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Expression of Herpes Simplex Virus β and γ Genes Integrated in Mammalian Cells and Their Induction by an α Gene Product

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The proteins of herpes simplex virus type 1 (HSV-1) form three kinetic groups termed α, β, and γ, whose synthesis is regulated in a cascade fashion. α products are synthesized first during infection, and they are required for synthesis of β and γ proteins. To examine the expression of several HSV-1 β and γ genes in the absence of α functions, we transferred into mammalian cells a plasmid containing a region of the HSV-1 genome that codes for only β and γ genes (0.315 to 0.421 map units). We found stable integration of at least one copy of the intact plasmid in each cell line. Four HSV-1 transcripts of the β and γ classes were transcribed constitutively in the cells, including the genes for glycoprotein B and DNA-binding protein. No constitutive synthesis of these two proteins could be demonstrated, however. The integrated HSV-1 genes responded to viral regulatory signals in that they could be induced by infection with HSV-1 mutants resulting in a high level of synthesis of both glycoprotein B and DNA-binding protein. The HSV-1 α gene product ICP4 was necessary for this induction, and it was found to be most efficient at a low multiplicity of infection. Functional expression of four genes was demonstrated in that the cell lines complemented infecting HSV-1 temperature-sensitive mutants. The same genes were not available for homologous recombination with infecting virus, however, since no recombinant wild-type virus could be detected. These data demonstrate that HSV-1 β and γ genes can be transcribed in the absence of α functions in mammalian cells, but that they still respond to HSV-1 regulatory signals such as the α gene product ICP4.

The polypeptides specified by herpes simplex virus type 1 (HSV-1) are classified into three groups termed α, β, and γ, whose synthesis is coordinately regulated and sequentially ordered in a cascade fashion (21, 51). The α genes are expressed first after infection, and their transcription does not require de novo protein synthesis (21, 25). Functional α products are required for β gene expression, and functional β gene products are required for viral DNA synthesis and for γ gene expression (8, 22, 30). Control of expression appears to be at the transcriptional level (21, 22, 25, 30).

Important for an understanding of the mechanism of coordinate regulation is the elucidation of the differentiating characteristics of α, β, and γ genes. Studies with the HSV-1 thymidine kinase gene (*tk*) have suggested that there are structural differences in the regulatory regions of α and β genes. For example, the *tk* gene is normally expressed as a β gene, but it can be expressed as an α gene if its natural promoter is replaced with the promoter and upstream regulatory sequences of an α gene (33, 44). In addition, when Reyes et al. (48) fused the HSV-1 *tk* promoter to a human β-interferon cDNA, the human β-interferon was expressed as an HSV-1 β gene, responsive to α products during HSV-1 infection. These studies imply that the kinetic class of a gene is determined by its promoter and upstream regulatory sequences. On the other hand, the *tk* gene can also be expressed constitutively in the absence of α functions when it is separated from the viral genome and used to transform Ltk^- cells to the TK^+ phenotype (62). This may be a consequence of the strong selective pressure used to isolate and maintain this phenotype. Therefore β gene expression can occur without α gene products, but probably not by the usual mechanism.

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Since most previous studies have been performed with the *tk* gene, we wanted to look at the regulation of expression of other HSV-1 β and γ genes in the absence of α gene functions. To accomplish this we used protoplast fusion (53, 54) to introduce into Ltk$^-$ cells plasmids containing the HSV-1 *tk* gene linked to a region of the HSV-1 genome between coordinates 0.315 and 0.421. This region was chosen because it encodes only β and γ genes and because a number of well-characterized HSV-1 functions have been mapped to it (5, 7, 19, 39, 46, 47, 52, 61). Cell lines were selected for the TK$^+$ phenotype by growth in HAT medium, and we then analyzed for expression of the nonselected HSV-1 sequences which were introduced with the *tk* gene. Expression of these HSV-1 β and γ genes was monitored without manipulation, looking for constitutive expression, and after infection with mutants of HSV-1, looking for induced expression.

In this report we show that the nonselected HSV-1 sequences were present in the transformed cells. Functional expression of at least four HSV-1 products encoded by these sequences was shown by complementation of temperature-sensitive mutants. No constitutive synthesis of protein products from the glycoprotein B (gB) and DNA-binding protein genes, which map within the nonselected sequences, was detected in the transformed cell lines, although constitutive transcription of those two genes was observed. Induced expression of the gB and DNA-binding proteins was found after infection with mutants of HSV-1. The immediate early HSV-1 gene product ICP4 was necessary for this induction.

MATERIALS AND METHODS

Cells and viruses. Ltk$^-$ cells (27) were used as the recipients in the protoplast fusion experiments. Vero cells were used to prepare all virus stocks and for all titrations by the plaque assay method (1). The tk-8 cell line used as a control in the complementation and Northern hybridization experiments and the tk-6 cell line used as a control in the Southern blot experiments are TK$^+$ derivatives of Ltk$^-$ cells which were isolated by selection in medium containing HAT (15 μg of hypoxanthine, 1 μg of aminopterin, 5 μg of thymidine, 15 μg of glycine per ml [60]) after protoplast fusion transfer of plasmid pX1 (10) which contains the HSV-1 *tk* gene. Ltk$^-$ cells were grown in Eagle minimal essential medium supplemented with nonessential amino acids, 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 10% fetal calf serum (GIBCO Diagnostics). All TK$^+$ transformed cell lines were grown in the above medium supplemented with HAT.

The isolation of the temperature sensitive mutants of wild-type HSV-1 KOS (mutants *ts*-16, *ts*-17, *ts*-18, and *ts*-606) has been described previously (12), as has the isolation of the HSV-1 HFEM mutant *ts*-B5 (34). HSV-1 strain 17 mutants 17*ts*VP1201 (46) and 17*ts*VP1204 were kindly sent to us by Valerie Preston. HSV-1 (KOS), HSV-2 (333), and all viral mutants were grown by infection at low multiplicity in Vero cells, as described previously (12).

Construction of plasmids pSG384 and pSG393. The *Eco*RI F fragment of HSV-1 KOS was inserted into plasmid pX1 (10) between the *Eco*RI site downstream from the *tk* gene and the *Eco*RI site in the pBR322 moiety of the plasmid (see Fig. 1). This resulted in a deletion of 875 base pairs (bp) of the original plasmid between the two *Eco*RI sites. Because pX1 contains a third *Eco*RI site near the *tk* promoter (see Fig. 1), this site was protected by methylation before cutting pX1 with *Eco*RI. A partial methylation reaction was performed as follows. Plasmid pX1 DNA (10 μg) was cut with *Hin*dIII (Bethesda Research Laboratories) according to the supplier's instructions. The resulting linearized plasmid DNA was precipitated in ethanol and suspended in 100 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA–8.5 μM *S*-adenosylmethionine in a total volume of 500 μl. *Eco*RI methylase (15 U) was added, and the reaction was incubated at 37°C for 2 h. After extraction with phenol and then ether, the DNA was precipitated in ethanol and suspended in the buffer recommended for digestion with *Eco*RI (Bethesda Research Laboratories). After incubation for 1 h at 37°C with 10 U of *Eco*RI, the reaction was diluted 10-fold and heated to 65°C for 10 min. DNA was treated with calf intestinal alkaline phosphatase as described by Goodman and MacDonald (14). *Eco*RI fragment F was purified from low-melting-temperature agarose after digestion of plasmid pSG18 (13) with *Eco*RI. The 16.5-kilobase (kb) band was cut from the gel, and the agarose was liquefied at 65°C and then extracted with phenol four times and with ether four times. The DNA was precipitated with ethanol and then suspended in 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.0). Alkaline phosphatase-treated pX1 DNA and *Eco*RI-F DNA were combined in a ratio of 1:10 in ligation buffer (66 mM Tris-hydrochloride (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.4 mM ATP) to which was added 10 U of T4 DNA ligase (New England Nuclear), followed by incubation at 4°C for 16 h. Transformation of competent DH-1 bacteria was performed as described previously (D. Hanahan, J. Mol. Biol., in press). Ampicillin-resistant colonies were screened by rapid lysis (20).

Isolation of *Eco*RI-F-containing cell lines. Plasmids pSG384 and pSG393 were transferred to Ltk$^-$ cells by protoplast fusion as described previously (53). After 3 weeks of growth in medium containing HAT, individual colonies were picked and grown to mass culture.

Isolation of cellular DNA. DNA was isolated from each cell line after expansion to mass culture. Cells were harvested and suspended in lysis buffer containing 10 mM Tris-hydrochloride, 50 mM EDTA (pH 8.0), 1% sodium lauryl sulfate, and 1% sodium lauryl sarcosine. RNase (heated at 83°C for 20 min before use) was added to 500 μg/ml, and cell lysates were incubated at 37°C for 60 min. Pronase (heated at 83°C for 20 min before use) was added to 2 mg/ml, and the lysates were incubated at 37°C overnight. The lysates were extracted with phenol five to seven times, twice with chloroform-isoamyl alcohol (98:2), and then dialyzed extensively against 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.0). DNA samples were precipitated in ethanol

and suspended in 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.0).

Transfer of restriction endonuclease-digested DNA to nitrocellulose and hybridization. DNA was digested with each enzyme indicated at 4 U of enzyme per μg of DNA at 37°C for 6 h in the buffers recommended by the supplier (Bethesda Research Laboratories). After digestion, samples were precipitated in ethanol and suspended in 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.0). DNA samples (either 20 or 10 μg/slot as indicated in the figure legends) were electrophoresed in 1% agarose slab gels in 30 mM NaH_2PO_4–36 mM Tris–1 mM EDTA (pH 8.0) at 50 V for 20 h. DNA in the gels was denatured by soaking the gel in 0.5 M NaOH–1.5 M NaCl for 2 h, neutralized by immersion in 0.5 M Tris-hydrochloride–3 M NaCl (pH 5.0) for 2 h, and then transferred to nitrocellulose (56). Probes were nick translated as described previously (13), and hybridizations were performed in the presence of dextran sulfate in a buffer containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 6.8) at 65°C for 18 to 20 h, as described previously (13).

Immunoprecipitation and polyacrylamide gel electrophoresis. Ltk$^-$ cells and *Eco*RI-F-transformed cells were labeled with 20 μCi of [^{35}S]methionine per ml, as described by Holland et al. (18). In samples which were infected with HSV-1 mutants, [^{35}S]methionine was added 5 h after infection. At 18 h postinfection and at 24 h after the addition of label, infected cells and uninfected cells, respectively, were harvested and lysed as described previously (18). Immunoprecipitations and polyacrylamide gel electrophoresis were performed as described by Holland et al. (18), except that 10% (instead of 8.5%) acrylamide gels were used.

Isolation of RNA and Northern transfer hybridizations. Polyribosome-associated RNA was isolated from cells lysed with Triton X-100 by the Mg^{2+} precipitation method of Palmiter (41), followed by digestion with proteinase K (Boehringer-Mannheim) and phenol-chloroform extraction (58). Polyadenylated [poly(A)$^+$] RNA was purified by the use of oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.). RNA was denatured at 50°C for 1 h in a buffer containing 1 M glyoxal as described by McMaster and Carmichael (37). Samples (10 μg/well for uninfected cell RNA and 1 μg/well for RNA from HSV-1-infected cells) were electrophoresed in 1% agarose slab gels in 10 mM phosphate buffer, pH 7.0, at 30 V for 16 to 18 h. RNA was transferred to Gene Screen (New England Nuclear Corp.) according to the supplier's procedures without prior treatment of the gel. Blots were prehybridized and hybridized in a buffer containing 5× SSC and 50% formamide at 42°C for 22 to 24 h as described by Thomas (59). Dextran sulfate (10%) was included in the prehybridization treatment solution and in the hybridization solution. ^{32}P-labeled probes were nick translated and denatured as described previously (13). The RNA blots were washed in 2× SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature, followed by two washes in 0.1× SSC–0.1% SDS at 50°C as described by Thomas (59). Removal of hybridized probe before rehybridization with different probes was done by two successive washes in 0.05× wash buffer (1× wash buffer contains 50 mM Tris-hydrochloride [pH 8.0], 2 mM EDTA, 0.5% sodium pyrophosphate, and 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone [59]) and 50% formamide for 90 min each at 80°C with constant agitation. This was followed by a wash at room temperature in 0.05× wash buffer alone to remove formamide from the blot. Rehybridization was as described above.

RESULTS

Isolation of cell lines containing HSV-1 sequences. We showed previously that protoplast fusion was an efficient method for transferring cloned HSV-1 sequences to mammalian cells (53), resulting in stable transformants (R. M. Sandri-Goldin, A. L. Goldin, J. Glorioso, and M. Levine, submitted for publication). Therefore, this method was used to transfer plasmids containing the *tk* gene and the region between coordinates 0.315 and 0.421 to Ltk$^-$ cells. The region of the HSV-1 genome from 0.315 to 0.421 map units is contained within a 16.5-kb *Eco*RI fragment termed *Eco*RI-F (38). This fragment was covalently linked to the HSV-1 *tk* gene by insertion into plasmid pX1. Plasmid pX1 consists of the 3.5-kb *Bam*HI fragment of HSV-1 which includes the *tk* gene and pBR322 (10). The *Eco*RI F fragment was inserted between the *Eco*RI site downstream from the *tk* gene and the *Eco*RI site in pBR322, deleting an 875-bp region of the plasmid (Fig. 1). The *Eco*RI F fragment was inserted in the two possible orientations relative to the direction of transcription of the *tk* gene. The resulting plasmids, shown in Fig. 1, were termed pSG384 and pSG393. Four cell lines isolated by selection in HAT medium after transfer of pSG384 and one cell line isolated after transfer of pSG393 were selected for further analysis. We have termed these cell lines 384-1, 384-2, 384-4, 384-5, and 393-1.

Presence of the nonselected *Eco*RI-F sequences. To determine whether the nonselected *Eco*RI-F sequences were present in the transformed cells, Southern transfer hybridizations were performed on DNA isolated from cell lines 384-1, 384-2, 384-4, 384-5, and 393-1. The genomic DNA was digested with *Hin*dIII, which has no sites in plasmids pSG384 or pSG393. If intact plasmid DNA remained unaltered in the cells, a band comigrating with the original 23.8-kb supercoiled plasmid DNA would be expected. Bands migrating more slowly than the 23.8-kb supercoiled plasmid were present in hybridizations with ^{32}P-labeled pX1 (Fig. 2A). A single high-molecular-weight band was seen in cell lines 384-1, 384-2, and 384-5, suggesting a single integration of plasmid DNA into high-molecular-weight DNA in these cell lines. Cell lines 384-4 and 393-1 appeared to have two high-molecular-weight bands present, suggesting two integration events in these two cell lines. An identical blot was hybridized with gel-purified ^{32}P-labeled *Eco*RI fragment F (Fig. 2B). Bands of the same size as those seen in Fig. 2A were present in cell

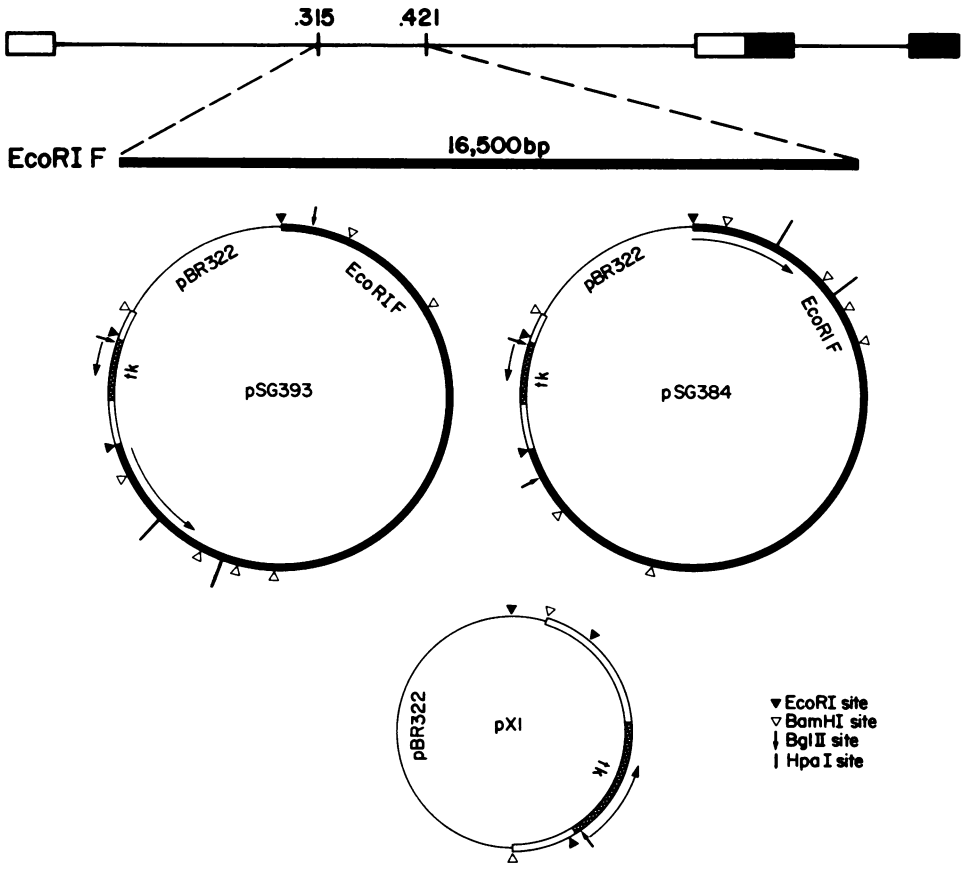

FIG. 1. Structure of plasmids containing the HSV-1 *tk* gene and fragment *Eco*RI-F. The location of the *Eco*RI fragment F in the prototype arrangement of HSV-1 is shown at the top. The cloned 16.5-kb fragment (13) was gel purified and inserted into the plasmid pX1 (10) at the two *Eco*RI sites (▼) downstream from the *tk* gene (crosshatched region), deleting that region of pX1. Plasmids were isolated containing the two possible orientations of the *Eco*RI F fragment relative to the direction of transcription of the *tk* gene. The fragment sizes generated by digestion with the indicated restriction endonucleases are as follows. For pSG393: *Eco*RI (▼), 2.4, 16.5, and 4.6 kb; *Bam*HI (▽), 3.65, 2.4, 0.86, 0.76, 8.0, 2.3, and 5.5 kb; *Bgl*III (↓), 18.1 and 5.4 kb; *Hpa*I (|), 21.8 and 1.7 kb. For pSG384: *Eco*RI (▼), 2.4, 16.5, and 4.6 kb; *Bam*HI (▽), 4.6, 2.3, 8.0, 0.76, 0.86, 2.4, and 4.6 kb; *Bgl*II (↓), 2.9 and 20.6 kb; *Hpa*I (|), 21.8 and 1.7 kb. The sizes correspond to the fragments in the clockwise direction, starting with the fragment containing the *tk* gene (9 o'clock position).

lines 384-1, 384-2, 384-5, and 393-1, indicating that *Eco*RI-F sequences along with the *tk* and pBR322 sequences had integrated into high-molecular-weight DNA. In cell line 384-4, only the lower band hybridized with *Eco*RI-F. Thus, although all of the plasmid sequences appeared to be represented at this integration site, only *tk* sequences or pBR322 sequences or both had integrated into the other site.

To determine whether at least one copy of all of the sequences in *Eco*RI-F was present in each cell line, genomic DNA was digested with *Bam*HI and hybridized to ^{32}P-labeled pSG18, a plasmid which contains the *Eco*RI F fragment in the vector pBR325 (13). As shown in Fig. 1, when pSG384 or pSG393 are cut with *Bam*HI, seven fragments are generated. The sizes of these fragments are given in the legend to Fig. 1. Figure 3 (lanes 1 and 2) shows the hybridization to the *Bam*HI fragments generated from pSG384 and pSG393 mixed with Ltk$^-$ DNA. Two of the fragments of pSG384 are the same size, 4.6 kb, and therefore cannot be distinguished from each other. Hybridization to the 3.65-kb fragment of pSG393 is considerably less intense than to the other bands because this fragment consists primarily of the *tk* region and has homology to only about 650 bp of the 22.5-kb probe. Comparison of DNA from the cell lines transformed with pSG384 with the plasmid PSG384 profile showed that the 8- and 4.6-kb bands were present. Whether both the 2.4- and 2.3-kb bands were

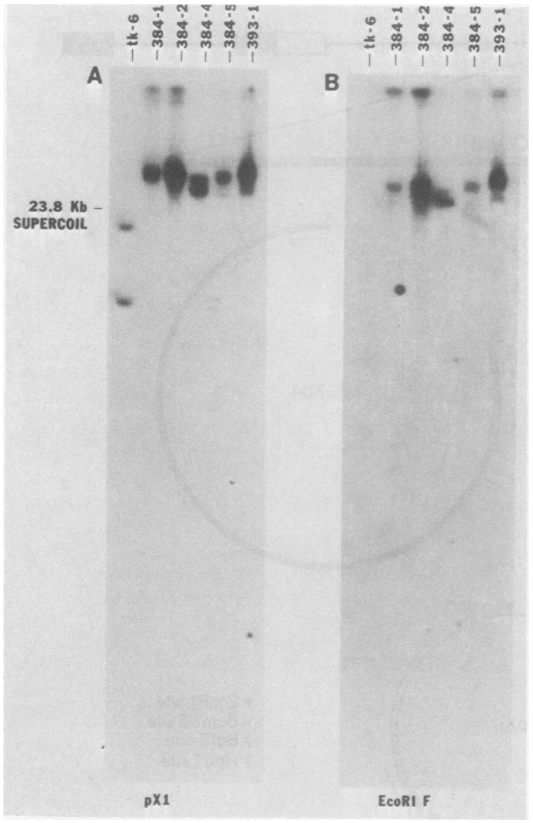

FIG. 2. Southern blot hybridizations of *Hin*dIII-digested DNA from the TK^+ cell lines isolated after protoplast fusion transfer of pSG384 or pSG393. (A) *Hin*dIII-digested DNA (10 μg each) from cell lines 384-1, 384-2, 384-4, 384-5, and 393-1 were fractionated in 1% agarose gels and transferred to nitrocellulose (56). tk-6 is a TK^+ cell line isolated by HAT selection after protoplast fusion transfer of pX1 to Ltk^- cells. DNA from tk-6 cells (10 μg) was included as a control. The position of the 23.8-kb supercoil was determined from its mobility relative to the fragments from tk-6, as analyzed previously (unpublished data). The blot was hybridized with ^{32}P-labeled pX1. (B) A blot identical to that in panel A was hybridized with ^{32}P-labeled *Eco*RI-F. The fragment was purified by *Eco*RI digestion of plasmid pSG18 (which contains *Eco*RI-F in pBR325 [13]), followed by fractionation in low-melting-point agarose. The DNA band was extracted as described in the text. The gel-purified fragment was then labeled with ^{32}P by nick translation.

present could not be distinguished in this hybridization. Also, the 0.86- and 0.76-kb bands could be visualized only in cell lines 384-1 and 384-2. Therefore, the blot was stripped and rehybridized with subfragments of *Eco*RI-F specific for the 2.4-, 0.86-, and 0.76-kb fragments. All three bands were present in each of the cell lines (data not shown). A similar comparison of cell line 393-1 to the pSG393 profile showed that the 8-, 5.5-, and 3.65-kb bands were present (Fig. 3). Again on rehybridization, the 2.4-, 0.86-, and 0.76-kb bands were seen (data not shown). In addition to the *Bam*HI digest, a *Bgl*II/*Hpa*I double digest was performed, and the blot was hybridized to ^{32}P-labeled pSG18. All four expected bands (Fig. 1) generated by this digestion were seen in each cell line (data not shown). We conclude that at least one copy of the entire plasmid, including the nonselected *Eco*RI-F and pBR322 sequences, was present in each cell line.

Since at least one complete copy of the plasmid unperturbed by integration appeared to be present in the transformed cell lines, it seemed likely that each plasmid had integrated as at least

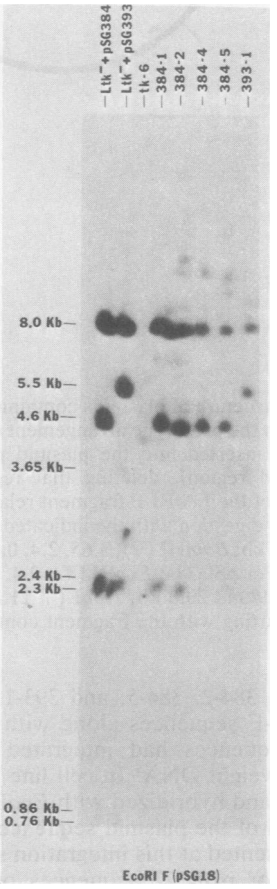

FIG. 3. Analysis of *Eco*RI-F sequences present in cell lines 384-1, 384-2, 384-4, 384-5, and 393-1. The indicated genomic DNAs (10 μg/slot) were digested with *Bam*HI and then fractionated in agarose gels and transferred to nitrocellulose. As controls, Ltk^- DNA (10 μg) mixed with 100 pg of pSG384 or PSG393 DNA were treated similarly. The blot was hybridized with ^{32}P-labeled pSG18 (13).

a dimer. To determine the copy number of HSV-1 sequences in each cell line, a reconstruction experiment was performed. pSG384 DNA was mixed with Ltk⁻ DNA in proportions equivalent to 1, 2, 5, and 10 copies per cell and then was digested with *Eco*RI. The blot was hybridized with gel-purified ^{32}P-labeled *Eco*RI-F. An intact 16.5-kb *Eco*RI-F band was seen in each cell line (Fig. 4). A comparison of the intensities of each band to the pSG384 bands in the reconstructions suggested that about 5 copies of *Eco*RI-F were present in 384-1, that 5 to 10 copies were present in 384-2, ca. 2 copies were found in 384-4, and probably only a single complete copy was present in 384-5. These results indicate that the plasmid DNA integrated as a multimer or at least as a dimer in these cell lines. A small amount of hybridization was also detected to a 4.6-kb band. This *Eco*RI fragment contains all of the pBR322 sequences as well as 650 bp from one end of the 3.4-kb *tk*-containing *Bam*HI fragment. This 650-bp region represents sequences common to the *tk-Bam*HI fragment (coordinates 0.295 to 0.320 in the HSV-1 genome, Fig. 1) and the *Eco*RI F fragment (coordinates 0.315 to 0.421). A number of other bands were observed which also hybridized less intensely than the *Eco*RI-F band. Some of these bands, such as the two bands seen in 384-1, may represent the junctions between plasmid and cellular DNA. Others, such as the multiple high-molecular-weight bands in 384-2, may represent rearrangements which occurred in some of the copies of integrated plasmid DNA.

The cell line 393-1 was compared with plasmid pSG393 in a reconstruction experiment that was the same as that described for pSG384-transformed cell lines. The copy number was similar to that observed for cell line 384-5, indicating that the plasmid had probably integrated as a dimer in cell line 393-1 (data not shown).

Complementation of HSV-1 *ts* mutants by viral sequences resident in the cellular genome. The region of the HSV-1 genome spanned by *Eco*RI-F has been shown to code for a number of viral functions of the β and γ class. The β functions include the major DNA-binding protein (ICP8) (31, 61), and gB, a glycoprotein required for virion infectivity (7, 19, 52). The viral DNA polymerase, another β gene, is encoded in part by sequences at the right end of *Eco*RI-F, although mutants defective in DNA polymerase also map to the region adjacent to *Eco*RI-F (5). The gene for the γ protein p40 (46) and the genes for at least two other polypeptides map in *Eco*RI-F (39). To determine whether any of these functions could be expressed in the cell lines containing the *Eco*RI fragment F sequences, we performed genetic complementation experiments using mutants from six complementation groups which map in the *Eco*RI-F region. The mutants, which were all temperature sensitive, included *ts*-18, which is defective in the DNA-binding protein at nonpermissive temperature (31); *ts*-B5, which has a lesion in the gB locus (34); *ts*-16, which is defective in the viral DNA polymerase (D. Parris, personal communication); and *ts*-17, a mutant which makes viral DNA in reduced amounts relative to wild type but whose lesion has not been associated with a specific gene product (unpublished data). In addition, mutant 17 *ts*VP1201, whose lesion lies in the region of the p40 gene (46), and mutant 17*ts*VP1204, which maps near 17*ts*VP1201 but whose specific defect has not been elucidated (V. Preston and B. Matz, personal communication), also were used.

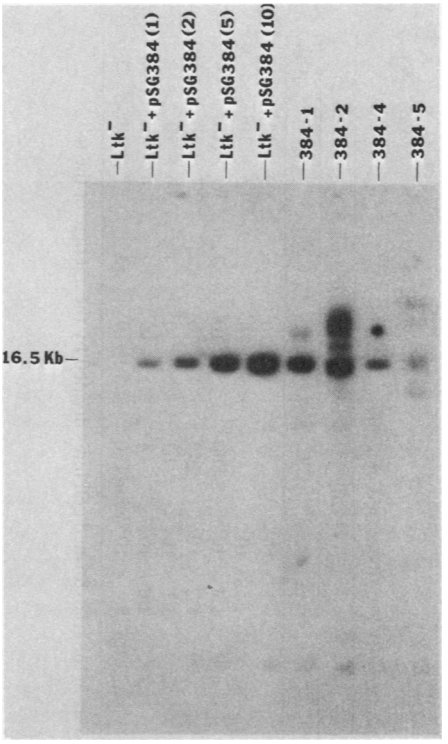

FIG. 4. Determination of the copy number of *Eco*RI-F sequences present in the cell lines. DNA (10 μg/slot) from cell lines 384-1, 384-2, 384-4, and 384-5 was cut with *Eco*RI. DNA from pSG384 was mixed with Ltk⁻ DNA to approximate 1 copy per cell (30 pg of pSG384 DNA per 10 μg of Ltk⁻ DNA), 2 copies per cell (60 pg/10 μg), 5 copies per cell (150 pg/10 μg), and 10 copies per cell (300 pg/10 μg). The blot was hybridized with ^{32}P-labeled *Eco*RI-F purified from pSG18 as described in the legend to Fig. 2B.

The cell lines 384-1, 384-2, 384-4, 384-5, and 393-1 were infected with each *ts* mutant at 39°C, the nonpermissive temperature, for 48 h at a multiplicity of infection of 5. In addition to the

TABLE 1. Complementation of HSV-1 *ts* mutants in *Eco*RI-F-transformed cell lines

Cell line	KOS		*ts*-18		*ts*-B5		*ts*-17		17*ts*VP1201		17*ts*VP1204		*ts*-16	
	Titer	C.I.[a]	Titer	C.I.	Titer	C.I.	Titer	C.I.	Titer	C.I.	Titer	C.I.	Titer	C.I.
Ltk$^-$	1.1×10^6		3.8×10^1		2.5×10^1		4.3×10^2		5×10^1		$<5 \times 10^1$		5.9×10^2	
tk-8	8.5×10^5		7.5×10^1		5.0×10^1		6.0×10^2		5×10^1		$<5 \times 10^1$		9.5×10^2	
384-1	2.3×10^6	2	1.4×10^4	248	1.8×10^3	48	1.9×10^6	3,689	1.4×10^5	2,800	$<5 \times 10^1$	1	3.1×10^3	4
384-2	5.0×10^5	0.5	7.7×10^4	1,363	2.0×10^5	5,330	3.6×10^5	699	1.2×10^4	240	$<5 \times 10^1$	1	7.1×10^2	0.9
384-4	3.0×10^5	0.3	5.0×10^1	0.9	1.5×10^2	4	1.4×10^5	272	3.1×10^3	62	$<5 \times 10^1$	1	3.6×10^2	0.5
384-5	6.0×10^5	0.6	3.0×10^2	5	6.0×10^2	16	7.7×10^5	1,495	2.0×10^2	4	$<5 \times 10^1$	1	8.4×10^2	1
393-1	1.1×10^6	1	5.0×10^3	88	6.2×10^2	17	1.2×10^6	2,330	8.5×10^3	170	$<5 \times 10^1$	1	2.2×10^3	3

[a] C.I., Complementation index, which equals the titer of the virus progeny from infections in the transformed cell lines divided by the average of the titer of virus progeny from infections in Ltk$^-$ cells and in tk-8 cells.

transformed cell lines, parallel control infections were performed in Ltk$^-$ cells and tk-8 cells. tk-8 is a cell line which was transformed with the plasmid pX1 and contains the HSV-1 *tk* gene but no *Eco*RI-F sequences. Progeny virus from the infections were assayed at 34°C, the permissive temperature. The virus yields from the transformed cell lines were compared to the yields from control infections, and complementation indexes were computed by dividing the titer of the virus progeny from infections in each transformed cell line by the average of the progeny titer from infections in Ltk$^-$ cells and in tk-8 cells. A complementation index of >10 was taken to indicate complementation.

Four mutants (*ts*-18, *ts*-B5, *ts*-17, and 17*ts*VP1201) were complemented with indexes ranging from 16 to 5,330 in some or all of the transformed cell lines (Table 1). This indicates that the viral DNA sequences resident in these cells were expressed on infection since they supplied the functions defective in these mutants, allowing them to grow to signficantly higher titers than in the control infections. Two mutants, *ts*-16 and 17*ts*VP1204, were not complemented by any of the cell lines (Table 1). As stated above, only part of the coding sequence for DNA polymerase is contained in *Eco*RI-F; therefore, it is not surprising that the cell lines could not provide a functional polymerase to complement the defective polymerase of *ts*-16. Mutant 17*ts*VP1204 maps near the left end of *Eco*RI-F (V. Preston and B. Matz, personal communication), a region to which we have mapped two transcripts which span or initiate very close to the left *Eco*RI site (unpublished data). Although the specific defect in 17*ts*VP1204 is not known, it is possible that the mutation lies in a gene coding for one of these two transcripts. In that case, like the polymerase locus, the entire coding sequence or a portion of the regulatory region might not be present in the *Eco*RI F fragment, so the transformed cell lines could not provide a functional gene product.

Although three of the cell lines, 384-1, 384-2, and 393-1, routinely complemented the mutants *ts*-18, *ts*-B5, *ts*-17, and 17*ts*VP1201, two of the cell lines, 384-4 and 384-5, did not complement all of these mutants. Cell line 384-4 did not complement *ts*-18 or *ts*-B5, and 384-5 did not complement *ts*-18 or 17*ts*VP1201. All of the cell lines supported the growth of HSV-1 KOS, the wild-type strain from which *ts*-16, *ts*-17 and *ts*-18 were isolated, to about the same extent (Table 1). Similarly, all of the cell lines supported the growth of wild-type strain 17, from which 17*ts*VP1201 and 17*ts*VP1204 were derived, to the same extent (data not shown). Therefore, the inability of the *ts* mutants to grow in 384-4 and 384-5 suggests that the corresponding product

was not expressed in these cell lines or was made at levels too low to allow viral growth.

Since HSV-1 undergoes recombination to high levels during infection (42), we wanted to determine whether any wild-type recombinants had formed as a result of homologous recombination between the infecting virus and the viral sequences resident in the cell lines. In all of the complementation experiments the progeny virus were also assayed at 39°C to score any wild-type virus. The number of plaques at 39°C was equivalent to the number of plaques scored when the parental mutant stocks were plated at 39°C (data not shown). Therefore, a recombination frequency above the reversion frequency for each mutant ($<2 \times 10^{-5}$) was not detected, indicating that recombination between the infecting viral DNA and homologous viral sequences integrated in the cellular genome is a rare event.

Immunoprecipitation of gB in a cell line containing sequences from the *Eco*RI F fragment. The complementation data demonstrated that various HSV-1 functions were expressed in the transformed cells on infection with *ts* mutant virus. Those experiments do not distinguish between induced or constitutive expression, however. To determine whether any of the proteins encoded by the sequences spanned by *Eco*RI-F were made constitutively in the cell lines, we looked at the synthesis of one of the proteins in this region gB. We also looked at gB expression after infection. To distinguish between gB specified by the infecting virus and that encoded by the viral sequences resident in the cellular genome, infections were performed with two different mutants. At 39°C the temperature-sensitive mutant *ts*-B5 fails to make the fully processed form of gB, which has an apparent molecular weight of 126,000, but does accumulate gA, a precursor to gB which has an apparent molecular weight of 119,000 (9, 16). Since gB cannot be made by the infecting virus at 39°C, any gB detected will have been specified by the viral sequences resident in the cell. The second mutant is an antigenic variant which produces fully functional gB. However, this mutant, *mar* B1.1, fails to bind to the monoclonal antibody B1 directed against gB, although it does bind to at least two other anti-gB monoclonal antibodies (18; unpublished results). Only gB specified by the viral sequences resident in the cellular genome will be precipitated using the monoclonal antibody B1.

The cell line 384-2, which most strongly complemented *ts*-B5, and Ltk$^-$ cells were either left uninfected or were infected with *ts*-B5 at 39°C or *mar* B1.1 at 37°C, each at a multiplicity of infection of 2. Cells were then labeled with [^{35}S]methionine, and after 18 h, the cells were lysed and the lysates were immunoprecipitated with the monoclonal antibody B1. Precipitates were electrophoresed in an SDS-polyacrylamide gel. As expected, gB was precipitated from Ltk$^-$ cells infected with wild-type HSV-1 KOS (Fig. 5, lane 2), and no gB was precipitated from uninfected Ltk$^-$ cell lysates (lane 1) or from lysates of Ltk$^-$ cells infected with *mar* B1.1 (lane 3) or *ts*-B5 at nonpermissive temperature (lane 4). The lower-molecular-weight precursor gA was precipitated from *ts*-B5-infected Ltk$^-$ cells (Fig. 5, lane 4). No gB was precipitated in uninfected 384-2 cell lysates (Fig. 5, lane 5). This indicates that gB may not be made constitutively in this cell line. It is possible that gB was made in amounts too low to detect by immunoprecipitation, or that it was not processed sufficiently to allow binding to monoclonal antibody B1. To determine whether a partially processed form of gB was made in cell line 384-2, [^{35}S]methionine-labeled cell lysates of line 384-2 were immunoprecipitated with a pool of monoclonal antibodies to gB which recognizes at least three

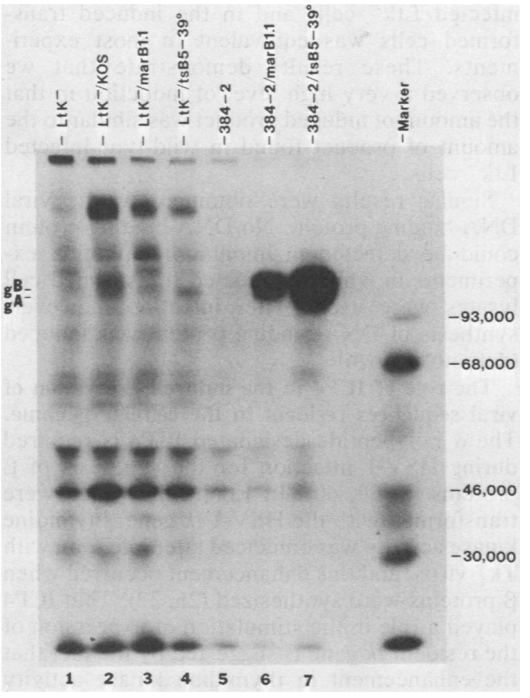

FIG. 5. SDS-polyacrylamide gel profiles of cell line 384-2 lysates immunoprecipitated with a monoclonal antibody to gB. [^{35}S]methionine-labeled lysates from uninfected 384-2 cells (lane 5) and Ltk$^-$ cells (lane 1) and from cells infected with HSV-1 KOS (lane 2) or mutants *mar* B1.1 and *ts*-B5 at a multiplicity of infection of 2 (lanes 3, 4, 6, and 7) were immunoprecipitated with a monoclonal antibody to gB (B1). The position of gB (molecular weight, 126,000) and its precursor form gA (molecular weight, 119,000) are indicated.

different antigenic sites (unpublished data). No gB nor any bands which could have represented precursor forms were found (data not shown). Therefore, constitutive production of gB was not detected in cell line 384-2.

In contrast to the result observed with uninfected 384-2 cells, a large amount of gB was precipitated from 384-2 cells infected with *mar* B1.1 (Fig. 5 lane 6) or *ts*-B5 at 39°C (lane 7), demonstrating that gB is strongly induced after infection. Therefore, although no constitutive gB synthesis was observed in this cell line, the viral sequences resident in the cells could be induced to produce gB by viral products made during infection. These data further show that viral sequences, which are part of the cellular genome, were recognized as HSV-1 specific by the infecting virus. It should be noted that it appears that considerably more gB was synthesized by the resident viral sequences after induction (Fig. 5, lanes 6 and 7) than was made by wild-type virus in Ltk$^-$ cells (Fig. 5, lane 2). This result was not found in other experiments. The amount of gB detected in HSV-1 (KOS)-infected Ltk$^-$ cells and in the induced transformed cells was equivalent in most experiments. These results demonstrate that we observed a very high level of induction in that the amount of induced product was similar to the amount of product found in wild-type-infected Ltk$^-$ cells.

Similar results were obtained for the viral DNA-binding protein. No DNA-binding protein could be detected in immunoprecipitation experiments in which uninfected transformed cell lysates were used. After infection, however, synthesis of DNA-binding protein was induced (data not shown).

The role of ICP4 in the induced expression of viral sequences resident in the cellular genome. The α polypeptide designated ICP4 is required during HSV-1 infection for the synthesis of β proteins (8, 29, 60). In Ltk$^-$ cells which were transformed with the HSV-1 *tk* gene, thymidine kinase activity was enhanced after infection with Tk$^-$ virus, and this enhancement occurred when β proteins were synthesized (26, 32). That ICP4 played a role in the stimulation of expression of the resident *tk* gene is suggested by the fact that the enhancement in thymidine kinase activity did not occur when an ICP4 mutant was used as the infecting virus (28). To determine whether ICP4 was required for the induced expression of gB, the immunoprecipitation experiment described above was repeated with a *ts* mutant defective in ICP4. Cell line 384-2 and control Ltk$^-$ cells were infected with *ts*-606, an ICP4 mutant (12), and then labeled with [^{35}S]methionine (Fig. 6). Cell lysates were again immunoprecipitated with the monoclonal antibody B1. A *ts*-606 infection does not make any β or γ proteins, including gB, at nonpermissive temperature (12), so that any gB detected would be due to expression of the resident viral sequences. As expected, gB was not precipitated from infections of Ltk$^-$ cells with *ts*-606 at 39°C (Fig. 6, lanes 8 and 9). Also, as described earlier, no gB was detected in uninfected 384-2 cells (Fig. 6, lane 10). In infections of 384-2 cells with *ts*-606 at 34°C, gB and the precursor gA specified by both the infecting virus sequences or the resident viral sequences or both were precipitated (Fig. 6, lanes 15 and 16). However, no gB was detected when 384-2 cells were infected with *ts*-606 at 39°C (Fig. 6, lanes 17 and 18). These data demonstrate that ICP4 is required for the induction of gB synthesis in this transformed cell line.

The effect of multiplicity on the expression of viral sequences in the transformed cells. Leiden et al. (32) showed that the enhancement of thymidine kinase activity resulting from HSV-1 infection of HSV-1-transformed Ltk$^+$ cells was multiplicity dependent. At multiplicities higher than 2, the enhancement was not observed. To determine whether the multiplicity of infection affected the expression of gB, we infected the cell line 384-2 with the mutants *mar* B1.1. and *ts*-B5 at multiplicities of 1 and 15. Infected cells were labeled with [^{35}S]methionine and cell lysates were immunoprecipitated with the monoclonal antibody B1 as described above. The amount of gB precipitated was greatly reduced at the higher multiplicity (Fig. 6, lanes 11 through 14). In contrast, in infections with *ts*-606 at 34°C (Fig. 6, lanes 15 and 16), the amount of gB increased at the higher multiplicity. This finding can be explained because *ts*-606 makes a wild-type gB at the permissive temperature so that gB specified by the infecting virus was precipitated. The increase in gB production during infection might be expected as the number of viral genomes increased. In contrast, expression of the viral genes resident in the cell was decreased as the number of the infecting viruses increased.

The multiplicity effect also was demonstrated for the expression of the DNA-binding protein, ICP8. A complementation experiment was performed in the transformed cell lines with the ICP8 mutant *ts*-18. The multiplicity of infection was varied from 0.6 to 18. Progeny virus were assayed, and the yield was compared to yields from control infections in Ltk$^-$ and tk-8 cells. Complementation indexes were computed as described previously. The complementation index decreased as the multiplicity increased (Table 2). This is seen most strikingly with cell line 393-1, in which a 35-fold decrease in complementation index was observed, and with 384-5, in which complementation was detected only at the

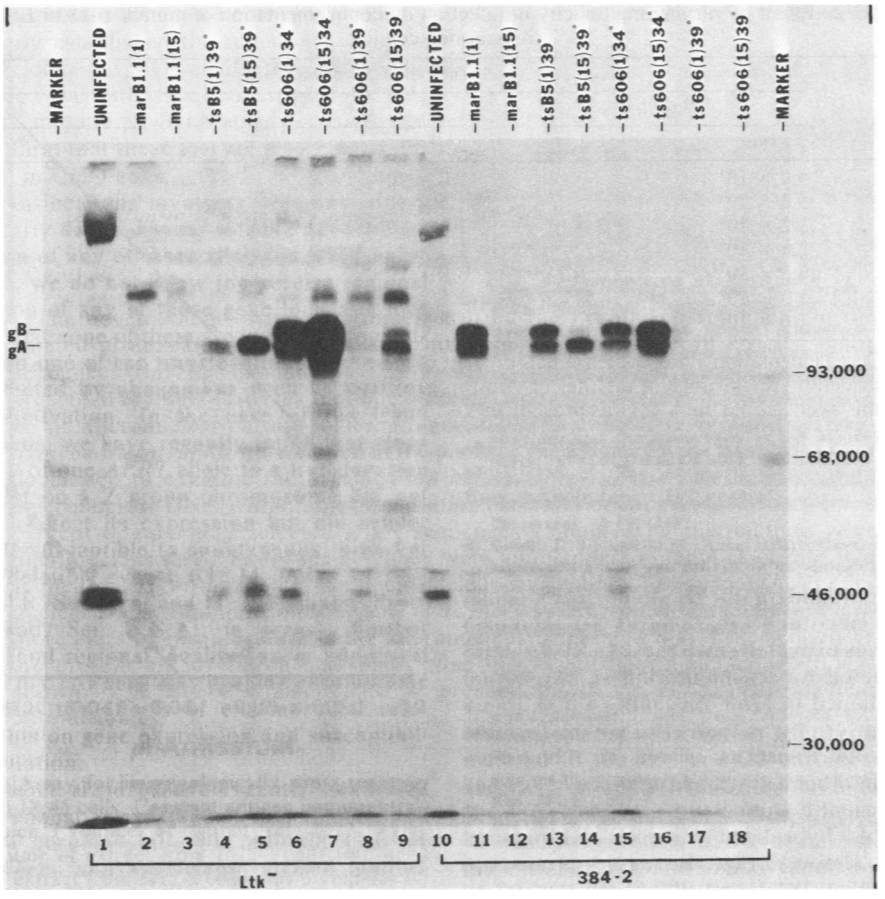

FIG. 6. The role of ICP4 and the effect of the multiplicity of infection on the expression of gB in cell line 384-2. Cell line 384-2 was infected with an ICP4 mutant *ts*-606 at 39°C (nonpermissive temperature) (lanes 17 and 18) and 34°C (permissive temperature) (lanes 15 and 16). [^{35}S]methionine-labeled lysates were immunoprecipitated with a monoclonal antibody to gB (B1) to assess the role of ICP4 in the induced synthesis of gB. Control infections were done in parallel in Ltk$^-$ cells (lanes 6 through 9). 384-2 cells also were infected with the gB mutants *mar* B1.1 and *ts*-B5 at two different multiplicities of infection, 1 and 15 (lanes 11 through 14), and then immunoprecipitated with monoclonal antibody B1 to determine whether multiplicity affects the stimulation of gB production seen after infection in 384-2 cells. Control infections were performed in Ltk$^-$ cells (lanes 2 through 5). Samples were electrophoresed in a 10% SDS-polyacrylamide gel.

lower multiplicity. In the previous complementation experiment (Table 1), performed at a multiplicity of infection of 5, complementation of *ts*-18 in 384-5 also was not observed. Because, in that experiment, complementation of *ts*-B5 and *ts*-18 was not observed in cell line 384-4 and complementation of 17*ts*VP1201 was not observed in cell line 384-5, infection of those cell lines was repeated with multiplicities of infection of 0.5 and 2. No complementation of *ts*-18 or *ts*-B5 was observed in 384-4 cells nor of 17*ts*VP1201 in 384-5 cells, even at the lower multiplicities (data not shown). Thus, the failure to demonstrate complementation in these latter cases is not a consequence of multiplicity.

Detection of HSV-1-specific transcripts in the transformed cell lines in the absence of infection. Although we were unable to detect either gB or DNA-binding protein by immunoprecipitation in the absence of infection, we wanted to determine whether transcription of any of the viral sequences in the transformed cell lines was constitutive. Therefore, we looked for HSV-1-specific transcripts. Polyribosomal poly(A)$^+$ RNA isolated from uninfected 384-2 and 393-1 cells was compared to poly(A)$^+$ RNA from uninfected Ltk$^-$ and tk-8 cells and from HSV-1-infected Ltk$^-$ cells by Northern blot hybridization.

Figure 7A shows a blot which was hybridized

TABLE 2. Effect of varying multiplicity of infection on complementation of mutant *ts*-18 in *Eco*RI-F-transformed cell lines

Cell line	Titer and C.I.[a] at MOI[b] of:					
	0.6		6		18	
	Titer	C.I.	Titer	C.I.	Titer	C.I.
Ltk$^-$	5.0×10^1		5.0×10^1		1.5×10^2	
tk-8	5.0×10^1		1.5×10^2		1.0×10^2	
384-1	3.2×10^4	348	2.6×10^4	282	1.7×10^4	185
384-2	2.5×10^4	272	1.7×10^4	185	8.5×10^3	93
384-4	6.0×10^1	0.7	8.8×10^1	1.0	1.5×10^2	1.6
384-5	5.0×10^3	54	2.5×10^2	2.7	2.0×10^2	2.2
393-1	1.6×10^5	1,740	1.8×10^4	196	4.4×10^3	48

[a] C.I., Complementation index, which equals the titer of the virus progeny from infections in each transformed cell line divided by the average of the titer of virus progeny from infections in Ltk$^-$ cells and in tk-8 cells. To calculate this average, the titers of virus progeny in Ltk$^-$ and tk-8 cells at all three multiplicities were used. That average was 9.2×10^1.

[b] MOI, Multiplicity of infection.

with the 3.5-kb *Bam*HI HSV-1 fragment containing the *tk* gene. The 1.6-kb poly(A)$^+$ *tk* transcript (35) found in the HSV-1-infected Ltk$^-$ cells was also found in 384-2, 393-1, and tk-8 cells. This is expected since these three cell lines were selected for thymidine kinase expression and maintained in HAT medium. In addition, a 1.1-kb transcript was found in 384-2, 393-1, and tk-8 cells. A transcript of this size has been detected in other Tk$^+$-transformed cell lines and may represent a truncated *tk* message (49). An identical blot hybridized with an *Eco*RI fragment F probe is shown in Fig. 7B. A 1.4-kb transcript was found in 384-2 and 393-1 cells, a 3.3-kb transcript was found in cell line 384-2, and a 4.2-kb transcript was found in cell line 393-1. Transcripts of similar sizes were observed in HSV-1-infected Ltk$^-$ cells. In addition to these transcripts, a 1.0-kb transcript was observed in the transformed cells which is present at very low levels in infected cells (Fig. 7F).

In experiments mapping infected cell transcripts which hybridize to *Eco*RI-F (unpublished data), we have found that a 4.2-kb transcript hybridized to the region of *Eco*RI-F to which we have mapped a number of DNA-binding protein mutants; a 3.3-kb transcript mapped to the region of the gB gene; and a 1.4-kb transcript mapped to the left of the 3.3-kb transcript (Fig. 8). To determine more precisely the regions within *Eco*RI-F to which the HSV-1-specific transcripts from 384-2 and 393-1 cells hybridized, we stripped the blots shown in Fig. 7A and B and rehybridized with two probes within *Eco*RI-F. The blot shown in Fig. 7C was hybridized to a 2.75-kb *Sal*I fragment (Fig. 8) which spans a region containing at least a portion of the gB gene (7, 19) and at least a portion of the region of the 1.4-kb transcript. The 3.3-kb transcript from 384-2 cells was seen to hybridize to the *Sal*I fragment, suggesting that this transcript may represent the gB message. Likewise, the 1.4-kb transcripts from 384-2 and 393-1 cells hybridized to the *Sal*I probe. In the blot shown in Fig. 7D, the RNA was rehybridized with a 2.3-kb *Bam*HI fragment (Fig. 8) which spans a portion of the region to which a number of DNA-binding protein mutants have been mapped (61; unpublished data). The 4.2-kb transcript from cell line 393-1 hybridized to this region (Fig. 7D), and a faint band also was seen with the 384-2 RNA, suggesting that the message for DNA-binding protein was transcribed in these cell lines. These results indicate that although the corresponding protein products could not be detected in uninfected 384-2 or 393-1 cells, transcripts which probably encode gB and DNA-binding protein, both β polypeptides, were made constitutively in these cell lines. Also, a 1.4-kb transcript of the γ class (unpublished data) was made constitutively.

The *Eco*RI F fragment was inserted in two different orientations in the plasmids pSG384 and pSG393 (Fig. 1). To determine whether there were any differences in the transcripts found near one end of the *Eco*RI F fragment as a result of the orientation difference, we again stripped the blots shown in Fig. 7C and D and rehybridized with two additional probes. In Fig. 7E the blot was hybridized with pBR322 DNA alone to determine what transcripts, if any, represent vector sequences. A 2.2-kb transcript in cell line 384-2 and a 1.4-kb transcript in cell line 393-1 were found to be pBR322 specific (Fig. 7E). The blot in Fig. 7F was hybridized with a 2.4-kb *Eco*RI/*Sal*I fragment from the left end of *Eco*RI-F (Fig. 8). This fragment, cloned in pBR325, was not separated from the cloning vector before nick translation, so that both the viral insert and the pBR325 moiety were labeled.

In addition to the pBR322-specific bands seen in Fig. 7E, a 1.0-kb HSV-1-specific transcript was present in both cell lines (Fig. 7F). A transcript of the same size was also seen in the infected cell control lane, suggesting that still another HSV-1-specific transcript was made constitutively in the cell lines. This transcript was also seen in Fig. 7B. Apart from the size differences in the transcripts which hybridized to the pBR322 probe, no other differences were seen between the two cell lines.

It should be noted that the amount of constitutive message we detected was considerably less than was seen in the HSV-1-infected cell controls. One-tenth as much poly(A)$^+$ RNA was loaded in the slot for infected cells, and the exposure time for the hybridization to infected cell RNA was 1 day versus 2 weeks for RNA from transformed cells. Based on comparisons of hybridization intensities of the messages from transformed cells with the corresponding messages from infected cells, there may be 1/100 to 1/500 times less message in the transformed cells. Therefore, the level of constitutive transcription in the transformed cells was considerably lower than the level of transcription of HSV-1 messages during the infection.

DISCUSSION

In our experiments we showed that nonselected HSV-1 sequences linked to the *tk* gene were integrated and expressed in mammalian cells. We observed two types of expression of the genes encoded by the nonselected *Eco*RI-F sequences. First, low-level constitutive transcription of at least four HSV-1 genes, including those for gB and DNA-binding protein, was observed. However, synthesis of these two proteins from these transcripts could not be detected. Second, there was a high level of induced synthesis of gB and DNA-binding protein after HSV-1 infection. This induction required the α gene product ICP4.

The apparent absence of constitutive protein synthesis of gB and DNA-binding protein in the presence of constitutive transcription of these genes might be explained by the greater sensitivity of our detection procedures for RNA than for proteins. The transcribed mRNA species may be translated at levels too low to be detected by immunoprecipitations. We are currently using Western transfers as a more sensitive assay for protein synthesis. Another explanation for the low level of constitutive protein synthesis could be that the HSV-1 mRNA molecules compete poorly with host messages during translation. This would imply that during infection, HSV-1 either modifies the host translational machinery (55, 57) or degrades cellular mRNA to permit more efficient translation of viral mRNA (23, 40).

The studies of Post et al. (44), Mackem and Roizman (33), and McKnight and Kingsbury (36) have suggested that there are two regulatory regions in HSV-1 genes which control the quality and quantity of transcription. The first region is the promoter sequence or TATA box (for review, see reference 4), the region immediately upstream from the site of transcription initiation. This region is primarily responsible for the specificity and fidelity of transcription initiation. In our transformed cells we found four transcripts of equivalent size to four HSV-1-infected cell transcripts which map in the same viral genomic regions. Although we cannot exclude the possibility that the transcripts made in the transformed cell lines were slightly altered from their counterparts found in infected cells, no differences between the two were detectable in Northern transfer hybridizations. These results suggest that the cellular RNA polymerase II, which normally transcribes HSV-1 sequences during infection (6), was capable of recognizing the promoters of β and γ genes and transcribed these genes at a low level in the absence of α products. It is possible that the HSV-1 sequences were actually transcribed at a high level in the absence of α products but that the mRNA did not associate with the ribosomes. Because we analyzed polysome-associated poly(A)$^+$ RNA rather than total cytoplasmic RNA, our results do not exclude this alternative.

The second regulatory region in HSV-1 genes may be separate from the promoter region and probably modulates the quantitative level of transcription of a given gene. The elegant studies of Post et al. (44) and Mackem and Roizman (33) on the regulatory role of sequences upstream from the promoter in HSV-1 α genes and of McKnight and Kingsbury (36) on the regulatory role of upstream sequences in the β *tk* gene indicate that this second region does play a crucial role in the regulation of transcription. In our transformed cells, the expression of the HSV-1 β and γ genes occurred only at low levels in the absence of α gene products, and the presence of ICP4 during viral infection greatly induced expression of the same genes. This induction of β and γ genes by α functions is the usual course of events during HSV-1 infection (21, 22, 43), so that these genes integrated into the cell are still capable of responding normally. Others have previously shown that ICP4 is essential for this induction (29, 45, 60), and this is also the case in our transformed cells. Post et al. (44) and Mackem and Roizman (33) presented evidence for a *trans*-acting regulatory signal that presumably interacts with the upstream sequences of α genes. This regulatory signal may

be a protein which is injected along with the virus (2). The requirement for ICP4 for the induction of β genes suggests that ICP4 is a *trans*-acting regulatory signal for β genes. This implies that there are structural differences between the upstream regulatory sequences of α and β and γ genes.

The induction of expression of the viral sequences resident in the transformed cells by α products during infection was multiplicity de-

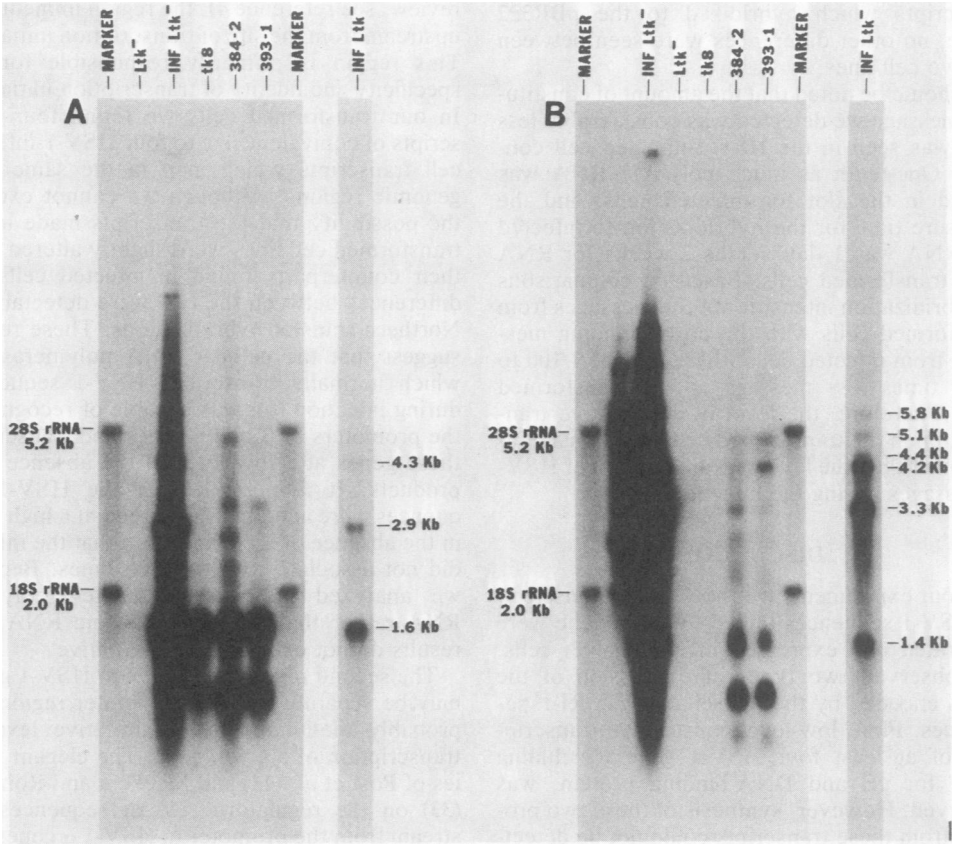

FIG. 7. Northern blot hybridizations of poly(A)$^+$ RNA isolated from 384-2 and 393-1 cells. (A) Poly(A)$^+$ RNA samples (10 μg/slot) isolated from 384-2 cells, 393-1 cells, tk-8 cells, uninfected Ltk$^-$ cells, and Ltk$^-$ cells infected with HSV-1 (KOS) for 8 h at a multiplicity of infection of 5 (1 μg/slot) were denatured in glyoxal (37), fractionated in 1% agarose gels, and then transferred to Gene Screen (New England Nuclear Corp.). The blot was hybridized with the *tk* gene-containing 3.5-kb *Bam*HI fragment purified from plasmid pX1 and labeled with ^{32}P as described for *Eco*RI-F in the legend to Fig. 2B. ^{32}P-labeled rRNAs were included as molecular weight markers. Two exposures of the slot containing HSV-1-infected cell RNA are shown. The first was a 2-week exposure. The lighter exposure, at the right, was for 1 day. (B) Identical blot as that shown in Fig. 7A hybridized with ^{32}P-labeled *Eco*RI fragment F purified as described in the legend to Fig. 2B. (C) The blot in Fig. 7A was stripped of the hybridization probe as described in the text and was rehybridized with a 2,750-bp *Sal*I fragment (shown in Fig. 8) which was gel purified from plasmid pSG18-S84 (19) as described above and then labeled with ^{32}P by nick translation. (D) The blot shown in Fig. 7B was stripped and rehybridized with a 2,300-bp *Bam*HI fragment (shown in Fig. 8) which was gel purified as previously described from a plasmid containing this fragment cloned in pBR322. (E) The blot in Fig. 7C was stripped and rehybridized with ^{32}P-labeled pBR322 to determine whether any transcripts arose from pBR322 sequences. Single-stranded DNA size markers were derived from a mixture of restriction digests of pBR325 DNA and were included in the lanes containing the rRNA markers (Fig. 7A through D). These size markers are now visualized by their hybridization to the pBR322 probe. The DNA markers were added at 40 to 200 pg per band per slot. (F) The blot in Fig. 7D was stripped and rehybridized with a 2,400-bp *Eco*RI-*Sal*I fragment from the left end of *Eco*RI-F (shown in Fig. 8). This fragment, cloned in pBR325, was not gel purified before labeling with ^{32}P so that hybridization occurred to the pBR322 markers and to the transcripts homologous to pBR322 seen in Fig. 7E as well as to HSV-1-specific transcripts.

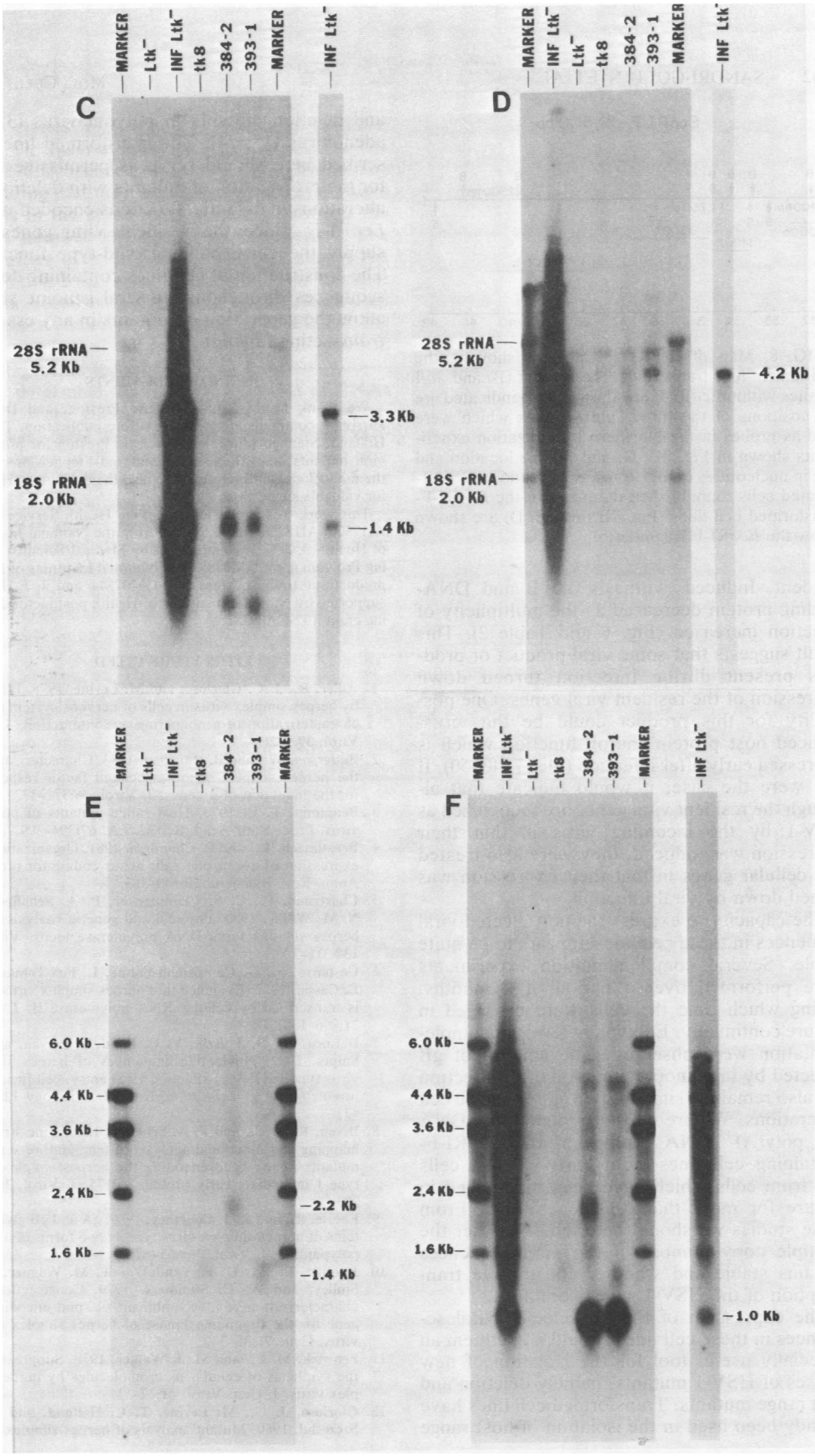

FIG. 7—*Continued*

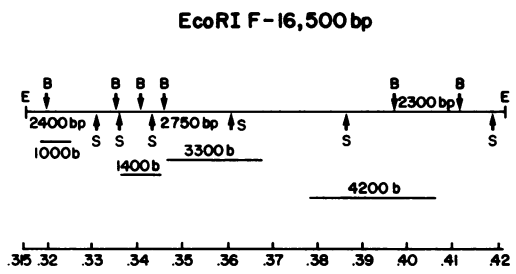

FIG. 8. Map of *Eco*RI F fragment showing the positions of four transcripts. The *Bam*HI (B) and *Sal*I (S) sites within *Eco*RI-F are shown. Also indicated are the positions of the three subfragments which were used as probes in the Northern hybridization experiments shown in Fig. 7C, D, and F. The location and size in nucleotides of four transcripts found in HSV-1-infected cells (unpublished data) and in the *Eco*RI-F-transformed cell lines (Fig. 7B through D) are shown below the *Eco*RI-F fragment.

pendent. Induced synthesis of gB and DNA-binding protein decreased as the multiplicity of infection increased (Fig. 6 and Table 2). This result suggests that some viral product or products present during infection turned down expression of the resident viral genes. One possibility for this product could be the virus-induced host protein shutoff function which is expressed early after infection (11, 17, 40, 50). If this were the case, it would indicate that although the resident viral genes are recognized as HSV-1 by the incoming virus in that their expression was induced, they were also treated like cellular genes in that their expression was turned down by viral infection.

The capacity to express the nonselected viral sequences in these cell lines appears to be quite stable. Several complementation experiments were performed over a period of 7 months, during which time the cells were passaged in culture continually. Equivalent levels of complementation were observed. The amount of gB detected by immunoprecipitation after infection has also remained similar after more than 60 cell generations. We are currently comparing DNA and poly(A)$^+$ RNA profiles of the *Eco*RI-F-containing cell lines from early-passage cells and from cells which have been maintained in culture for more than 100 generations. From these studies we should determine whether the multiple copy number of the viral sequences remains stable and whether constitutive transcription of the HSV-1 genes continues.

The expression of the nonselected viral sequences in these cell lines should make them an especially useful tool for the isolation of new classes of HSV-1 mutants, namely deletion and host range mutants. Transformed cell lines have already been used in the isolation of host range and deletion mutants in polyomavirus (3) and adenovirus (15, 24). The transformed lines described here should serve as permissive hosts for the propagation of mutants with deletions or alterations in essential functions encoded within *Eco*RI-F, since the resident viral genes can supply the corresponding wild-type functions. The construction of cell lines containing defined sequences throughout the viral genome should allow the generation of mutants in any essential *trans*-acting function.

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