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Open reading frame P—a herpes simplex virus gene repressed during productive infection encodes a protein that binds a splicing factor and reduces synthesis of viral proteins made from spliced mRNA

(serine/arginine-rich proteins/colocalization/posttranslational processing/latency)

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ABSTRACT **The open reading frame P (ORF P) is located in the domain and on the DNA strand of the herpes simplex virus 1 transcribed during latent infection. ORF P is not expressed in productively infected cells as a consequence of repression by the binding of the major viral regulatory protein to its high-affinity binding site. In cells infected with a mutant virus carrying a derepressed gene, ORF P protein is extensively posttranslationally processed. We report that ORF P interacts with a component of the splicing factor SF2/ASF, pulls down a component of the SM antigens, and colocalizes with splicing factors in nuclei of infected cells. The hypothesis that ORF P protein may act to regulate viral gene expression, particularly in situations such as latently infected sensory neurons in which the major regulatory protein is not expressed, is supported by the evidence that in cells infected with a mutant in which the ORF P gene was derepressed, the products of the regulatory genes α0 and α22 are reduced in amounts early in infection but recover late in infection. The proteins encoded by these genes are made from spliced mRNAs, and the extent of recovery of these proteins late in infection correlates with the extent of accumulation of posttranslationally processed forms of ORF P protein.**

Herpes simplex virus 1 (HSV-1) causes two kinds of infection. The first, exemplified most dramatically after first exposure to the virus, results in productive infection at the portal of entry of the virus into the body. In productive infection, approximately 80 genes are expressed, viral protein and DNA are made, viral progeny is assembled and, ultimately, the cell is destroyed. Latent infection takes place only in sensory neurons populated by viruses brought to that sites by retrograde transport along the axons from the portal of entry. In latently infected cells, viral DNA is maintained as an episome, and the only products detected to date are transcripts arising from two copies of a 8.5-kb domain of the DNA. Recombinants lacking sequences encoding the promoters and 5′ domains of these RNAs, however, are capable of establishing latent infections. These finding suggested that either as yet unknown viral gene products or cellular gene products are responsible for the establishment of the latent state (for a review, see ref. 1). Inasmuch as the genes expressed during productive infection encode numerous functions related not only to viral replication but also to increasing the pool of susceptible cells, to enabling more efficient spread of infection from cell to cell, and to ablating host response to infection (2), it seemed rather unlikely that establishment of latency, a vital function for the perpetuation of the virus in human populations, would solely rely on functions expressed by the host cell. Experiments designed to test the hypothesis that a set of genes mapping within the genome domain expressed during latency can be expressed, led to the discovery that an open reading frame (ORF), designated as ORF P is repressed during productive infection, but can be expressed under conditions under which either the repressor is not made or the site of binding of the repressor is ablated (3, 4). Specifically, ORF P is located in the inverted repeats flanking the unique long (U_L) sequences of HSV DNA antisense to a gene designated $\gamma_1$34.5 (see Fig. 1*A*). The transcription initiation site of ORF P coincides with the location of a high-affinity binding site of infected cell protein 4 (ICP4; ref. 4). This protein functions both as a transactivator and as a repressor (ref. 1 and references therein). The repression of ORF P is particularly strong as evidenced by the observation that the products of the ORF P are observed only under conditions in which ICP4 is not functional or its binding site at the transcription initiation site of the ORF P is ablated by mutagenesis (3, 4).

ORF P expressed by HSV-1 strain F is predicted to contain 248 amino acids. Inasmuch as it is almost entirely antisense to the $\gamma_1$34.5 gene, it contains a short amino-terminal domain, followed by 3 amino acids repeated 10 times, and a long carboxyl-terminal domain (see Fig. 1*B*). In Vero cells infected with viruses carrying a derepressed ORF P, the nascent protein designated ORF Pa is posttranslationally processed to slower migrating forms ORF Pb and ORF Pc and late in infection the protein appears to aggregate into dense structures that do not colocalize with the sites of viral DNA synthesis or late gene transcription. These observations led to the suggestion that the posttranslational processing of ORF P inactivates its function (4). In this report we show that in cells infected with a mutant carrying a derepressed ORF P, (*i*) ORF P protein interacts with and colocalizes with splicing factors, and (*ii*) the accumulation of ICP0 and ICP22, two regulatory proteins made from spliced mRNAs, is reduced early in infection but recovers late in infection, particularly in cells in which ORF P protein is posttranslationally modified.

MATERIALS AND METHODS

Cell Lines and Viruses. HeLa, SK-N-SH, and HEp-2 cell lines were obtained from the American Type Culture Collection. HSV-1(F) is the prototype HSV-1 strain used in this laboratory (5). In recombinant R7530, the ICP4 binding site at the transcription initiation site of ORF P has been ablated by base substitution mutagenesis (6).

Plasmids. pRB5105 contains the ORF P domain from amino acids 85–248 fused in-frame with the DNA-binding

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Abbreviations: HSV, herpes simplex virus; ORF, open reading frame; ICP, infected cell protein; GST, glutathione *S*-transferase; FITC, fluorescein isothiocyanate; SR protein, serine/arginine-rich protein.

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domain of GAL4 in pGBT9 (CLONTECH). pRB4966 contains the same ORF P domain fused in-frame with the glutathione *S*-transferase (GST) in pGEX4T-1 (Pharmacia).

Two-Hybrid System. pGBT9-ORFP was transformed into yeast strain HF7c (CLONTECH). Transformants were grown to large-scale (1 liter) and transformed with 0.5 mg of an Epstein–Barr virus-transformed human peripheral blood lymphocyte cDNA library in pACT (CLONTECH). Double transformants were selected in medium lacking tryptophan, leucine, and histidine. Colonies were restreaked on the same medium and then tested for LacZ activity by filter assay (as recommended by CLONTECH). LacZ-positive colonies were grown 24 hr in medium lacking leucine, and total yeast DNA was extracted and electroporated into *Escherichia coli* HB101. Bacterial transformants were selected on M9 minimal medium (7) containing ampicillin (50 μg/ml), proline (40 μg/ml), 1 mM thiamine, 0.4% glucose, and an amino acid mixture lacking leucine (as described in CLONTECH protocol). Minipreparations of plasmids were made by the alkaline lysis method (7) and retransformed into yeast alone or with various GAL4 DNA-binding domain fusion proteins, and LacZ activity was tested by filter assay.

Purification of the ORF P Antibody. The preparation of the antibody to the amino-terminal domain of ORF P has been described elsewhere (6). The anti-ORF P IgG was purified as follows: 0.5 ml of serum was loaded on a protein A column (ImmunoPure IgG purification kit, Pierce) and approximately 12 mg of IgG was recovered following the procedures recommended by the manufacturer. Of this amount, 3 mg was mixed with 3 mg of GST–ORF P fusion protein coupled to CNBr-activated Sepharose 4B (Pharmacia), and the mixture was incubated for 1.5 hr at 4°C. The beads were rinsed with 10 mM Tris·HCl (pH 7.5) and then with the same buffer but containing 0.5 M NaCl. The antibodies were eluted first with 100 mM glycine (pH 2.5) and then with 100 mM triethylamine (pH 11.5), dialyzed against PBS, and stored at −20°C.

RESULTS

ORF P Interacts with Components of the Cellular RNA Splicing Machinery. The domain of the ORF P gene encoding amino acids 85 to 248 (Fig. 1*B*) was cloned into pGBT9 and transformed into the yeast strain HF7c to yield HF7c/ORFP. Control experiments showed that the product of the chimeric gene did not transactivate the chromosomally located LacZ gene in yeast (data not shown). To screen for interacting proteins, HF7c/ORFP was transformed with a cDNA library from an Epstein–Barr virus-transformed human peripheral blood lymphocyte cell line and transformants were selected. Of approximately 700,000 transformants, 9 displayed strong expression of LacZ. The plasmids containing the cDNAs were isolated and retransformed with various fusion proteins. The results were that expression of LacZ could be detected only in presence of the ORF P fusion protein, but not in the presence of irrelevant chimeric genes (such as ICP22, p53, and lamin) or by itself (data not shown). The cDNAs were sequenced and all 9 were shown to match completely p32, a subunit of the essential non-small nuclear ribonucleoprotein splicing factor SF2/ASF (8, 9).

Of the various proteins forming the transcriptional machinery of the cells, we had available antibodies to SC35, a splicing factor belonging to the same superfamily of arginine/serine-rich splicing factors (SR proteins) as SF2/ASF (for a review, see ref. 10), and to the SM antigens, the protein components of the small nuclear ribonucleoproteins (11). To test the reactivity of the ORF P protein with splicing factors, a GST fusion protein to ORF P was made, reacted with cell extract from uninfected HeLa cells, and processed for detection of either SC35 or the SM splicing factors by immunoblot analysis. The ORF P–GST fusion specifically bound the B/B′ components of the SM antigens, whereas GST alone or an irrelevant fusion did not bind any of the SM proteins (Fig. 2). The same results were obtained with extracts of HSV-infected cells, except that the intensity of the B/B′ doublet was weaker possibly because of a general shut-off of host macromolecular synthesis induced by the HSV-1 infection. Since the SC35 antibodies did not react with denatured antigens, we could not test whether the GST–ORF P captured SC35 proteins. The results of the experiment with the anti-SM antibody suggest that ORF P also interacts with that factor.

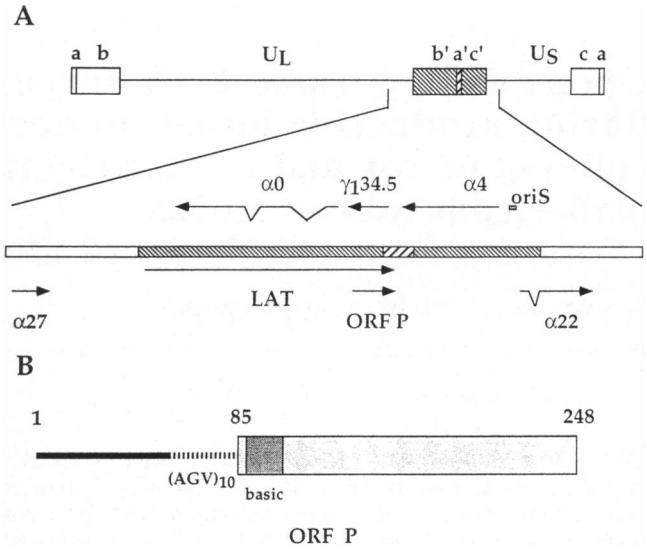

FIG. 1. Schematic representation of the sequence arrangement of the HSV-1 genome and of the location and polarity of the ORF P gene. (*A*) Upper line represents the HSV-1 genome. The thin lines represent the unique long (U_L) and unique short (U_S) sequences. The rectangles represent the inverted repeats sequeneces *ab* and *b'a'* flanking U_L and *a'c'* and *ca* flanking U_S. Lower line presents an expanded section of the internal inverted repeat and adjacent sequences. Shown are genes relevant for these studies. ORF P is almost completely antisense to the $\gamma_1$34.5 gene and maps in the *b* sequence of the inverted repeats. (*B*) The domains of the ORF P protein consisting of a amino-terminal domain (black line), 3 amino acids (Ala-Gly-Val) repeated 10 times (striped line), a short basic domain, followed by a longer carboxyl-terminal domain. The stippled box is the region used in the two-hybrid system.

ORF P and Splicing Factors Colocalize in the Nucleus of Infected Cells. The results described above suggest an interaction between ORF P and components of the splicing machinery. To further verify this interaction, cells were infected with a recombinant virus, R7530, in which the ICP4 binding site at the transcription start site of ORF P was mutagenized to derepress the gene (6). At 18 hr after infection, the cells were fixed, reacted with antibody, and examined by immunofluorescence for the localization of ORF P and the proteins SC35 and SM. The results were that ORF P colocalized with both SC35 and SM proteins (Fig. 3 and data not shown). A problem that emerged during the preliminary examination was that the amounts of Texas Red fluorescence indicative of ORF P protein was lower than that of the splicing factors. To ensure that fluorescence was not due to spillover, infected cells were reacted with anti-ORF P alone, anti-SC35 alone, or both antibodies. The infected cells were then photographed with the same settings for both Texas Red (ORF P) and fluorescein isothiocyanate (FITC) (SC35). As shown in Fig. 3, only the infected cells reacted with both antibodies showed yellow structures characteristic of colocalization of Texas Red- and FITC-conjugated antibodies.

Overexpression of ORF P Results in a Reduction in the Level of Expression of ICP0 and ICP22. ORF P maps in the genome domain transcribed during latency and is expressed

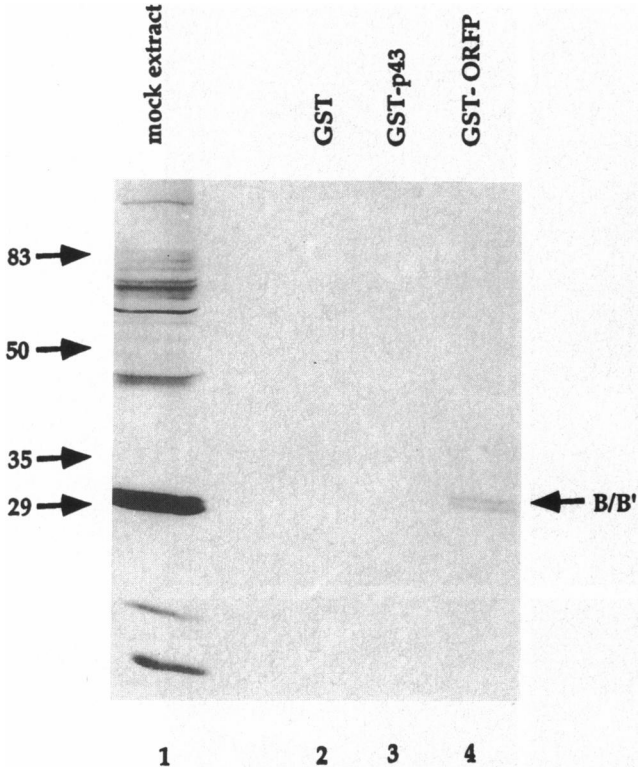

FIG. 2. Photographic image of cell proteins bound to GST fusion proteins, electrophoretically separated in denaturing gels, and reacted with a human sera to the Sm proteins. Recombinant pGEX vectors were transformed into *E. coli* BL21 and fusion proteins were isolated from a large-scale culture as recommended by the manufacturer (Pharmacia). HeLa cells from a 150-cm^2 flask were scraped into PBS, washed with PBS, and resuspended in 600 μl of PBS* (1% deoxycholate/1% Nonidet P-40/10 μm tolylsulfonyl phenylalanyl chloromethyl ketone/10 μm α-tosyl-L-lysine chloromethyl ketone in PBS. The cell extract (150 μl) was reacted with 2–5 μg of GST or GST–ORF P proteins bound to glutathione-agarose (Sigma) for 3–4 hr at 4°C. The beads were then collected by low-speed centrifugation, washed three times with 1 ml of PBS*, and resuspended in 60 μl of 2× disruption buffer (100 mM Tris·HCl, pH 6.8/200 mM dithiothreithol/4% sodium dodecyl sulfate/0.2% bromphenol blue/20% glycerol). Proteins were separated in 10% denaturing polyacrylamide gels, transferred to nitrocellulose, blocked, reacted with the human Sm antisera (ANA human serum 5, Centers for Disease Control, Atlanta) and then with a goat anti-human antibody conjugated to alkaline phosphatase, and processed as described by the manufacturer (Bio-Rad). Lanes: 1, cell extract from HeLa cells; 2–4, HeLa cell extract bound to GST, GST–p43 (an irrelevant fusion protein; unpublished data), and GST–ORF P, respectively. B/B′ refers to the position of the B (28 kDa) and B' (29 kDa) polypeptides. Molecular masses (in kDa) are shown on the left.

only in absence of functional ICP4. In the preceding section, we have shown that ORF P colocalizes and interact with cellular splicing factors. If ORF P plays a role in the establishment of the latent state, we could expect that it would function to down-regulate the expression of α regulatory proteins that are crucial for the initiation of productive infection. Of the six α genes (α0, α4, α22, α27, α47, and U_S1.5) (1, 12), three (α0, α22, and α47) yield spliced mRNAs (see Fig. 1*A*). The 5′ untranslated transcribed domain of α22 and α47 genes are identical and contain identical introns. It would therefore be expected that if accumulation of ORF P affects the synthesis of proteins encoded by spliced mRNAs, the effects would be directly demonstrable by examination of the levels of ICP0 and ICP22 in cells infected with wild-type and recombinant R7530 encoding derepressed ORF P genes. Since ICP27 and ICP4 arise from unspliced mRNAs, they would discriminate between an overall effect on protein synthesis and one that specifically affects the levels of accumulation of proteins encoded by spliced mRNAs.

The results of studies on HeLa and SK-N-SH cell lines are shown in Fig. 4. In this series of experiments, the cells were harvested at 8 and 18 hr after infection, solubilized, subjected to electrophoresis in denaturing polyacrylamide gels, transferred to a nitrocellulose sheet, and reacted with antibodies to ICP4, ICP0, ICP22, and ICP27. The results may be summarized as follows. In both HeLa and SK-N-SH cell lines at both 8 and 18 hr after infection with either virus, the levels of accumulated ICP4 and ICP27 were similar if not identical. At 8 hr after infection of HeLa cells with R7530, the amounts of ICP0 and ICP22 were reduced relative to those of wild-type infected cells. This difference was also apparent at 18 hr after infection. In SK-N-SH cells at 18 hr after infection with R7530, the levels of ICP0 were nearly identical to that of wild-type infected cells whereas the level of ICP22 was still reduced relative to that of wild-type infected cells. These results suggest that ORF P exerts its activity through the host splicing machinery.

The Extent of Posttranslational Modification of ORF P Varies Significantly in Different Cell Lines and Correlates with ORF P Activity. The results described so far suggest that the activity of ORF P is regulated differently in the two cell lines. In an earlier report (4), this laboratory showed that ORF P is extensively modified late in infection and suggested that these modifications could be a mechanism by which the activity of ORF P could be modulated. To test whether the phenotypes observed in the two different cell lines could be correlated to the extent of ORF P modification, lysates of infected HeLa or of SK-N-SH cells were solubilized, electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and probed with the anti-ORF P antibody. The results, Fig. 5, show that ORF P was barely detectable in HeLa cell extracts 8 hr after infection. At 18 hr after infection, only the unmodified form of ORF P (ORF Pa) was detectable in thes cells (see Fig. 5*A*, lane 4). In SK-N-SH cells only the nascent form of ORF P (ORF Pa) was detected 8 hr after infection. At 18 hr however, the lysates of SK-N-SH cells contained multiple forms of ORF P, including the slower migrating ORF Pb and ORF Pc (Fig. 5*B*, compare lanes 2 and 4). ORF P was not detected in lysates of cells infected with the wild-type virus (Fig. 5 *A*, lanes 1 and 3, and *B*, lanes 1 and 3).

DISCUSSION

ORF P is expressed under conditions in which ICP4, the major regulatory protein of HSV-1, is not present or not functional or if the gene is derepressed by mutagenesis of the ICP4 binding site (3, 4). ORF P expressed from a derepressed promoter during productive infection is extensively posttranslationally processed (4). ORF P therefore fits the prediction of a gene whose product, if expressed, could reduce the expression of α (immediate early) genes of HSV-1 and thereby enable the establishment of the latent state but be modified and rendered inactive after the switch from latent to productive infection (4, 15). The key observations made in our studies is that in an attempt to define the function of the ORF P protein, we have shown that (*i*) ORF P protein interacts in the two-hybrid system with p32, a component of the non-small nuclear ribonucleoprotein splicing factor SF2/ASF, (*ii*) GST–ORF P chimeric protein pulls down a protein from the unrelated SM antigens, and (*iii*) consistent with that observation, ORF P protein colocalizes with splicing factors in nuclei of infected cells, suggesting that it is a component of the spliceosomes. SF2/ASF belongs to the growing family of SR proteins (10). SR proteins are essential splicing factors and are also involved in the selection of alternative splice sites (16, 17). Interestingly, two SR proteins, SF2/ASF and SC35, have been shown to play a role in the repression of splicing of certain viral

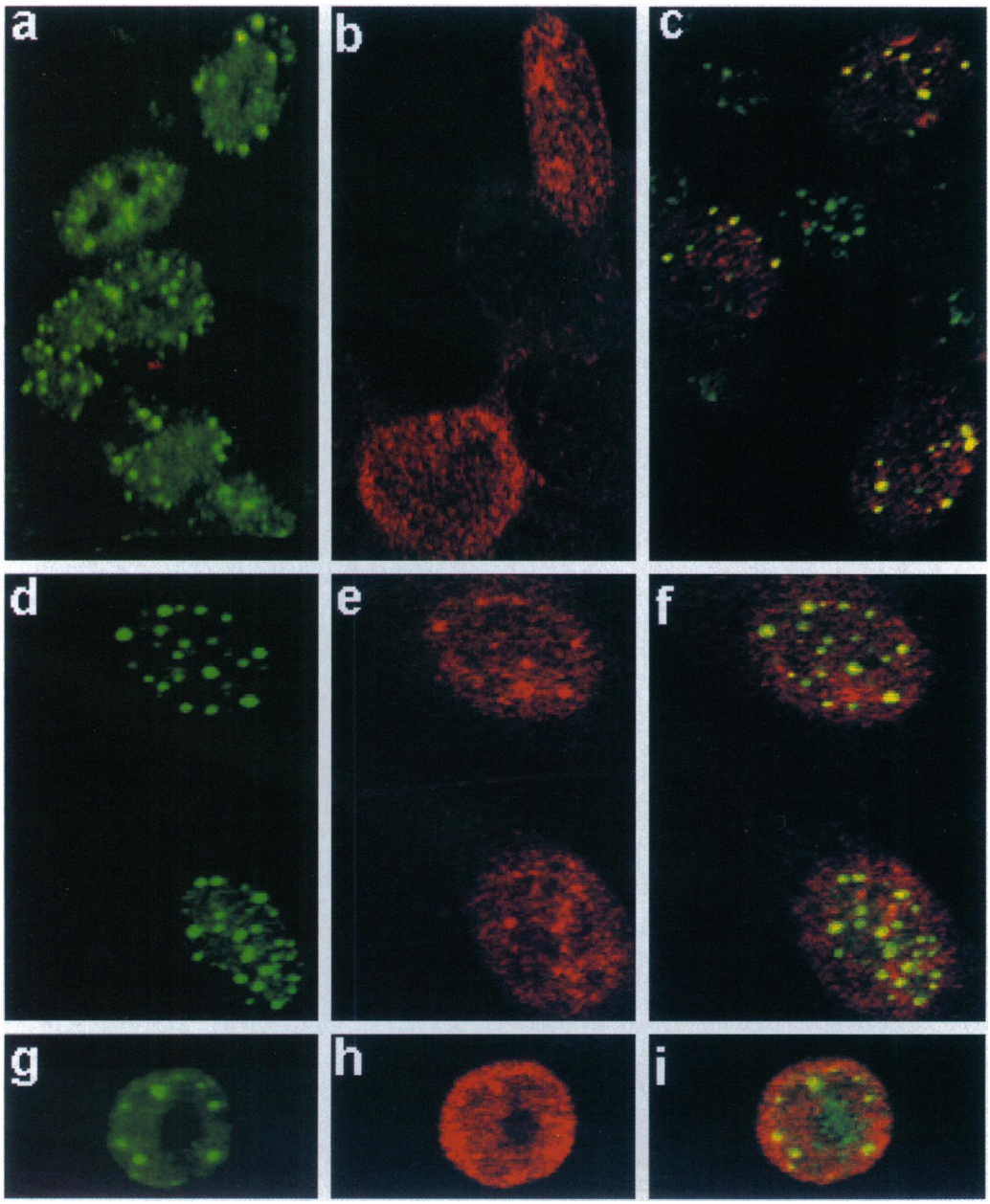

FIG. 3. Photomicrographs of Hep-2 (*a–c*), SK-N-SH (*d–f*), and HeLa cells (*g–i*) infected with virus R7530 and reacted with antibodies to SC35 alone (*a, d,* and *g*), to ORF P alone (*b, e,* and *h*), and to both ORF P and SC35 (*c, f,* and *i*). Confluent cells grown on wells on 1 × 3 inch slides (1 inch = 2.54 cm) were infected with approximately 10^6 plaque-forming units of virus, incubated at 37°C, and fixed in methanol for 20 min at −20°C. The cells were reacted for 30 min with PBS containing 20% normal human serum and 1% BSA at room temperature, rinsed once with PBS, and reacted overnight at 4°C with the primary antibody diluted in PBS containing 10% normal human serum and 1% BSA. The final dilutions were 1:40 for purified anti-ORF P antibody and 1:2000 for anti-SC35 antibody (Sigma). Cells were rinsed three times in PBS and reacted for 1 hr with goat anti-rabbit IgG conjugated to Texas Red (Molecular Probes) or goat anti-mouse IgG conjugated to FITC (Sigma), rinsed again three times with PBS, and mounted in PBS containing 90% glycerol and 1 mg of *p*-phenylenediamine per ml. The slides were examined under a Zeiss confocal fluorescence microscope, digitized images of the fluorescent-antibody-stained cells were acquired with software provided with the microscope and printed by a Tektronix 440 phaser printer. Single color images were acquired by excitation using an argon/krypton laser at 488 nm (FITC) or 568 nm (Texas Red). Double-stained images were obtained by acquiring a split image of both fluorochromes filtered by 515- to 540-nm band pass (FITC) and 590 nm long pass (Texas Red) filters and subsequent overlay of the two color images.

mRNAs (18, 19). Thus, the interaction of ORF P with SF2/ASF, or with SR proteins in general, may interfere with or disturb the activity of spliceosomes. HSV encodes only four genes known to yield spliced mRNAs. These include the three α genes α0, α22, and α47, as well as the late or γ gene U_L15. The introns of α22 and α47 are located within identical inverted repeats, and therefore, the splice donor and acceptor sites would be identical. If ORF P plays a role in regulation of viral gene expression, it could be expected that the effect of expression of ORF P during productive infection would be on genes whose mRNA is spliced. In this report we show that in cells infected with the mutant carrying the derepressed ORF P, the amounts of ICP22 and ICP0 are reduced relative to those accumulating in wild-type-virus-infected cells. We also show that the levels of ICP4 and ICP27, the products of α genes that yield nonspliced mRNAs, are unaffected by ORF P. Lastly, we show that late in infection, the levels of ICP22 and ICP0 recover and approach those of wild-type infected cells. The extent of recovery, however, correlates with the accumulation of posttranslationally modified ORF P protein.

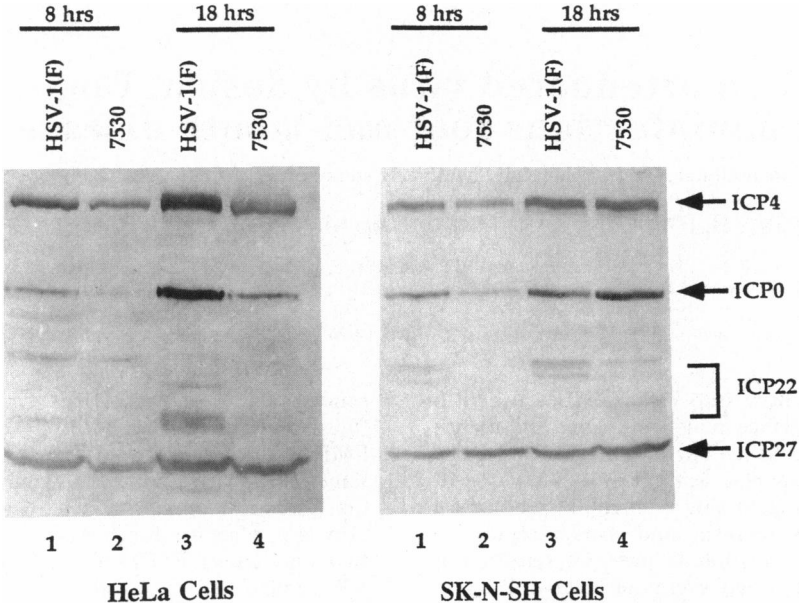

FIG. 4. Photographic image of infected cell proteins electrophoretically separated in denaturing gels and reacted with antibodies to α proteins. Cells grown in 25-cm^2 flasks were infected with 10 plaque-forming units of virus per cell, incubated for 8 or 18 hr at 37°C, scraped into PBS, and resuspended in 200 μl of 2× SDS-disruption buffer. Approximately 60–100 μl of the cell lysate was electrophoretically separated in 7% denaturing polyacrylamide gels cross-linked with bisacrylamide, electrically transferred to a nitrocellulose sheet, blocked in 5% skim milk in PBS, reacted with the indicated antibody and then with the secondary goat anti-mouse or anti-rabbit antibody conjugated to alkaline phosphatase, and processed for colorimetric detection with alkaline phosphatase. (*Left*) HeLa cells infected with either HSV-1(F) (lanes 1 and 3) or recombinant R7530 (lanes 2 and 4). (*Right*) SK-N-SH cells infected with either HSV-1(F) (lanes 1 and 3) or R7530 (lanes 2 and 4). The antibodies were a rabbit polyclonal antibody R77 to ICP22 and mouse monoclonal antibodies to ICP0 (1083), ICP4 (H640), and ICP27 (H1113) described by Ackermann *et al.* (13, 14).

The results of these studies support the hypothesis that ORF P can repress the expression of two regulatory genes, and by extension, of productive infection, a process essential for the establishment of latency. We should point out, however, that the direct role of ORF P protein in splicing of RNA remains to be shown and that ORF P protein has not been demonstrated as yet in sensory neurons harboring latent virus. We should also note that ORF P may not be the sole factor involved in establishment of latency inasmuch as viruses lacking ORF P are capable of establishing latent infections but at a reduced rate (G. Randall and B.R., unpublished results). An interesting property of HSV is that key functions such as entry into cells, regulation of gene expression, viral DNA synthesis, packaging of viral DNA into capsids, and egress from infected cells involve several gene products (1, 2). We may expect that additional genes, some as yet unknown, play a role in the establishment of latent infections.

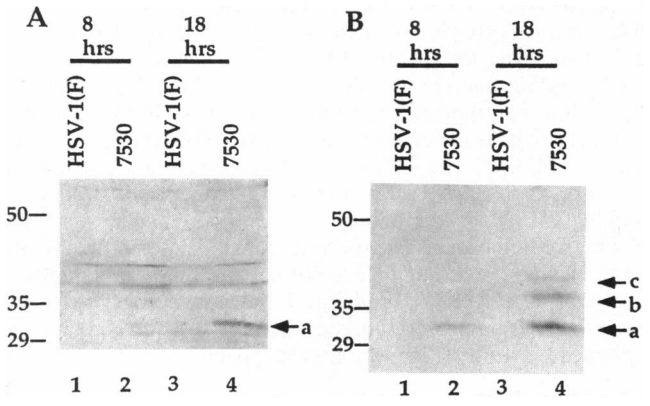

FIG. 5. Photographic image of infected cell proteins electrophoretically separated and reacted with an antibody to ORF P. HeLa cells (*A*) and SK-N-SH cells (*B*) were infected with HSV-1(F) (lanes 1 and 3) or with R7530 (lanes 2 and 4), harvested after 8 hr (lanes 1 and 2) and 18 hr (lanes 3 and 4), electrophoretically separated, transferred to nitrocellulose, and reacted with an antibody to ORF P. The different forms of ORF P are designated by the letters on the right. Numbers on the left are molecular mass markers (kDa).

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