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SHORT COMMUNICATIONS

Stable Induction of a 51K Cellular Protein in Neuronal Cells Surviving Herpes Simplex Virus Type 1 Infection

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A series of survivor cell lines derived by infection of B103 rat neuroma cells with active wild-type herpes simplex virus type 1 (HSV-1) (M. Levine, A. L. Goldin, and J. C. Glorioso, *J. Virol.* **35**, 203–210 (1980)) has been isolated. The survivor cells produced no infectious virus, yet they continued to react with HSV-1 antiserum for over 100 cell generations following the initial infection. The reactivity of the survivor cells with HSV-1 antiserum is characterized as being due to expression of a 51K protein. The 51K protein reacted with antiserum prepared against HSV-1 virions and was not detectable in the parental B103 cells. A protein of the same molecular weight was seen in productively infected B103 and HEL cells. The protein detected in the survivor cells comigrated with that seen in the infected cells on two-dimensional gel electrophoresis, indicating that they represent similar proteins. Despite the presence of the 51K protein reactive with HSV-1 antiserum, the survivor cells contain no detectable HSV-1 DNA sequences. They do contain DNA sequences which cross-hybridize with HSV-1 DNA, but similar cross-hybridizing sequences were also present in the parental B103 cells. No hybridizing polysomal, polyadenylated RNA species were present in the survivor cells that were not present in the parental B103 cells when probed with the cross-hybridizing HSV-1 restriction fragments. Therefore, the 51K protein evidently represents a cellular protein induced by the HSV-1 infection.

Herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2) interact uniquely with neuronal cells *in vivo* to result in latent infections (*1, 3, 2*). These latent infections have been demonstrated experimentally in mice, rabbits, and guinea pigs and represent the usual course of events in humans (*4, 3*). The mechanism by which a latent infection is maintained is unknown, but it has been demonstrated that the dormant virus is harbored in the neuronal cell bodies of sensory ganglia (*5, 3*).

We have been studying the interaction of HSV-1 with a rat neuroma cell line called B103 (*6*). B103 cells were nonpermissive for growth of the KOS strain of HSV-1 (*7*), and this nonpermissivity was most pronounced at elevated temperature and low multiplicity of infection (*8*). When B103 cells were infected with HSV-1 (KOS) under the least permissive conditions, about 10% of the cells survived the infection. These survivor cells ceased to produce any infectious virus by 14 days postinfection, and grew as well as the original B103 cell line. The survivor cells were distinguishable from the parental B103 cells, however, in that they reacted with HSV-1 antiserum as measured by immunofluorescence. This apparent hereditary change, plus, preliminary data suggesting complementation of superinfecting temperature-sensitive mutant vi-

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rus and the presence of DNA sequences which hybridize to viral DNA, led us to propose that at least a portion of the HSV-1 genome is harbored in these survivor cells (*8*).

To determine what specific proteins in the survivor cells were reactive with HSV-1 antiserum, the cell extracts were analyzed by the Western transfer technique (*9*). The survivor cell lines used in this experiment were an uncloned survivor cell population, KOS-survivor-1, and a cloned survivor cell line, KOS-Survivor-2.7, derived from KOS-Survivor-2. Cellular lysates prepared for protein analysis were electrophoresed in SDS–polyacrylamide gels and then electroblotted onto nitrocellulose. The resulting filters were reacted with antiserum prepared against HSV-1 virions, then treated with ^{125}I-labeled *Staphylococcus aureus* protein A and autoradiographed (Fig. 1). Neither uninfected B103 cells nor uninfected HEL cells contained any proteins reactive with the anti-HSV-1 antibody. Both survivor cell lines expressed a series of proteins that reacted with the HSV-1 antiserum. One predominant protein migrating with the apparent molecular weight of 51K was observed, along with three minor species between 46K and 49K. A protein that comigrated with the 51K protein was also present in all of the cell lines infected with HSV-1 including B103- and HEL-infected cells. A similar protein has not been observed in uninfected B103 cells, however, even when the autoradiograph was exposed for 2 weeks instead of the 4-day exposure shown in Fig. 1. As a control, preimmune serum failed to react with a 51K protein from either survivor or infected cells (data not shown).

To confirm that the 51K protein seen in infected cells was the same as that seen in survivor cells, lysates of infected and survivor cells were examined by two-dimensional gel electrophoresis followed by Western transfers. The lysates were electrophoresed in isoelectric focusing tube gels followed by a second-dimension SDS-polyacrylamide gel electrophoresis. The two-dimensional gels were then transferred to nitrocellulose and probed with HSV-1 antiserum as before (Fig. 2). The circled spots are ^{14}C-labeled protein markers included as points of reference.

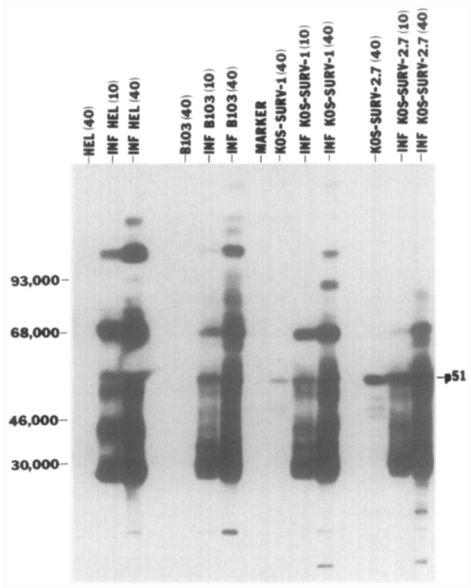

FIG. 1. Western transfer analysis of B103 and survivor cell proteins. Cellular lysates were electrophoresed in a 10% polyacrylamide–SDS gel, electroblotted onto nitrocellulose, probed with antiserum against HSV-1 virions (diluted 1:100) followed by addition of ^{125}I-labeled *S. aureus* protein A (2×10^5 dpm/ml) and autoradiographed as described previously (*27–29, 9*). The samples consisted of 40 μg of lysate from uninfected HEL, B103, KOS-Survivor-1, and KOS-Survivor-2.7 cells, and either 10 or 40 μg of lysate from each cell line infected with HSV-1 (KOS) at an m.o.i. = 5 for 24 hr at 37°. The markers consist of ^{14}C-labeled molecular-weight standards (New England Nuclear), and are too faint to be visible on this autoradiograph, which was exposed for 4 days. The location of the 51K protein is indicated (p51). Antiserum against HSV-1 (KOS) virions was prepared by hyperimmunization of New Zealand white rabbits as described previously (*33*). The extracellular virus released into the medium from 20 roller bottles of Vero cells infected with HSV-1 (KOS) at an m.o.i. of 0.1 were purified by differential centrifugation and sucrose gradient centrifugation. The purified virions (10^{10} PFU) were resuspended in 5 ml of PBS and uv irradiated at 12 ergs/mm^2/sec for 20 min. Aliquotes (0.25 ml each) were diluted 1:1 in complete Freund adjuvant and injected intramuscularly into the rabbits at 2-week intervals, for a total of eight injections, after which serum was collected. The serum was heated at 56° for 30 min to inactivate complement.

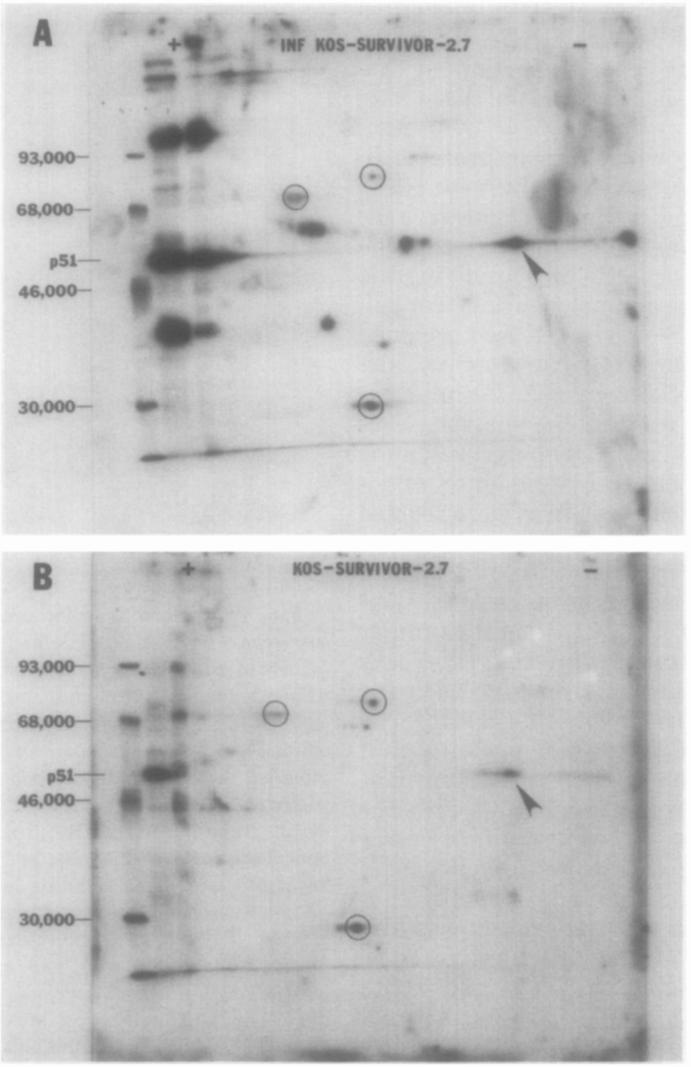

FIG. 2. Two-dimensional electrophoresis and Western transfer analysis of B103 and survivor cellular proteins. Lysates for two-dimensional gel electrophoresis were prepared as described by Haarr and Marsden (*30*). Two-dimensional gel electrophoresis was performed by modifications of the procedures of O'Farrell (*31*), and O'Farrell *et al.*, (*32*), as described by Haarr and Marsden (*30*). Lysates from each cell line were electrophoresed first in 3-mm tube isoelectric focusing gels and then in 10% polyacrylamide–SDS slab gels, electroblotted onto nitrocellulose, and probed with antiserum against HSV-1 virions followed by ^{125}I-labeled *S. aureus* protein A and autoradiography as described in the legend to Fig. 1. The samples consisted of (A) KOS-Survivor-2.7 cells infected with HSV-1 (KOS) at an m.o.i. = 5 for 24 hr at 37°, (B) uninfected KOS-Survivor-2.7 cells, (C) B103 cells infected with HSV-1 (KOS) at an m.o.i. = 5 for 24 hr at 37°, and (D) uninfected B103 cells. On the left are shown conventional one-dimensional SDS-polyacrylamide gel profiles of ^{14}C-labeled molecular-weight standards (New England Nuclear) and lysate in one-sixth the amount loaded onto the isoelectric focusing gels. The acidic end of the isoelectric focusing gel is at the left. The spots with circles around them are ^{14}C-labeled markers included as points of reference. The arrow points to the location of the 51K protein. These autoradiographs were exposed for 3 weeks.

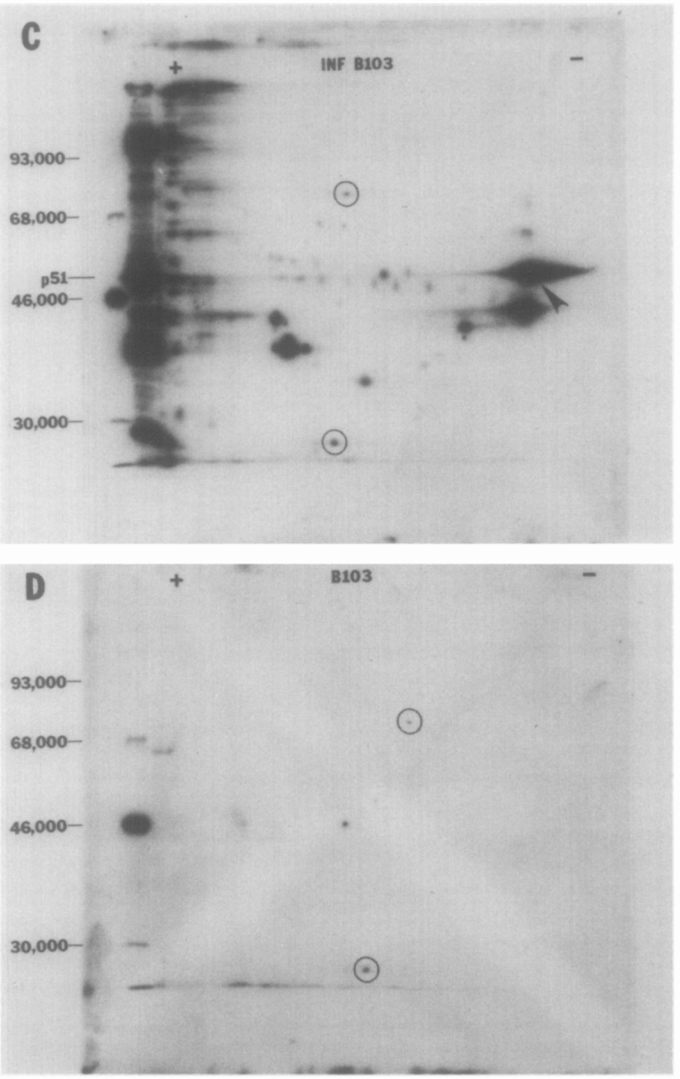

FIG. 2—*Continued.*

In the uninfected KOS-Survivor-2.7 cell pattern there is a spot migrating at 51K, indicated by the arrow in Fig. 2B. A more intense spot in a similar location can be seen in the infected KOS-Survivor-2.7 cell pattern (Fig. 2A), indicating that this protein, presumably the 51K protein detected in the one-dimensional Westerns, is induced during HSV-1 infection. Similar induction of a protein migrating to the same position can be seen in the infected B103 cell pattern (Fig. 2C). The greater number of spots present in the infected B103 cell lysate compared to the infected KOS-Survivor-2.7 cell lysate reflects the more productive infection of B103 versus survivor cells. As expected, no proteins reactive with the antiserum are apparent in the uninfected B103 cell pattern (Fig. 2D). Therefore, a 51K protein that is constitutively expressed in the survivor cells also appears to be induced by lytic HSV-1 infection in both the B103 and survivor cells.

This stable change in the survivor cells suggested that the survivor cells had ac-

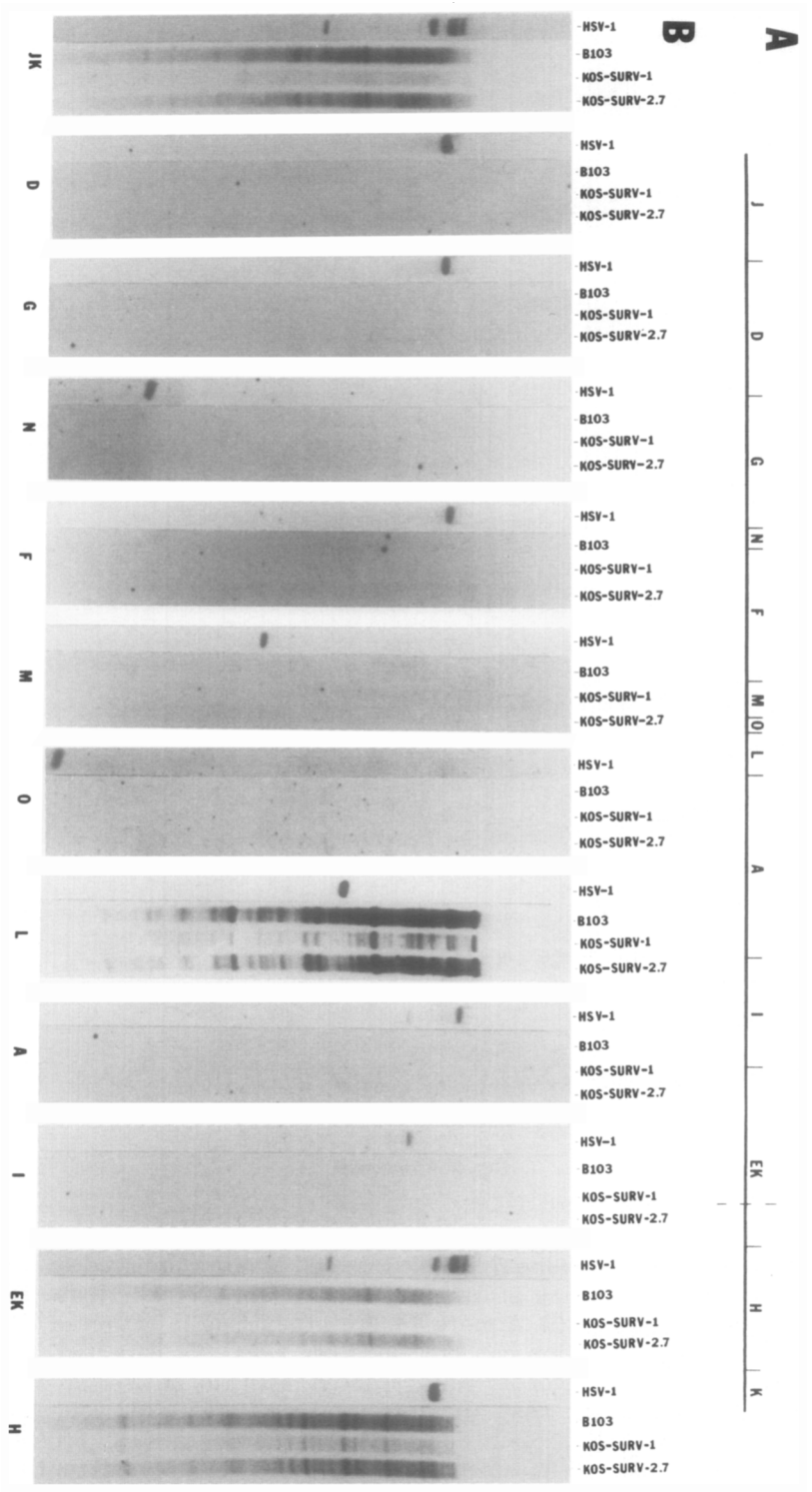

FIG. 3. Southern blot hybridizations of survivor cell DNA with cloned fragments spanning the HSV-1 genome. (A) *Eco*RI restriction map of HSV-1 (KOS) showing the restriction fragments which were used as hybridization probes. (B) Hybridizations of the restriction fragment probes to *Eco*RI-digested B103 and survivor cell DNA. Ten micrograms of DNA from B103, KOS-Survivor-1, and KOS-Survivor-2.7 cells were digested with *Eco*RI, electrophoresed in 1% agarose,

quired and were expressing HSV-1 DNA sequences. To test this, DNA from the two survivor cell lines was analyzed for the presence of HSV-1 DNA sequences by Southern transfer hybridizations. Twelve cloned *Eco*RI restriction fragments representing the entire HSV-1 genome (*10*), were used as hybridization probes (Fig. 3A). The terminal fragments E, J, and K were not used for hybridization since they were represented in the joint fragments EK and JK. B103 and survivor cell DNA were digested with *Eco*RI, fractionated in 1% agarose gels, and transferred to nitrocellulose. As a control, unlabeled HSV-1 DNA was added to *Escherichia coli* DNA at a level equivalent to one copy per haploid mammalian cell genome. Figure 3B shows the results of hybridizations of parental B103, KOS-Survivor-1, and KOS-Survivor-2.7 DNA with gel-purified inserts from the 12 cloned probes. The only fragments that hybridized with the survivor cell DNA were EK, JK, H, and L. These four fragments also hybridized to a similar extent with B103 parental cell DNA (Fig. 3B). These four restriction fragments of HSV-1 contain sequences which hybridize to mammalian DNA from a variety of species (*11*, *12*, unpublished results). No additional hybridization bands other than those representing the cross-hybridization cellular sequences were detected. A similar analysis was performed using four additional survivor cell lines which reacted with HSV-1 antiserum and in which p51 was detected (data not shown). Results similar to those described in Fig. 3B were obtained. No HSV-1 DNA sequences were detected in any of these survivor cell lines.

Although the survivor cells did not appear to contain any newly acquired HSV-1 DNA sequences, they did contain DNA sequences which cross-hybridized to HSV-1. The complex hybridization patterns of these sequences made it difficult to rule out the possibility that some hybridization bands representing acquired HSV-1 DNA might be present. Since any HSV-1 sequences responsible for the reactivity of the survivor cells with HSV-1 antiserum would be transcribed into messenger RNA, presumably polyadenylated, it should be possible to detect the RNA transcripts of those sequences if they were present in the survivor cells. Therefore, polysomal oligo(dt) purified RNA was isolated from B103 and KOS-Survivor-2.7 cells and analyzed by Northern transfer hybridizations. The RNA was glyoxalated, fractionated in 1% agarose gels, transferred to Gene Screen (New England Nuclear Corp.), and hybridized to the *Eco*RI fragments EK, JK, H, and L. No hybridizing transcripts unique to the survivor cells were observed with any of the four HSV-1 probes (data not shown). This indicates that the survivor cells are not expressing any HSV-1 DNA sequences from these regions of the viral genome.

The sensitivity of the Southern hybridizations probing for HSV-1 sequences in the survivor cells depends upon the copy number of the sequences in the cells and the size of the hybridization probes. The analysis was performed on a cloned survivor line, KOS-Survivor-2.7, so that any HSV-1 sequences that were acquired would be present in every cell, equivalent to a level of at least one copy per cell. The largest hybridization probe employed in the analysis of the survivor cells was *Eco*RI fragment A, a 21-kb fragment. In previous hybridizations using a DNA probe of similar size we were able to detect a single-copy fragment at least 10% of the size of the hybridization probe (*13*). This level of sensitivity was demonstrated to be at least 6% the size of the probe in other experiments (unpublished results). Therefore, the *Eco*RI A fragment should

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and transferred to nitrocellulose as described previously (*13*). Also included was a positive control, indicated as HSV-1, which consisted of unlabeled HSV-1 DNA added to *E. coli* DNA at a level equivalent to one copy per haploid mammalian cell genome (2×10^{-4} $\mu g/\mu g$). The 12 HSV-1 *Eco*RI fragments contained in the previously described chimeric plasmids (*10*), were gel purified (*13*), ^{32}P-labeled by nick translation (*10*), and used as hybridization probes. Each probe is indicated below the corresponding filter.

detect an HSV-1 DNA fragment of about 1.3 kb present as a single copy in the survivor cells. The other hybridization probes should have detected correspondingly smaller HSV-1 fragments. Although further hybridizations with smaller probes would be necessary to completely exclude the possibility of retained HSV-1 DNA in the survivor cells, it does seem unlikely that KOS-Survivor-2.7 cells contain HSV-1 DNA fragments larger than about 1.3 kb. A protein of 51K molecular weight would consist of approximately 460 amino acids, requiring a coding capacity of 1380 nucleotide pairs. Although not rigorously proven, it is unlikely that an HSV-1 DNA fragment this large is present in the KOS-Survivor-2.7 cells. Consistent with this, no hybridizing polysomal, polyadenylated RNA species were present in the survivor cells that were not also present in the parental B103 cells when probed with the cross-hybridizing HSV-1 restriction fragments.

In a previous publication (*8*), we interpreted the appearance of the new protein phenotype in the survivor cells as due to the persistence of HSV-1 genes in these cells. This interpretation is clearly at variance with the findings reported here. Preliminary findings suggested that the survivors could complement temperature-sensitive mutants of HSV-1 (KOS) and contained sequences hybridizing to HSV-1 DNA. The complementation indices observed were more than 2 but less than 10, the minimum now accepted as indicating complementation. The preliminary hybridization experiments were undoubtedly confused by the hybridization effects reported in this paper.

A 51K protein, which appears to comigrate with the 51K protein of survivor cells, is observed in productively infected B103 cells and survivor cells. These proteins migrate to the same position in two-dimensional gel electrophoresis followed by Western transfers, indicating a similarity between them. These results suggest that the 51K protein is a cellular protein that has been induced by the initial HSV-1 infection and is stably synthesized in the survivor cells. There are at least two explanations why such a cellular protein might react with antiserum prepared against HSV-1 virions. First, HSV-1 virions acquire a membranous envelope by budding through virus-modified patches of the nuclear membrane (*14, 15*), so that cellular membrane proteins are present in the viral envelope (*16*). If the 51K protein represents a cell-specific membrane protein induced by HSV-1 infection it would likely be contained in the viral envelope and, consequently, be present in the HSV-1 antigen preparation. The second explanation is that the inoculations of virus particles into the rabbit could have resulted in a low level HSV-1 infection. As detailed in the legend to Fig. 1, purified virions were uv irradiated, then aliquots containing the equivalent of 5×10^8 plaque-forming units (PFU) were injected intramuscularly. Although no infectious virus was detected in this uv-irradiated preparation, sufficient infectious virus may have been injected to cause a low-level HSV-1 infection, even though the rabbit was not noticeably ill. The rabbit would then be likely to produce antibodies to any cellular proteins induced during the HSV-1 infection.

SV40 and adenovirus induce a 53K cellular protein during transformation (*17–19*). This protein, termed p53, has also been detected in spontaneous transformants (*20, 21*) and in cells transformed by chemical mutagenesis (*22*). p53 may be a cell-cycle, transformation-specific protein (*23*). Epstein-Barr virus induces a cellular protein of 53K (*24, 25*), but this protein is not the same p53 induced by SV40 and adenovirus (*26*). The 51K protein induced by HSV-1 in the survivor cells is not related antigenically to the SV40-induced p53. No immunoreactive protein in the molecular-weight range of 50,000 to 55,000 was detectable on Western transfers of lysates from either of two SV40-transformed cell lines, using the HSV-1 antiserum (data not shown). There are no data concerning the relationship of the 51K protein to the Epstein-Barr virus-induced p53. The function of that p53 is not known, but it also appears to be related to transformation, as the pro-

tein is present in Epstein-Barr virus-transformed cells and binds Epstein-Barr virus nuclear antigen (*25*). The HSV-1 induced 51K protein was not found in one HSV-1 transformed cell line (data not shown). Additional HSV-1 transformants will have to be tested to confirm its absence.

In conclusion, we have demonstrated that B103 cells which survived HSV-1 infection express a 51K cellular protein that reacts with HSV-1 antiserum. This protein was not detected in uninfected B103 cells, but was induced during lytic HSV-1 infection of those cells and was stably induced in the survivor cell lines. No function has yet been demonstrated for the 51K protein, but it does not appear to be related to transformation by HSV-1. Further studies will be required to determine the function and significance of the 51K protein induced by HSV-1 in these neuronal survivor cell lines.

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