Expression of Cloned Herpesvirus Genes

I. Detection of Nuclear Antigens from Herpes Simplex Virus Type 2 Inverted Repeat Regions in Transfected Mouse Cells

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Three different recombinant plasmids containing the entire 15-kilobase L and S inverted repeat sequence of herpes simplex virus type 2 DNA have been introduced into cultured Ltk⁻ or BSC cells by both the calcium and DEAE-dextran transfection procedures. In each case, after 24 h approximately 1% of the cells gave strongly positive nuclear staining when assayed by immunofluorescence with hyperimmune antisera made against early and immediate-early infected-cell polypeptides. The nuclear fluorescence pattern and intensity mimicked that observed within 2 to 3 h after infection of Ltk⁻ cells with either herpes simplex virus type 1 or type 2 wild-type virus. Herpes simplex virus type 1 (KOS Δ tsB2)-infected Ltk⁻ cells under nonpermissive conditions did not express these antigens in the nucleus. Therefore, we conclude that either one or both of the 185,000- and 110,000-molecular-weight immediate early proteins, or some other as yet unknown gene product encoded entirely within the inverted repeats, can be transiently expressed in large amounts in transfected cells in the absence of other viral genes or accompanying virion components. Permanent mouse cell lines derived from transfection with these plasmids by using the thymidine kinase coselection procedure did not express sufficient nuclear antigen to be detectable by immunofluorescence.

RNA and protein synthesis in productive herpes simplex virus (HSV) infections proceeds in a complex regulated process that can be subdivided into at least three stages referred to as immediate-early (alpha), delayed-early (beta), and late (gamma) (15). The immediate-early (IE) class genes are defined as those that are transcribed at the onset of infection, even in the presence of cycloheximide to block new viral protein synthesis. Only six immediate-early genes are recognized at present, and the two that give rise to the most abundant species of mRNA are duplicated genes mapping within the inverted repeats in both HSV type 1 (HSV-1) and type 2 (HSV-2) (2, 7, 17). The well-characterized 175,000-molecular weight (175K)/185K product of gene IE-IV, also known as ICP-4, is a DNA-binding, phosphorylated protein, localized predominantly within the nucleus (2, 4, 30). This protein is known to be directly involved in transcriptional activation of at least some of the delayed-early genes, including that for the thymidine kinase (TK) enzyme (18, 21, 33), and its expression appears to be needed continuously throughout infection: for example, after shift-up

to nonpermissive conditions with the *tsk* or *tsB2* mutants, which synthesize a thermosensitive 175K product, the transcription pattern in an infected cell reverts to the immediate-early pattern even at late times (6, 38). The other immediate-early proteins, with the exception of 136K (gene IE-II, ICP6), are also thought to function predominantly in the nucleus and are chromatin-associated DNA-binding proteins (12). Two of the immediate-early mRNAs from HSV-1 are known to be spliced, but the introns are very small (39), and recent evidence about the location of the 5'-capped sites in HSV-1 immediate-early mRNA suggests that each immediate-early gene may have its own promoter and that the mRNAs lack long leader sequences (22).

To study the roles and mechanisms of action of regulatory genes in the HSV infection process, we have embarked on a series of studies both to isolate and clone individual intact genes and establish experimental systems in which individual genes either are expressed constitutively or are able to be switched on or off by the introduction of other viral genes or gene products. Transfection with purified HSV DNA and

isolated or cloned viral DNA fragments has been used previously to demonstrate infectivity and focus formation (8, 10, 34, 36) or to rescue Ltk⁻ cells in HAT medium with the isolated HSV TK gene (14, 34, 40). Similarly, isolated DNA fragments have been employed for recombination in marker rescue experiments along with intact HSV DNA from *ts* mutants or specific plaque-type virus (27, 37). However, with the exception of TK (25, 26), there have been no reports of attempts to demonstrate expression of individual cloned genes in the absence of the rest of the viral genome. We have initially attempted to clone overlapping sets of viral DNA fragments that are sufficiently large to be able to demonstrate the action of individual genes or combinations of genes after transfection, either in short-term assays involving immunofluorescence, or by establishing permanent cell lines by linked coselection with the viral TK gene (14; G. Reyes, D. Ciuffo, and G. S. Hayward, manuscript in preparation). In the present report, we show that at least one viral nuclear protein can be expressed from cloned DNA fragments after short-term transfection in either Ltk⁻ mouse cells or BSC monkey cells.

MATERIALS AND METHODS

Cells and viruses. BSC-1 cells were supplied by Greg Milman. The simian virus 40 (SV40)-transformed NBE cell line was obtained from Solon Rhode III. LMTk⁻ cells were originally obtained from Saul Silverstein. LH₂p18 is a TK⁺ transformant derived in this laboratory by transfection of LMTk⁻ cells with the pGR18 plasmid. HSV-1(MPc/226) and HSV-2(333c/6) are our standard plaque-purified prototype strains routinely grown and titered in Vero cells. A seed stock of HSV-1(KOS_{ts}B2) was kindly provided by Priscilla Schaffer. All cells and virus used were shown to be free of mycoplasma contamination by the Hoesch stain test or by cultivation assays performed by Flow Laboratories.

Plasmids and DNA. The procedures used for molecular cloning of *Hind*III, *Bgl*II, and *Sall* fragments from total digests of HSV-1 or HSV-2 DNA in pBR322 are described elsewhere (G. Reyes, Ph.D. Thesis, Johns Hopkins University, Baltimore, Md., 1982) and adhered to the Institutional and National Institutes of Health Guidelines for Recombinant DNA Research in effect at the time. All recombinant plasmids were grown in *Escherichia coli* HB101 by standard procedures. The DNA was purified by banding to equilibrium in cesium chloride-ethidium bromide density gradients. The structures of pGH3, pGR90, pGR91a, pGR91b, pGR150, pGR151a, and pGR151b are described in the text and illustrated in Fig. 2. They were all selected and identified by colony hybridization with the isolated *Sall*-A fragment from HSV-2(333) as a probe. The TK vehicle pGR18 contains a *Hind*III-*Sall* fragment from coordinates 0.287 to 0.320 in HSV-2(333) DNA (14; G. R. Reyes, K.-T. Jeang, and G. S. Hayward, J. Gen. Virol., in press). pGR60 contains

the *Bgl*II-N morphological transforming fragment from HSV-2(333), and pGR116 contains the *Bgl*III-I fragment from HSV-2(MP) (34). The pSVB21 plasmid containing SV40 DNA cloned into the *Bam*HI site of pBR322 was a gift from Keith Peden (28).

Antisera. The principal antisera used in this work were prepared in Glasgow in Hooded Lister rats from a highly inbred colony. Immunization protocols involved 3 weekly intracutaneous injections of sonicated cell extracts in phosphate-buffered saline (PBS), followed by a final boost 10 to 14 days later. The extracts were derived from secondary monolayers of rat embryo cells infected with HSV at 10 to 20 PFU/cell. The immediate-early IE¹ and IE² antigens were produced in rat cells infected with HSV-1(17) or HSV-2(HG-52) in the presence of 50 µg of cycloheximide (Sigma) per ml for 7.5 h, then released from this block (after three washes with PBS) and incubated for 2 h in the presence of 5 µg of actinomycin D (Merck, Sharp & Dohme) per ml. The early E¹ and E² antigens were produced in cells infected with HSV-1(17) or HSV-2(HG-52) for 7.5 h in the presence of cycloheximide, followed by washing and incubation in growth medium without inhibitors for 3 h to permit additional synthesis of delayed-early proteins. Immediate-early *ts*K¹ antigens represented rat cells infected with HSV-1(17_{ts}K) at the nonpermissive temperature (38.5°C). Only immediate-early proteins are produced by this virus at nonpermissive temperature (33). Goat antisera against purified HSV-1 and HSV-2 virions were obtained from Mette Strand. Hamster anti-SV40 T-antigen serum was supplied by Sondra Lazarowitz. Samples of HSV monoclonal antibodies produced by mouse hybridoma cell lines were obtained from Berge Hampar (anti-136K, 2S; anti-132K, 39S) and Tony Minson (anti-65K, LP-1). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat immunoglobulin G (IgG) and other appropriate FITC-conjugated IgGs were obtained from commercial suppliers.

Transfection procedures. For short-term calcium transfections, washed Ltk⁻ mouse cells at 50% confluence in wells on glass slides were treated with uncut pGR150 DNA at 150 µg/ml without added carrier DNA. The plasmid DNA mix in 0.125 M CaCl₂, Dulbecco modified Eagle medium, and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) saline buffer (pH 7.05) (10) was incubated for 30 min at 22°C and then allowed to absorb to the cells for 40 min at 37°C (30 µl per well). Dulbecco modified Eagle medium plus 10% fetal calf serum (200 µl per well) was added for 3 h at 37°C; the cells were then washed with PBS and incubated further in fresh medium. DEAE-dextran transfections were usually performed with BSC-1 monkey cells and again by direct adsorption onto the monolayers. The plasmid DNA mix, at up to 300 µg/ml in Dulbecco modified Eagle medium, 0.04 M HEPES (pH 7.4), and 200 µg of DEAE-dextran per ml was added to PBS-rinsed cells for 30 min at 22°C. The slides were then washed with PBS and incubated overnight in fresh Dulbecco modified Eagle medium plus 10% fetal calf serum at 37°C. Long-term transfections of Ltk⁻ cells for establishing TK-coselected cell lines from colonies selected in HAT medium were performed essentially as described by Graham et al. (10) and Wigler et al. (40). Details of the quantities of coselected DNA, TK plasmid, and carrier DNA used and the nomenclature of the cell lines are given in

Table 3. HAT medium was changed fresh every 3 days; colonies were counted and subcloned at 14 days.

Immunofluorescence. Near-confluent cultures of Ltk⁻ cells growing in wells on glass slides were infected with HSV-2(333) virus at a multiplicity of 10 PFU/cell. At either 2 or 6 h after completion of the absorption step, the cells were washed with PBS and fixed in absolute methanol at -20°C . Transfected cells were fixed after a 24-h incubation period. For immunofluorescence, each well was first incubated with 30 μl of rat anti-HSV serum (usually at a 1:50 to 1:200 dilution), rinsed, and then incubated with 30 μl of FITC-conjugated mouse anti-rat IgG diluted into a rhodamine contrast stain. After a final washing with Tris-MgCl₂, the cells were mounted under a cover glass in PBS-glycerol and observed under oil immersion in a Leitz epifluorescence UV-microscope. Photographs were taken on 400 ASA color film by using a 40 $\times$ objective and narrow-band FITC filters.

Protein labeling and immunoprecipitation. Infected BHK cells were labeled for 2-h periods at appropriate times after infection with either [³⁵S]methionine at 40 $\mu\text{Ci/ml}$ in methionine-free medium or ³²P_i at 100 $\mu\text{Ci/ml}$ in phosphate-free medium. The cultures were harvested and lysed by sonication at 10⁷ cells per ml in precipitation buffer containing 150 mM NaCl, 0.5% Nonidet P-40, and 20 mM sodium phosphate (pH 7.2). For immunoprecipitation, the clarified antigen sample was thoroughly mixed with an appropriate dilution of clarified antiserum in 100 μl and incubated at 37 $^{\circ}\text{C}$ for 30 min and then overnight at 4 $^{\circ}\text{C}$. Protein A-Sepharose beads (6 mg in 50 μl of precipitation buffer) were added with thorough mixing for 30 min and then pelleted and washed four times in 0.6 M NaCl–20 mM sodium phosphate buffer at 4 $^{\circ}\text{C}$ and twice in 20 mM Tris-hydrochloride (pH 7.2). Finally, the mixture was disaggregated by heating to 90 $^{\circ}\text{C}$ for 10 min in 0.5% sodium dodecyl sulfate–0.5% β -mercaptoethanol–20 mM Tris-hydrochloride (pH 7.2) and separated from the Sepharose beads by centrifuging. The samples were analyzed by polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Recombinant plasmids containing the inverted repeats. Representative cleavage patterns and physical maps of the relevant plasmids employed in this study are presented in Fig. 1 and 2. A total of eight different clones containing the junction region between the L and S segments plus varying additional portions of the inverted repeats or adjacent unique sequences were derived from shotgun experiments with total *Hind*III-, *Bgl*II-, or *Sal*I-cleaved HSV-2 DNA. Although these plasmids are relatively large and somewhat difficult to grow, they appear to be quite stable and did not undergo rearrangement.

Plasmids pGR91a and pGR91b contain the 17.3-kilobase (kb) *Hind*III-JK fragment from HSV-2(333) DNA inserted in different orientations into the *Hind*III site in the tetracycline resistance gene of pBR322. They would be expected to encode the complete body of mRNA species IE-I, IE-IV, and IE-V (see Fig. 2). Simi-

larly, pGR90 possesses a complete copy of the entire inverted L and S repeat sequence, but differs from pGR91 in that the S-unique portion comes from the right-hand side of the S segment rather than the left-hand side: this 18.8-kb *Hind*III-JM fragment insert should contain the intact genes IE-I, IE-IV, and IE-VI. A quarter-molar *Bgl*II fragment (13) from HSV-2(333), cloned into pBR322 at the *Bam*HI site, yielded the plasmids referred to as pGR150, pGR151a, and pGR151b. Restriction site mapping of pGR150 and pGR151b revealed that they contained different orientations of the 23-kb *Bgl*II-HM species, which would be expected to encode the intact genes IE-III, IE-I, and IE-IV and at least part of IE-V. pGR151a proved to be similar to pGR151b except that it contained an additional copy of the terminally redundant "a" sequence at the L-S junction. Finally, the 11.5-kb *Sal*I-A fragment, representing an internal

FIG. 1. Sample cleavage patterns of cloned "inverted repeat" segments from HSV-2 DNA. Plasmid DNAs were cleaved with appropriate restriction enzymes, and the resulting fragments were resolved by agarose gel electrophoresis followed by staining with ethidium bromide. Channels 1 to 7, 1.2% agarose gel: 1, *Eco*RI digest of phage lambda DNA for size references; 2 to 4, *Hind*III plus *Xba*I digests of pGR90, pGR91a, and pGR91b; 5 to 7, *Bam*HI plus *Sal*I digests of pGR90, pGR91a, and pGR91b. Channels 8 to 15, 1.0% agarose gel: 8, *Bam*HI digest of pGR150 for size references; 9 to 11, pGR150b cleaved with *Bam*HI, *Eco*RI, or *Hind*III; 12, pGR151a, *Bam*HI; 13 to 15, *Hind*III plus *Bam*HI digests of pGR151a, pGR151b, and pGR150b.

FIG. 2. Physical map illustrating the original location within the HSV-2 genome of molecularly cloned DNA fragments containing all or part of the inverted repeat sequences. Only the right-hand half of the genome is drawn, from map coordinates 0.55 to 1.00. The map position, orientation, and relative abundances of the currently recognized immediate-early mRNAs are indicated by the solid horizontal arrows. The positions of the L and S inverted repeats and the junction between the L and S segments (13) are denoted by hatched regions and the broken vertical line, respectively. The most commonly assigned molecular weights of the HSV-1 equivalent protein products and their predominant nuclear or cytoplasmic locations are also given for each IE gene. In the case of IE-IV the HSV-2 protein has a slightly larger molecular weight and is referred to as 185K in this paper. Only the inserted viral portions of the four major plasmid types used in this work are illustrated (open and hatched bars). The key for confirmed restriction enzyme cleavage sites is as follows: *Bam*HI, ○; *Bgl*II, ●; *Eco*RI, ◇; *Hind*III, □; *Hpa*I, ☆; *Kpn*I, ▽; *Sal*I, ▲; *Xba*I, ★.

subsection of the HSV-2 inverted repeat from coordinates 0.775 to 0.848, is carried in opposite orientations in plasmids pGH3 and pGH4.

Characterization of early antisera by immunofluorescence. Neither monoclonal nor monospecific antisera that immunoprecipitate HSV-2 immediate-early nuclear gene products are available as yet. For initial studies, to examine whether or not any early viral gene products could be expressed in transfected cells without supplying selective pressures, hyperimmune sera prepared against unfractionated infected cells (and therefore likely to react with as many early gene products as possible) seemed to be the most useful. The rat sera used in these experiments react against two classes of antigens: (i) predominantly nuclear species generated within 3 h after HSV-1 or HSV-2 infection, and (ii) cytoplasmic granular material present in

many morphologically transformed cells but not in Rat-1, BALB/c-3T3, or other nontransformed cells. Preimmune normal rat serum showed neither reactivity. The cytoplasmic granular fluorescence was removed by absorption against fixed homologous transformed cells (Ltk⁻ or BSC), whereas the viral nuclear antigens were unaffected by absorption. Four different sera (23) generated in rats against sonic extracts of rat cells infected with HSV-1 or HSV-2 under various different antibiotic or nonpermissive conditions (see Materials and Methods) gave essentially identical results.

Typical nuclear antigen staining patterns in HSV-2-infected Ltk⁻ cells at 6 h after infection, obtained with the absorbed anti-E² serum, are shown in Fig. 3a. Table 1 summarizes the properties of all sera tested with regard to detection of nuclear immunofluorescence in infected and

FIG. 3. Comparison of nuclear immunofluorescence patterns obtained with infected or transfected Ltk^{-} mouse cells. Rat antiserum against early (immediate-early plus delayed-early) stage HSV-2-infected rat cells (anti- E^2) was diluted 1:100 and absorbed against fixed uninfected Ltk^{-} cells and then used for indirect immunofluorescence with FITC-conjugated anti-rat IgG antiserum. (a) Ltk^{-} cells 6 h after infection with HSV-2(333) virus. (b) and (c) Ltk^{-} cells 24 h after transfection by the calcium procedure with circular pGR150 DNA at 150 $\mu\text{g}/\text{ml}$ without added carrier DNA.

TABLE 1. Comparison of different antisera for detection of nuclear antigens in HSV-infected cells

Cell	Virus strain (time)	Nuclear fluorescence ^a with antiserum against:				
		E ^{2b}	IE ²	IE ¹	tsK ¹	SV40 T-antigen
Ltk ⁻	333 (2 h)	++	+	+	+	-
Ltk ⁻	333 (6 h)	+++	++	++	++	-
Ltk ⁻	MP (2 h)	+	+	+	+	-
Ltk ⁻	MP (6 h)	++	++	++	++	-
Ltk ⁻	None	-	-	-	-	-
NBE (SV40) ^c	None	-	-	-	-	+++
LH ₂ p18 (TK)	None	-	-	-	-	-
BSC-1	333 (6 h)	+++	-	-	-	-

^a -, Negative response in all cells; +, ++, +++, relative intensity of positive response in a majority of the cells. Superscripts 1 and 2 denote HSV-1 or HSV-2 origin of antigen.

^b Absorbed against fixed Ltk⁻ cells.

^c SV40-transformed human fibroblast cells.

control cells. The anti-E² serum derived from HSV-2 infection of cells harvested 3 h after reversal from a cycloheximide block gave the strongest reaction and was used in most of the transfection experiments described below. Anti-IE¹ and anti-IE² sera prepared from infected cells harvested after 2 h in actinomycin D upon reversal from a cycloheximide block, and an anti-tsK¹ serum from cells infected with an IE-IV ts mutant of HSV-1 at nonpermissive temperature, all gave very similar but less intense nuclear fluorescence against 2-h and 6-h HSV-2-infected Ltk⁻ or BSC cells.

Several samples of antisera prepared against purified virions or late-stage-infected cells proved negative for nuclear fluorescence on infected cells at these early times (not shown), indicating that we are unlikely to be observing input virion components in these studies. Similarly, the HSV early sera did not react with SV40-transformed cells, nor did SV40 T-antisera react with the HSV-infected cells (Table 1).

Permissiveness of Ltk⁻ cells for HSV infection. In other experiments we have constructed sets of transposition plasmids containing large portions of the HSV-1 and HSV-2 genomes directly linked to an HSV-2 TK gene in the pGR18 plasmid vehicle and have used these to establish panels of LTK⁺ cell lines (14; Reyes et al., in preparation). However, none of the coselected cell lines tested that received delayed-early or late viral DNA regions expressed viral antigens detectable by immunofluorescence with the antisera described here (not shown). The semipermissive nature of mouse cells for HSV infections was of some concern in this regard. For exam-

ple, neither HSV-1(MP) nor HSV-2(333) formed plaques on these cells, and the yield of HSV-1 infectious virions (assayed on Vero cells) from a single-step growth experiment at a multiplicity of infection of 10 PFU/cell was 100-fold lower in Ltk⁻ cells than in Vero cells. Since we have found that only a single immediate-early viral protein is synthesized during the nonpermissive infection of mouse cells by human and simian cytomegaloviruses (R. L. LaFemina and G. S. Hayward, submitted for publication; K.-T. Jeang, G. Chin, and G. S. Hayward, Virology, in press), we considered it necessary to confirm that normal quantities of HSV immediate-early and delayed-early proteins are synthesized in HSV-infected Ltk⁻ cell cultures. Protein labeling studies indicate that most or all of the HSV-specific immediate-early and delayed-early polypeptides could be detected and that the transition from the immediate-early to delayed-early class occurs at the appropriate time (not shown). Therefore, the inefficient step in production of infectious progeny in these cells presumably occurs at some later point in the infection cycle, and the lack of detectable delayed-early and late viral antigens in the LTK⁺ transfected cell lines is more likely to involve the absence of appropriate viral "switch-on" regulatory functions than an inability of the mouse cells to synthesize these proteins (35).

Coselected cell lines. In the hope of establishing permanent cell lines that might express the HSV-2 immediate-early antigens continuously, we attempted to introduce pGR150 and pGR91a plasmid DNA into mouse cells by unlinked coselection with the cloned HSV-2 TK gene by following the general procedures introduced by Pellicer et al. (29). The derivation and use of the pGR18 TK vehicle to generate TK⁺ colonies in HAT medium after calcium transfection of Ltk⁻ cultures is described in detail elsewhere (14; Reyes et al., in press). In these experiments, linearized plasmids were transfected together with a smaller quantity of supercoiled pGR18 DNA (with or without carrier Ltk⁻ DNA) into Ltk⁻ cells. The number of colonies obtained in HAT medium and the subcloned cell lines derived from them are summarized in Table 2. These procedural variations give 10- to 20-fold lower transfection efficiencies than when uncleaved plasmid DNA is used, but are employed routinely in our laboratory in an attempt to obtain single-copy transformants with the maximal size of integrated nonselected sequences and also to minimize complications from the acquisition of carrier DNA sequences (Reyes et al., in preparation). Within three passages in mass culture after colony isolation most of the subclones listed (12 in all) that received either pGR151 or pGR91a DNA (LH2p151 and

TABLE 2. Calcium transfection of Ltk⁻ cells in HAT medium

Coselection		HSV-2 TK pGR18 (μ g)	Ltk ⁻ carrier (μ g)	No. of colonies per dish	TK ⁺ subclones	Gene expression (no. positive/no. tested)	
Plasmid (3 μ g)	Treat- ment					HSV nuclear antigen ^a	Human β -inter- feron ^b
pGR151	<i>Bgl</i> III	0.5	5.0	5	LH ₂ p151(1-5)	0/5	— ^c
pGR151	<i>Bgl</i> III	0.5	None	18	LH ₂ p151(6-8)	0/1	—
pGR91a	<i>Hind</i> III	0.5	5.0	9	LH ₂ p91a(1-4)	0/4	—
pGR91a	<i>Hind</i> III	0.5	None	6	LH ₂ p91a(5-6)	0/2	—
pGH3	<i>Hind</i> III	0.5	5.0	5	LH ₂ p3(1-5)	0/3	—
pGH3	<i>Hind</i> III	0.5	None	7	LH ₂ p3(6-9)	0/4	—
pHF β cDNA	<i>Hind</i> III	0.5	5.0	35	LH ₂ pCIF(5, 6)	—	2/2
pHF β cDNA	<i>Hind</i> III	0.5	None	15	LH ₂ pCIF(7-10)	—	2/2
None		0.5	5.0	5	LH ₂ p18-8	0/1	0/1
None		0.5	None	10	None	—	—
None		None	5.0	0		—	—
None		None	None	0		—	—

^a Tested by immunofluorescence with anti-E² serum.^b After polyriboninosinic acid:polyribocytidylic acid induction (Pitha et al., in press).^c —, Not done.

LH₂p91a) were tested for expression of viral nuclear antigens by immunofluorescence with the anti-E² serum. All proved negative by this assay. A number of pooled cultures representing 26 additional independent colonies also failed to give detectable nuclear immunofluorescence in any cells.

Detection of nuclear antigens in short-term transfection assays. Our inability to detect expression of immediate-early nuclear products in unlinked coselected cell lines may have been related to unstable integration or counterselection as a result of viral gene products that may be incompatible with cell survival. Therefore, we turned to short-term transfection procedures and asked for transient expression rather than stable constitutive expression of the antigens. In these experiments, introduction of pGR150 plasmid DNA at 150 μ g/ml resulted in strong nuclear fluorescence in 0.5 to 3% of the cells after 24 h. Positive results were obtained in both Ltk⁻ and BSC cells by using either the calcium phosphate (9) or DEAE-dextran procedures (24, 26, 36). Similar nuclear fluorescence was also obtained with the pGR90 and pGR91a plasmids, but not with pGR3. Several other HSV-2 plasmids tested, derived from elsewhere in the genome, were also negative (Table 3). Examples of the fluorescent nuclei obtained by short-term transfections of Ltk⁻ cells by using the anti-E² serum are shown in Fig. 3b and c. Note the close similarities between the antigen staining patterns observed in the transfected cells and those in the nuclei of infected cells, especially with the giant multi-nucleated cell seen in Fig. 3c. Carrier DNA was not used in either the calcium or DEAE-dextran procedures, and the supercoiled plasmid DNAs were used directly without re-

striction enzyme cleavage. The HSV-2 nuclear antigens tended to be much brighter with the calcium procedure than with DEAE-dextran, perhaps reflecting differences in the total amount of DNA acquired per cell. Again the anti-IE² serum also gave positive but less intense nuclear fluorescence in a similar proportion of the pGR91a- or pGR150-transfected cells. The percentage of nuclei that stained positive for SV40 T-antigen in parallel transfections with cloned SV40 plasmid DNA was usually lower than that with the HSV plasmids by a factor of

TABLE 3. Assays for nuclear antigens in transfected cells

Procedure	Cell	Plasmid	Nuclear fluorescence ^a with antiserum against:		
			E ²	IE ²	SV40 T- antigen
Calcium phosphate	Ltk ⁻	pGR150	++	+	—
	Ltk ⁻	pGR91a	++	+	—
	Ltk ⁻	pGR90	++	—	—
	Ltk ⁻	pGH3	—	—	—
	Ltk ⁻	pGR116	—	—	—
	Ltk ⁻	pGR60	—	—	—
	Ltk ⁻	pSVB21	—	—	++
	Ltk ⁻	None	—	—	—
DEAE- dextran	BSC	pGR90	+	—	—
	BSC	pSVB21	—	—	+++
	BSC	None	—	—	—
	Ltk ⁻	pGR150	+	+	—
	Ltk ⁻	None	—	—	—

^a Positive or negative response at 24 h in approximately 1% of the cells.

TABLE 4. Comparison of nuclear fluorescence in HSV-1(KOStsB2)-infected cells under permissive and nonpermissive conditions

Infection conditions	Fluorescence ^a with antiserum against:				
	E ^{2b} N	tsK ¹ N	136K ^{1,2} C	132K ¹ N	65K ^{1,2} C+N
Ltk ⁻ , HSV-1(MP)					
37°C, 3 h	++	++	+	—	(±)
37°C, 8 h	++	++	+++	+++	+++
Ltk ⁻ , HSV-1(KOStsB2)					
39.5°C, 3 h	(±)	(±)	+	—	(±)
39.5°C, 8 h	(±)	(±)	++	—	(±)
33.5°C, 3 h	+	+	+	—	(±)
33.5°C, 12 h	++	++	++	+	++
Ltk ⁻ , mock infection					
37°C, 8 h	—	—	—	—	(±)

^a Intensity of nuclear (N) or cytoplasmic (C) fluorescence in the majority of cells. Superscripts 1 and 2 refer to HSV-1 or HSV-2 serotype from which the antiserum was derived, or, in the case of the monoclonal antibodies, to serotype specificity.

^b Absorbed against Ltk⁻ cells.

two to three in the calcium experiments but better by a factor of two to three for DEAE-dextran. The HSV-2 antisera were negative on SV40-transfected cells and vice versa.

Do the nuclear antigens represent immediate-early gene products? Direct proof that the viral gene products synthesized in short-term transfected cells are immediate-early proteins might be possible with large quantities of high-titer antisera which immunoprecipitate these proteins. However, characterization of the rat antisera used in the previous transfection experiments by immunoprecipitation of proteins from either [³⁵S]methionine- or ³²P-labeled, infected BHK cell cultures (not shown) revealed the following. (i) Both the tsK¹ and E² antisera predominantly precipitated the 136K protein from extracts containing all five of the ³²P-labeled immediate-early gene products (175K, 136K, 110K, 68K, and 63K) that were synthesized by HSV-1(KOStsB2) at the nonpermissive temperature (39.5°C). Nevertheless, other studies indicate that both of these sera immunoprecipitate small quantities of the 175K/185K and 110K proteins under certain conditions (J. C. M. Macnab, unpublished data). (ii) In addition, the E² serum, and to a lesser extent the tsK¹ serum, precipitated many ³⁵S-labeled delayed-early viral polypeptides (such as the 132K major DNA binding protein) labeled within 6 h after infection. (iii) The proportions of the 155K major capsid protein, the 65K late phosphoprotein, and the virion glycoproteins, relative to the delayed-early proteins precipitated with these sera, were greatly reduced in comparison with the results obtained with hyperimmune sera prepared against later-stage-infected cells or purified virions. In conclusion, although these sera

are enriched for antibodies against immediate-early and delayed-early viral gene products (especially the largely cytoplasmic phosphorylated 136K protein) and contain only low reactivities against virion components, they do not immunoprecipitate proportional quantities of the nuclear immediate-early proteins.

As an alternative indirect approach to examine whether or not the nuclear immunofluorescence observed is likely to represent antibody recognition of viral immediate-early gene products, we compared the immunofluorescence results obtained after infection of Ltk⁻ cells with the HSV-1(KOStsB2) mutant at permissive (33.5°C) and nonpermissive temperatures (39.5°C). The tsB2 mutant synthesizes a thermosensitive 175K immediate-early polypeptide (5) whose processing and compartmentalization are aberrant at 39.5°C (1, 6, 33). The other immediate-early polypeptides are all overproduced at the nonpermissive temperature, but only trace amounts of a subclass of the delayed-early and late proteins are synthesized (30). Table 4 summarizes the results of our immunofluorescence assays on HSV-2-infected Ltk⁻ cells with the anti-E² and anti-tsK¹ rat sera and three reference monoclonal mouse hybridoma antisera. At both 3 and 8 h after absorption, the anti-E² and anti-tsK¹ sera each gave bright nuclear fluorescence in the HSV-1(MP)-infected cells, but only a trace of nuclear fluorescence was observed in the nonpermissive tsB2 infections (although the latter displayed cytoplasmic and perinuclear fluorescence with both antisera). In contrast, the monoclonal antibody against 136K gave typical uniform cytoplasmic and perinuclear fluorescence in some cells at 3 h and in most cells at 8 h, even after infection with tsB2 at 39.5°C. The

monoclonal sera against the 65K phosphoprotein and the 132K major DNA binding protein gave negative results in all cells with both viruses at 3 h and also at 8 h with *tsB2* at 39.5°C, but displayed their characteristic whole-cell or irregular intranuclear patterns, respectively, in most cells at 8 h after HSV-1(MP) infection.

In conclusion, the nuclear antigen(s) detected by immunofluorescence with both anti-E² and anti-*tsK*¹ sera at 3 h after infection by wild-type virus represent newly synthesized viral gene products which are presumably of the immediate-early or delayed-early class. In addition, infection of Ltk⁻ cells with *tsB2* at nonpermissive temperature resulted in either aberrant compartmentalization or lack of synthesis of the major nuclear antigen detected by our sera (probably the 175K protein itself) and failed to proceed to synthesize more than a trace of delayed-early or late proteins. Synthesis of the IE-II 136K cytoplasmic immediate-early protein, variously described elsewhere as an $\alpha\beta$ or β_1 class gene product (30), occurred at the same rate and timing in *tsB2* infection at 39.5°C as in HSV-1(MP) infection at 37°C and therefore appears not to be dependent on an active 175K protein (6).

DISCUSSION

Our observations represent the first demonstration that an isolated HSV-2 gene or genes, other than that for the TK enzyme, can be expressed in transfected cells. As far as we are aware, the T-antigens of SV40 and polyoma virus are the only other viral gene products that have been demonstrated to be synthesized directly from subgenomic or cloned fragments by short-term transfection procedures (20, 26). The effects of cloned early adenovirus genes have been demonstrated in assays for adeno-associated virus helper functions by both microinjection and short-term transfection (16, 35), and the gene E1A product of adenovirus is expressed in permanent TK-coselected cell lines although in an aberrant manner (11). However, there have been no reports of other adenovirus type 2 proteins being expressed in the absence of the E1A gene product, either constitutively in transfected cell lines or in short-term assays.

The physical map location in the HSV-2 genome of the fragments used for our transfections greatly limits the possibilities of which viral genes are being expressed (7). For example, the 136K immediate-early protein (map coordinates, 0.56 to 0.60) and also the major 132K DNA-binding protein from the delayed-early class (0.38 to 0.41) are both clearly eliminated (3, 7, 32). Since all three positive plasmids gave identical fluorescence patterns, the major com-

ponents of the nuclear antigens expressed must be coded by regions common to all three. This further restricts the possibilities to either the two known major immediate-early genes, IE-I and IE-IV coding for the 110K and 185K phosphoproteins, respectively, or to some other unidentified early gene product that also maps within the L- or S-inverted repeats. Proteins encoded by IE-III, IE-V, or IE-VI may each be synthesized from one or another of the plasmids, but those genes could not be present in all three inserts. Therefore, it seems reasonable to assume that IE-IV 185K and possibly also IE-I 110K are the major components of the viral antigens expressed in our transfected cells. Both are known to be major immediate-early, nuclear, DNA-binding proteins, and together their mRNAs (4.2 and 3.4 kb) comprise approximately 50% of the maximum single-stranded coding capacity of the inverted repeats.

The nuclear fluorescence pattern observed in all of our transfection experiments strongly resembled that produced within 2 to 3 h after viral infection of the same cell type and was similar to that described by Cabral et al. (1) in their ultrastructural studies of HSV-1 immediate-early infected cells stained by the immunoperoxidase technique with monospecific antiserum to the HSV-1 175K protein. The lack of nuclear fluorescence with our sera after *tsB2* infection at nonpermissive temperature implies either that the protein recognized is the 175K/185K product itself, which is not transported to the nucleus efficiently under these conditions (1, 30, 33), or that it is an unknown delayed-early product dependent upon the regulatory action of 175K/185K for its synthesis. Since strong nuclear fluorescence was observed with these sera within 2 to 3 h after infection with wild-type virus under permissive conditions, at which time the immediate-early products were the most predominant proteins synthesized, the former possibility appears the most likely. Therefore, our results suggest that at least one of the early nuclear gene products from HSV-2 (probably the 185K protein) can be synthesized in large quantities in the absence of the remainder of the viral genome or of input virion components. The latter point is significant because Knipe et al. (19) have recently proposed an essential role for a virion structural polypeptide in initiating viral infection based on the properties of the HSV-1(HFEM-*tsB7*) mutant. The infectivity of purified HSV DNA (36) provides a strong argument against the necessity for a virion component in the infectious process, but does not rigorously exclude the possibility of a phenol-resistant protein tenuously bound to the DNA. However, the possibility of such a protein on bacterial plasmid DNA is remote. Post et al. (31) have also report-

ed evidence for some factor provided by input superinfecting virus that gives a greater than 10-fold enhancement of the synthesis of viral TK enzyme from a TK⁺ cell line in which the TK structural gene is under the transcriptional control of the IE-IV promoter sequence; they again postulated the existence of some "pre-immediate-early" function. The results presented here, which we interpret to show that an early nuclear gene product is made in equal abundance in transfected cells as in infected virus cells, might suggest alternatively that the 175K/185K gene product has a positive regulatory effect upon its own synthesis and perhaps that of other immediate-early genes also.

Cell lines that continuously express HSV-2 immediate-early gene products would be extremely useful for detailed studies of the mechanisms of HSV gene regulation and for constructing and selecting host-range virus mutants. However, our initial attempts to establish cell lines receiving the pGR150 or pGR91a plasmid DNA by unlinked TK coselection procedures did not yield any subclones expressing detectable viral nuclear antigens. Parallel experiments for unlinked coselection of an interferon cDNA plasmid demonstrate that the LH₂pCIF cDNA lines receiving the human β -interferon cDNA plasmid were also negative for constitutive interferon production, but all that were tested (four of four) expressed authentic human β -interferon after polyribonucleosinic acid:polyribocytidylic acid induction, indicating that our coselection procedure had at least succeeded in efficiently transferring the 560-base pair interferon coding sequence into the mouse cells (P. M. Pitha, D. M. Ciufo, M. Kellem, N. B. K. Raj, G. R. Reyes, and G. S. Hayward, *Proc. Natl. Acad. Sci. U.S.A.*, in press). However, the HSV-2 immediate-early genes are 6- to 10-fold larger than the interferon coding sequence. To establish permanent transfected cell lines that express the coselected HSV-2 immediate-early antigens constitutively, a large number of factors must be favorable. In addition to being taken up into the same cells as the TK gene, these large plasmids (15 kb of viral DNA) must be stably integrated in a relatively intact form, they must be capable of being expressed efficiently in the absence of other viral regulatory factors, and their gene products must be compatible with cell survival. To our knowledge this has only been accomplished successfully in other DNA virus systems with the papovavirus T-antigens, the hepatitis B surface antigen, and the gene E1A product from adenovirus. The absence of expression at the level of detection by immunofluorescence does not preclude the presence of the appropriate DNA sequences in the cell. However, because of inherent difficulties created by the extensive

homology between portions of the HSV inverted repeat sequences and repetitive elements in mammalian cell DNA (K. Peden, P. Mounts, and G. Hayward, submitted for publication), we did not consider it feasible at present to attempt to detect and map unambiguously the coselected viral DNA sequences in the LH₂p151 and LH₂p91a cell lines. Attempts to induce the expression of possible phenotypically silent HSV-2 immediate-early genes in the LH₂p151 cells by superinfection or to detect them by genetic procedures are in progress.

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