *Bam*HI-G-containing plasmids were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis either before or after immunoprecipitation with 10-E3 anti-ICP8 serum (HSV-1 and HSV-2). The results (Fig. 2) were as follows. (i) Active-plasmid-containing maxicells directed the synthesis of two closely migrating proteins (M_r 45,000 and 42,000) (Fig. 2, lane B). The reason why two forms of the product are synthesized is unknown. One form may represent premature translation termination or readthrough. Inactive-plasmid-containing maxicell lysates did not show a new product. We did, however, occasionally observe new proteins with molecular weights of 100,000 and 39,000 in lysates of maxicells containing the inactive, inverted-orientation plasmid (data not shown). (ii) Both the 45,000- and 42,000-dalton products were immunoprecipitated with 10-E3 serum, as was a protein that comigrated with ICP8 from HSV-1(mP)-infected cells. Interestingly, a potential cleavage product of ICP8, or an ICP8-associated protein as reported by Quinlan et al. (16), was precipitated with the serum from the infected Vero cell lysates.

Additional effects of ICP8-active plasmids in *E. coli*. To extend the utility of these methods, we examined two additional measurable properties of cells containing ICP8-active plasmids.

Changes in the ability to use β-glucosides ($Bgl^- \rightarrow Bgl^+$) have been observed in strains bearing mutations in genes that affect the superhelicity of DNA (4). Since we suspected that ΔICP8 activity in *E. coli* may also affect DNA superhelicity, we examined *Bam*HI-G-containing plasmids and a previously reported derivative plasmid of HSV-1 *Bam*HI-F that confers resistance to coumermycin A1 (12). The results indicated that *bgl* induction occurred in an *E. coli* K strain that contained either ΔICP8-active (Table 1) or coumermycin A1 resistance-inducing plasmids (data not shown).

Secondly, plasmids that encoded active ΔICP8 underwent predictable deletions in greater than 4% but less than 10% of the bacterial clones. The isolates containing plasmid dele-

tions were readily detected by their production of normal-size *E. coli* colonies when plated overnight on L agar plates containing ampicillin. *E. coli* cells bearing active *Bam*HI-G-containing plasmids produced smaller colonies after overnight incubation. A summary of the sequences contained in ΔICP8-active, ΔICP8-inactive, and two selected deletion plasmids is included in Fig. 3. Deletion plasmid iv lost at least two noncontiguous regions. The larger deletion included both the *tet* promoter region and the entire *Bam*HI-G sequences. The smaller deletion occurred in the region of the pBR322 origin of replication. Deletion plasmid v was present in a mixed plasmid preparation which also contained an intact *Bam*HI-G-containing pBR322 plasmid; it lost at least three noncontiguous regions. A deletion of 2.66×10^6 daltons was lost from the *Bam*HI-G sequences, a smaller deletion occurred near the pBR322 origin of replication, and a third deletion encompassed sequences of the ampicillin resistance gene. Interestingly, an endpoint of the deletion in both iv and v mapped within 17 base pairs (bp) of pBR322 map position 4360, which contains the endpoint of the transposon *3* (Tn*3*) sequences in pBR322 (20).

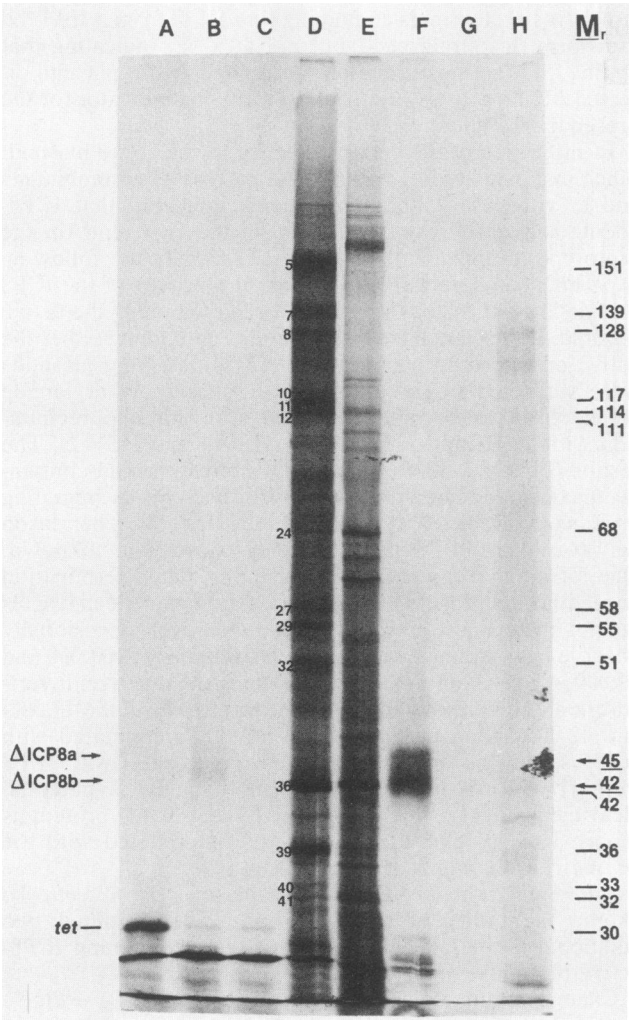

FIG. 2. Autoradiograph of proteins immunoprecipitated from labeled lysates of *E. coli* maxicell preparations (lanes A through C) and labeled lysates of HSV-1-infected Vero cells (lanes D through H). Lanes: A, pBR322; B, *Bam*HI-G in ΔICP8-active orientation; C, *Bam*HI-G in ΔICP8-inactive orientation; D, HSV-1-infected Vero cell lysate labeled for 5 to 7 h; E, mock-infected Vero cells; F, immunoprecipitate of active ΔICP8 *Bam*HI-G with 10-E3 anti-ICP8 monoclonal serum; G, immunoprecipitate of inactive *Bam*HI-G with 10-E3 anti-ICP8 monoclonal serum; H, immunoprecipitate of HSV-1-infected Vero cell lysate (labeled for 5 to 7 h) with 10-E3 anti-ICP8 monoclonal serum. M_r (in thousands) is shown at the right; ICP numbers are shown to the left of lane D.

TABLE 1. Phenotype of *E. coli* containing ICP8-active plasmids

Plasmid or plasmid insert	Growth inhibition	Temperature-sensitive growth inhibition	β-Glucoside utilization[a]
pBR322	No	No	No
*Bam*HI-G (G-V site near *tet* promoter)	Yes	No	Yes
*Bam*HI-G (inverted)	No	No	No
*Bam*HI-*Sal*I-GO (ΔO)	Yes	No	Yes
*Bam*HI-G [from HSV-1(mP) *ts*HA1]	Yes	Yes	Yes
New *Bam*HI-G-containing mutant plasmids			
*ts*9	Yes	Yes	Yes
*ts*4	Yes	Yes	Yes
*ts*18	Yes	Partial	Yes
*ts*3	Yes	Partial	Partial
*ts*8	Yes	Partial	Partial
*ts*1.8	Partial	Yes	No

[a] Performed in *E. coli* N99. Salicin utilization on indicator plates results in the production of an orange colony. Wild-type (Bgl^-) grows as grayish-white colonies.

Isolation of HSV-1(mP) *ts*HA1.8. Plasmid mutants were isolated after hydroxylamine mutagenesis (3) and selection for loss of the inhibitory activity described for *Bam*HI-G. The phenotypes of a selected group of these mutants are listed in Table 1. Mutant HSV-1(mP) *ts*HA1.8 was isolated after transfection of HSV-1(mP) DNA and mutant plasmid 1.8. This virus mutant exhibited a plating efficiency ratio (39°C/33°C) of $<10^{-6}$. Two experiments were performed to test for the presence of the mutant plasmid 1.8 lesion in *ts*HA1.8. In the first, marker rescue was performed. The progeny obtained after transfection of *ts*HA1.8 DNA with HSV-1(mP) *Bam*HI-G or *Bam*HI-*Sal*I-GO (ΔO) had plating efficiency ratios (39°C/33°C) of 4.7×10^{-3} and 4.6×10^{-4}, respectively. This indicated that the lesion was contained in the viral genome in the same position as it was in the mutated plasmid 1.8. Secondly, the cloned *Bam*HI-G from *ts*HA1.8 retained the original mutant plasmid phenotype in *E. coli* cells (Table 1), which indicated the successful transfer of a preselected mutation.

HSV-1 mutants containing lesions in the gene for ICP8 exhibit a characteristic reduction in late γ protein synthesis at the nonpermissive temperature. We examined the infected-cell proteins produced by *ts*HA1.8 and compared them with those observed for HSV-1(mP) and *ts*HA1 (3) by labeling infected HEp-2 cell cultures at intervals postinfection and analyzing the lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). From this experiment, it was apparent that the synthesis of the late γ proteins in *ts*HA1.8-infected cells at the nonpermissive temperature was greatly reduced. ICPs 15, 19, 20, and 32 were nearly absent from the lysate incubated at 39°C at 10 to 12 h postinfection. This result was comparable to that observed for lysates of *ts*HA1-infected cells (3) in tests

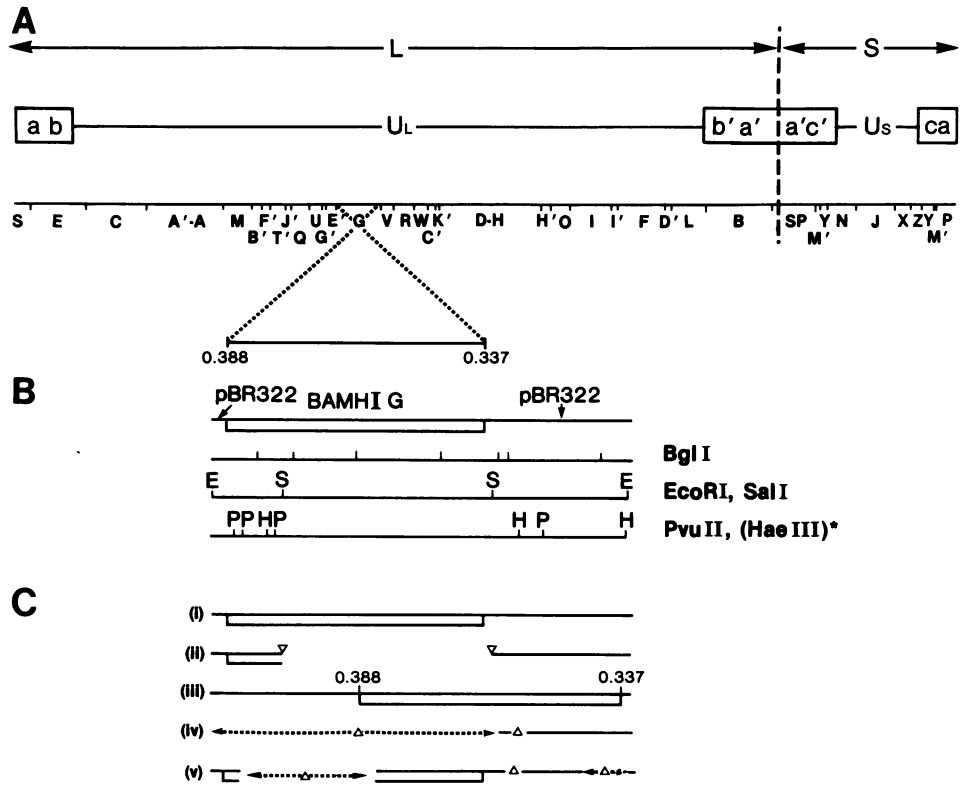

FIG. 3. Summary of HSV-1 genomic origin and sequences contained within ΔICP8-active and -inactive plasmids. (A) Genomic sequence components and genomic origin of *Bam*HI-G in the prototype arrangement. (B) Note that *Bam*HI-G is inverted relative to the prototype arrangement. The restriction endonuclease sites for *Bgl*I, *Eco*RI (E) *Sal*I (S), *Pvu*II (P), three selected *Hae*III* sites (H), and *Bam*HI are shown. These sites were used to map sequences of the plasmids shown in panel C. (C) *Bam*HI-G ΔICP8-active and -inactive plasmids. (i) *Bam*HI-G cloned with the *Bam*HI-G-V site nearest to the pBR322 *tet* promoter. (ii) Deletions of the sequences contained in *Sal*I-H, resulting in a plasmid containing part of the sequences from *Sal*I fragment O, i.e., ΔO. (iii) *Bam*HI-G cloned with the *Bam*HI-G-V site farthest from the pBR322 *tet* promoter. The plasmid is also drawn from the opposite direction solely to emphasize this fact. (iv) A spontaneously arising inactive plasmid with two deleted regions in pBR322 and in sequences from *Bam*HI-G. This plasmid contains the *Hae*III site at pBR322 bp position 4343 and does not contain the unique *Eco*RI site starting at pBR322 bp position 4360. (v) A plasmid arising spontaneously from active *Bam*HI-G with three deleted regions. The pBR322 *Hae*III site at bp position 4343 is absent, whereas the *Eco*RI site at bp position 4360 is present.

performed at the same time as this analysis of *ts*HA1.8 (Fig. 4).

DISCUSSION

ΔICP8 activity is most likely responsible for the inhibitory activity in strain HB101 and the inability of strain JM103 to survive after transformation with active *Bam*HI-G-containing pBR322. Since in vitro analyses reported by others have shown that ICP8 binding aids in the dissociation of synthetic DNA and exhibits preferential binding to single-stranded DNA (1, 14, 15), the ΔICP8 product probably affects the superhelicity of the *E. coli* chromosome. In agreement with this idea is the observation that the cryptic *bgl* operon was induced in these cells. DiNardo et al. (4) have shown that compensatory mutations that arise in *gyrA* or *gyrB* in response to *top* mutations activate this operon. Interpreted most simply, *bgl* operon activation is the result of a decrease in negative DNA superhelicity due to ΔICP8 activity. Work in progress (Pearson and Conley, unpublished data) indicates that ΔICP8 inhibits *E. coli* gyrase. Whether this is the only directly assayable function remains undetermined at present.

The expression of ΔICP8 in *E. coli* cells shows a number of properties which may be general characteristics of bacteria containing (some types of) functional eucaryotic proteins. We have observed both host strain restriction and host alterations of the resident plasmid when ΔICP8 is expressed. The survival of the clone depends on the genotypic background of the host bacterium and the bacterial promoter activity, as we have noted restriction with the host strain JM103 and the *lac* promoter (data not shown). In regard to bacterial modifications of the plasmid, spontaneous deletions similar to those in the ΔICP8 DNA may be a characteristic result of expressing heterologous proteins because of selective pressure. Also, *E. coli* mutants may be selected on the basis of expression of the heterologous proteins, since we have noted that putative mutant *E. coli* strains may be isolated on the basis of the ΔICP8 protein (Pearson and Conley, unpublished data). Although some of the phenotypes of ΔICP8 expression may be peculiar to the protein, others may be more general responses of *E. coli* to eucaryotic regulatory proteins. We can distinguish phenotypes directly related to ΔICP8 expression since we have found that a known HSV-1 temperature-sensitive mutant is also temperature sensitive for some phenotypes when expressed in *E. coli* cells.

E. coli has been successfully engineered to produce a number of eucaryotic proteins. In addition to production, *E. coli* can be used for the genetic and biochemical analysis of eucaryotic DNA sequences. As there are large collections of

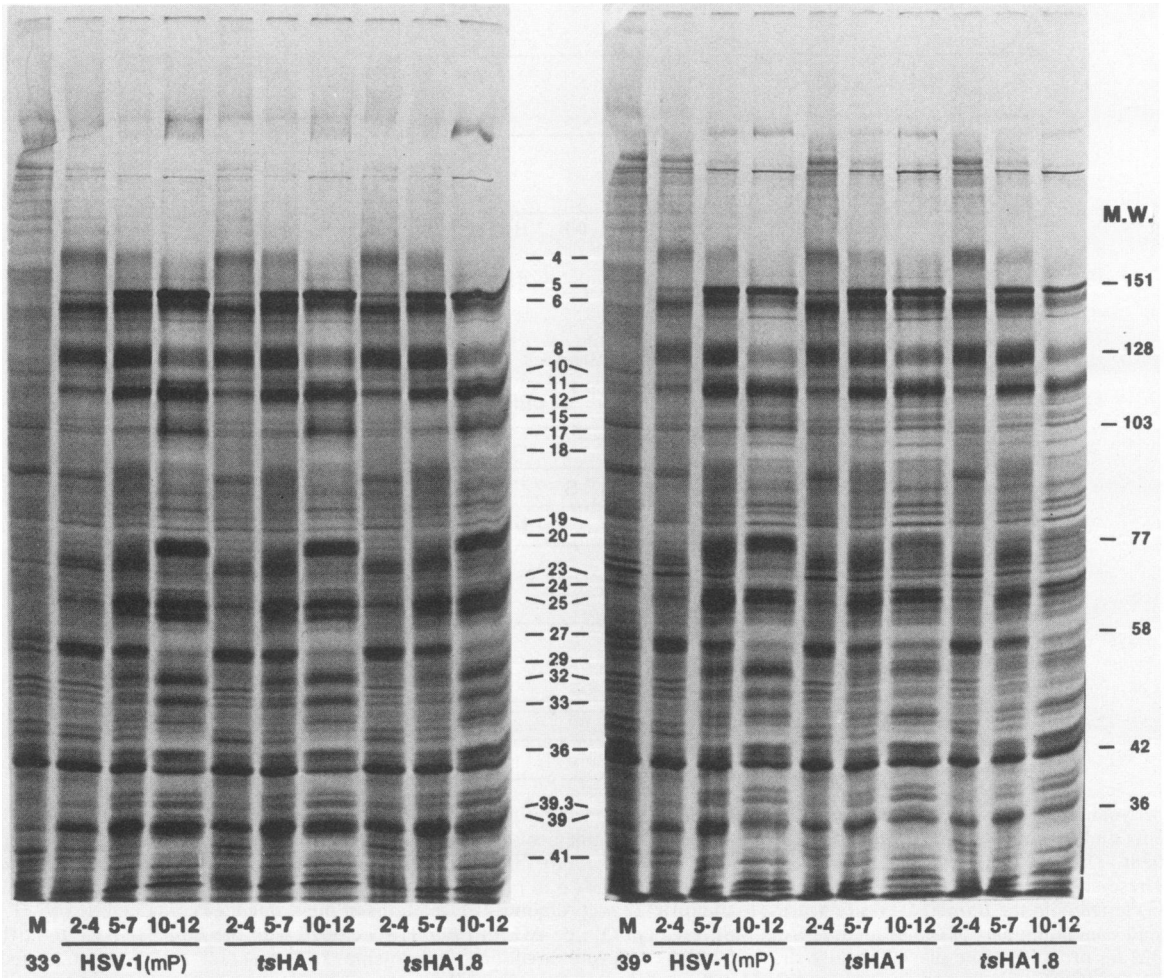

FIG. 4. Autoradiograph of lysates of cells infected with HSV-1(mP), HSV-1(mP) *ts*HA1, or HSV-1(mP) *ts*HA1.8 and labeled at 2 to 4, 5 to 7, or 10 to 12 h postinfection at the permissive (33°C) and nonpermissive (39°C) temperatures. The apparent molecular weight (in thousands) for a select group of infected-cell polypeptides is shown on the right. ICP numbers are shown in the center.

E. coli mutants, it should be easy to find a host that restricts the activity of a plasmid which encodes an expressed heterologous protein. On the basis of *E. coli* restriction, inferences can be made about the function of the heterologous protein in vivo, and methods can be developed for genetic analysis of the cloned insert. Biochemically, eucaryotic proteins can be studied effectively in these systems, as both host and plasmid can be used for in vitro experiments. Thus, systems of bacterial analysis should be useful tools before subsequent transfer of the expressed fragment back into the eucaryotic environment.

ACKNOWLEDGMENTS

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