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Identification of a Virus-Specific Polypeptide Associated with a Transforming Fragment (*BglII*-N) of Herpes Simplex Virus Type 2 DNA

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The *BglII* N fragment of herpes simplex virus type 2 (HSV-2) DNA (approximately 0.58 to 0.63 map unit) was examined for encoded products. Using plasmid pGZ59, which consists of *BglII*-N cloned in pAT153, in conjunction with hybrid-arrested translation, mRNA selection, and in vitro protein synthesis, we found that the major translated product of this region has an approximate molecular weight of 37,800. By further mapping, coding sequences for this polypeptide were located within the region of *BglII*-N representing approximately 0.58 to 0.61 genome map unit. To demonstrate immunological specificity, we used staphylococcal A protein immunoprecipitation with rabbit anti-HSV-1 or HSV-2 sera and antigens from HSV-1 or HSV-2 total mRNA translated in vitro and *BglII*-N-selected mRNA. The results show that the 37,800-dalton polypeptide has HSV-2 immunological specificity, as it is precipitated with anti-HSV-2 sera but not with anti-HSV-1 or control sera.

Inactivated herpes simplex virus type 2 (HSV-2) (2) and DNA from HSV-2 (6, 20) have been used successfully for the oncogenic transformation of cells in vitro. Recent studies, using specific restriction endonuclease-generated fragments of the HSV-2 genome, have identified two different regions of HSV-2 DNA that can transform cells in vitro (4, 7, 16). One region, referred to as *BglII*-N, is contained within the genome map coordinates 0.58 and 0.63 (1). Cells transformed by *BglII*-N form colonies in soft agar (16), contain viral DNA (16), and form tumors when injected into animals (4). Support for the association of this fragment with viral transformation has been provided by studies which used ^{32}P-labeled HSV-2 DNA fragments to demonstrate that the *BglII* N fragment was present in hamster cell lines transformed by inactivated HSV-2 (3). In view of this association between *BglII*-N and oncogenic transformation and because polypeptides specified by this fragment have not been identified, we examined the *BglII* N fragment for encoded products.

We made use of a hybrid plasmid containing the *BglII* N fragment of HSV-2 DNA to assist in the identification of polypeptides encoded by this DNA segment. This plasmid was used in conjunction with hybrid-arrested translation (12), mRNA selection (17), and immunoprecipitation (8); our results demonstrate that the major translated product from this DNA fragment has an approximate molecular weight of 37,800 and exhibits HSV-2 immunological specificity.

MATERIALS AND METHODS

Cells and virus. HSV-1 strain 17 and HSV-2 strain HG52 were grown and titrated in BHK-21 clone 13 cells (10).

RNA extraction. Total cytoplasmic RNA was extracted from BS-C-1 cells 6 h after infection as described previously (14).

Radiolabeling of infected cell polypeptides. Infected or control cell monolayers were washed with phosphate-buffered saline and pulse-labeled with phosphate-buffered saline containing 500 μCi of [^{35}S]methionine (Radiochemical Centre, Amersham, England) per ml. Cells were harvested and prepared for electrophoresis as described previously (11, 14).

Plasmid DNA. The four hybrid plasmids used in this study were constructed and identified by A. Davidson and I. Cameron (Medical Research Council Virology Unit, Glasgow, Scotland). Plasmid pGZ59 contains HSV-2 *BglII*-N inserted into the *BamHI* site of pAT153 (19). pGZ26 contains HSV-2 *HindIII*-A cloned in pAT153, and pGZ62 was prepared by cleavage of pGZ26 with *BglII*, followed by ligation of the fragment consisting of pAT153 plus the regions of *BglII*-C and *BglII*-H contained within *HindIII*-A. Plasmid pGZ15 contains HSV-2 *HindIII*-H cloned in pAT153. The *HindIII* and *BglII* maps of HSV-2 DNA are shown in Fig. 1, and the structures of plasmids pGZ26, pGZ59 and pGZ62 are illustrated in Fig. 4.

Isolation of DNA fragments generated by restriction endonuclease cleavage of pGZ59 was performed as described by Preston and McGeoch (15).

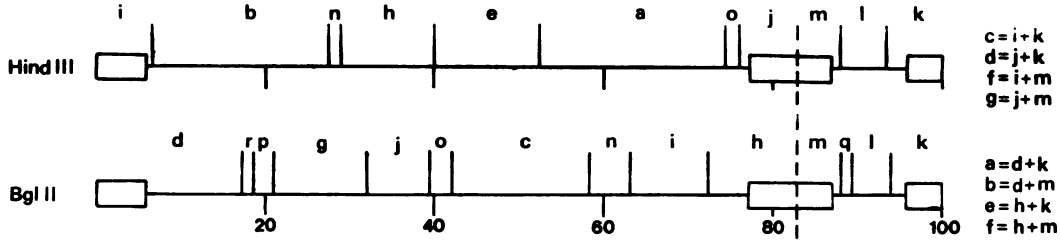

FIG. 1. *Cleavage maps of HSV-2 (strain HG52) for restriction endonucleases HindIII and BglII. The data are taken from Cortini and Wilkie (1).*

Hybrid-arrested translation. The method of Paterson et al. (12), modified for HSV DNA fragments by Preston and McGeoch (15), was used for hybrid-arrested translation. Twelve micrograms of *Eco*RI-restricted plasmid DNA, or an equivalent amount of isolated fragment, was denatured by incubation at 95°C for 5 min in 170 μl of 94% formamide containing 60 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid], pH 7.4. A 14-μl amount of cytoplasmic RNA (4 mg/ml), followed by 16 μl of 5 M NaCl, was added, and the reaction mixture was incubated at 58°C for 1 h. Polyadenylated RNA was selected on a small oligodeoxythymidylic acid cellulose column after the addition of 1 ml of HSB (500 mM KCl; 20 mM Tris-hydrochloride, pH 7.5; 1 mM EDTA). Polyadenylated RNA was ethanol precipitated with 16 μg of *Escherichia coli* rRNA, dissolved in deionized water, and used for in vitro translation either directly or after denaturation by heating at 95°C for 1.5 min.

mRNA selection. Hybrid plasmid DNA (100 μg) was immobilized on nitrocellulose filters (Schleicher & Scheull Co.) and used for mRNA selection as described by Ricciardi et al. (17), modified for HSV mRNA by Preston and McGeoch (15). Hybridization-selected mRNA fractions were purified by oligodeoxythymidylic acid cellulose chromatography before translation in vitro.

In vitro protein synthesis. Samples of RNA were translated in a micrococcal nuclease-treated fractionated reticulocyte cell-free system (13, 14).

Polyacrylamide gel electrophoresis. Protein samples were resolved on sodium dodecyl sulfate–6 to 15% polyacrylamide gradient gels (11). Radiolabeled proteins were either visualized by autoradiography on Kodirex film or processed for fluorography (9) and exposed to Kodak XH1 film.

Rabbit sera. Immune sera were prepared by injecting New Zealand white rabbits with primary rabbit kidney cells infected with HSV-1 or HSV-2 and emulsified in Freund complete adjuvant. The animals were test bled periodically and boosted before exsanguination.

Preimmune serum was obtained from each animal before immunization.

Immunoprecipitation. The *Staphylococcus aureus* Cowan I strain (SPA) was used as an immunoadsorbent to isolate antigen-antibody complexes of radiolabeled viral proteins and rabbit anti-HSV-1 or anti-HSV-2 sera (5, 8). The immune complexes were dissociated in solubilization buffer, and radiolabeled viral proteins were resolved by gradient polyacrylamide gel electrophoresis and visualized by fluorography (5).

Polypeptide nomenclature. Polypeptides synthesized in vitro and in vivo are classified according to their apparent weights in thousands and prefixed with "VI" or "Vmw," respectively.

Biological containment. All manipulations with bacteria containing plasmids with HSV-2 inserts were performed in a category II laboratory as recommended by the Genetic Manipulation Advisory Group.

RESULTS

HSV-2-induced polypeptides synthesized in vitro. The RNA preparation used for the experiments described here was extracted from BS-C-1 cells infected with HSV-2 strain HG52. Characteristic electrophoretic mobilities of HSV-2-induced polypeptides synthesized in vitro have been described previously (14), but those experiments used RNA from BHK-21-infected cells and were performed without the use of micrococcal nuclease to reduce endogenous protein synthesis in the reticulocyte cell-free system. Therefore, in initial studies polypeptides synthesized in vivo in BS-C-1 cells were compared with translation products of HSV-2-infected BS-C-1 cell RNA (Fig. 2).

A comparison of polypeptides synthesized by mock-infected and HSV-2-infected BS-C-1 cells showed the presence of 11 major virus-induced polypeptides which corresponded to species described previously (11). Eleven virus-induced polypeptides were also detected in in vitro translation products, seven of which comigrated with infected cell species synthesized in vivo (Fig. 2). The remaining four (VI 108, VI 78, VI 64, and VI 48) presumably represented precursors which were post-translationally modified in infected cells but not in the reticulocyte cell-free system.

Identification of polypeptides encoded by HSV-2 *Bgl*II-N. The specificity of mRNA's homologous to the *Bgl*II N fragment was determined by hybrid-arrested translation or mRNA selection with pGZ59 (Fig. 3). The first of these techniques relies on the inability of duplexed mRNA to be translated in vitro after hybridiza-

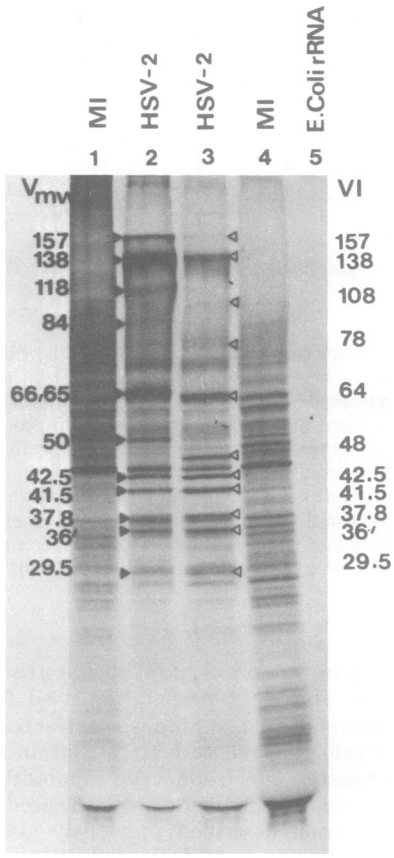

FIG. 2. *HSV-2 polypeptides synthesized in vivo and in vitro. Mock-infected (MI) or infected BS-C-1 cell polypeptides synthesized in vivo (lanes 1 and 2) or in vitro (lanes 3 and 4) are compared. Endogenous products of the cell-free system, synthesized after the addition of E. coli rRNA, are shown in lane 5. Virus-induced polypeptides synthesized in vivo (Vmw, ▶) and in vitro (VI, ◁) are labeled according to their apparent molecular weights in thousands (11, 14).*

tion to plasmid DNA. Two important controls for this technique are demonstration of recovery of mRNA activity upon denaturation of hybrids (14) and the use of unrelated DNA, or no DNA, since some HSV mRNA's are inactivated by mock hybridization (15).

Translation of RNA after hybridization to pGZ59 resulted in a reduction or inhibition of synthesis of polypeptides VI 41.5 and VI 37.8 (Fig. 3, lane 4). Their activities returned after denaturation of hybrids (Fig. 3, lane 5); therefore, these are candidates for *Bgl*II-N-specified polypeptides. When mock hybridization with no DNA was performed, however, synthesis of VI 41.5 was similarly reduced (Fig. 3, lanes 8 and 9), although no effect on VI 37.8 was noted. This suggests that the arrest of VI 41.5 synthesis was not due to *Bgl*II-N sequences. Plasmid pGZ15, which contains the HSV-2 *Hin*dIII H fragment (0.29 to 0.40 map unit), was used as a further control. The expected inhibition of synthesis of polypeptides VI 42.5 and VI 41.5, which has previously been mapped in this region (11), occurred (Fig. 3, lanes 6 and 7), but again no effect on VI 37.8 was observed.

Translation of *Bgl*II-N-specific RNAs selected by hybridization to nitrocellulose filters loaded with pGZ59 DNA is shown in Fig. 3, lane 3. The major product, apart from two endogenous reticulocyte bands (approximate molecular weights, 49,000 and 110,000), had an apparent molecular weight of about 37,800. Another minor *Bgl*II-N-specified band had an apparent molecular weight of 15,000, although size estimates in this region of the gel are unreliable due to overloading with globin (Fig. 3, lane 3). These two polypeptides were not observed when control pAT153 DNA-containing filters were used for mRNA selection (data not shown).

Therefore, the two mapping techniques, in

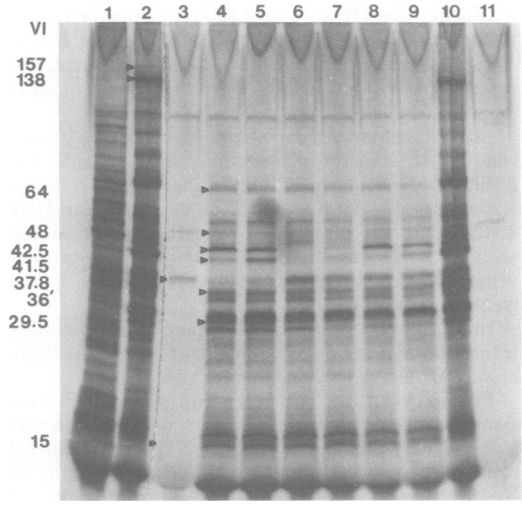

FIG. 3. *Hybrid-arrested translation and mRNA selection studies, using HSV-2 mRNA with pGZ59 DNA or pGZ15 DNA. The fluorogram shows proteins synthesized in vitro, using a fractionated reticulocyte system with the following additions: (1) mock-infected cell RNA; (2) HSV-2-infected cell RNA; (3) pGZ59-selected HSV-2 mRNA; (4) HSV-2 mRNA hybridized with pGZ59 DNA; (5) as (4) but denatured before translation; (6) HSV-2 mRNA hybridized with pGZ15 DNA; (7) as (6) but denatured before translation; (8) HSV-2 mRNA under hybridization conditions but no DNA present; (9) as (8) but denatured before translation; (10) as (2); (11) endogenous products of the translation system after the addition of E. coli rRNA. Numbers in the left margin are apparent molecular weights* $\times 10^{-3}$.

which different hybridization conditions are used, agree that the major mRNA homologous to *Bgl*II-N, in the cytoplasmic RNA preparation used, encoded a polypeptide which comigrates with Vmw 37.8 found in infected cells.

A more precise location of coding sequences for VI 37.8 was determined with restriction endonuclease fragments of pGZ59 and clone pGZ62, which contains sequences adjacent to *Bgl*II-N, in hybrid-arrested translation (Fig. 4).

Cleavage of pGZ59 with restriction enzyme *Hin*dIII plus *Bam*HI generated three bands upon electrophoresis. The largest of these contained pAT153 sequences plus *Bgl*II-N sequences representing 0.61 to 0.63 map unit on the HSV-2 genome (Fig. 4, solid lines). The smaller bands collectively contained *Bgl*II-N-derived sequences from 0.58 to 0.61 HSV-2 map unit plus a part of pAT153 (Fig. 4, broken lines). Hybrid-arrested translation revealed that the combined lower two bands arrested the synthesis of VI 37.8, whereas the upper band did not (Fig. 4, lanes 8 to 11). Plasmid pGZ62, containing the portion of *Bgl*II-C adjacent to *Bgl*II-N (approximately 0.53 to 0.58 map unit), failed to prevent the synthesis of VI 37.8 in hybrid-arrested translation (Fig. 4, lanes 6 and 7). Sequences essential for the synthesis of VI 37.8 in vitro, therefore, appear to lie exclusively within the portion of *Bgl*II-N representing 0.58 to 0.61 map unit on the HSV-2 genome.

Figure 4 (lanes 4 to 9) also shows some arrest of polypeptide VI 138 by plasmids pGZ59 and pGZ62 and the <0.58- to 0.61-map-unit region of *Bgl*II-N. A reduction in the intensity of VI 30 was noted in Fig. 3, lanes 4 and 5, and Fig. 4, lanes 4 and 5.

Immunological specificity of polypeptide VI 37.8. Having established that sequences encoding VI 37.8 lie within the *Bgl*II N fragment, we designed the next series of experiments to investigate the immunological specificity of this polypeptide.

Mock-, HSV-1-, and HSV-2-infected cell cytoplasmic RNA was translated in vitro and reacted with rabbit anti-HSV-2 serum. Immune complexes were isolated with SPA, and radiolabeled antigens were resolved by gradient polyacrylamide gel electrophoresis and visualized by fluorography. The results demonstrated that the anti-HSV-2 serum readily precipitated most of the HSV-2 polypeptides synthesized in vitro (Fig. 5, lane 4) and also cross-reacted with many of the HSV-1 polypepties synthesized in vitro (Fig. 5, lane 6). This serum did not, however, precipitate any of the polypeptides synthesized with RNA from mock-infected cells (Fig. 5, lane 7). When the anti-HSV-2 serum was reacted with products of pGZ59-selected RNA, VI 37.8 was the only polypeptide detectably precipitated (Fig. 5, lane 5). Preimmune serum did not precipitate VI 37.8 synthesized from pGZ59-selected or total cytoplasmic RNA (data not shown).

Although the above studies suggested that the polypeptide was virus specific, they did not indicate whether VI 37.8 shared cross-reacting antigenic determinants between HSV-1 and HSV-2. Therefore, viral proteins were synthesized in vitro from pGZ59-selected mRNA and cytoplasmic RNA from HSV-1- or HSV-2-infected cells. These proteins were incubated with anti-HSV-1 or anti-HSV-2 serum, and the reaction was monitored by the SPA-polyacrylamide gel electrophoresis-fluorography system as described above. When anti-HSV-2 serum was reacted with VI 37.8 synthesized from pGZ59-selected mRNA, the result was a positive precipitation (Fig. 6, lane 5). When this polypeptide was reacted with rabbit anti-HSV-1 serum, no precipitation was observed (Fig. 6, lane 4). These studies were continued with proteins synthesized in vitro from total HSV-1 or HSV-2 cytoplasmic mRNA as antigens. When these preparations were reacted with their homologous antibodies, most of the polypeptides were precipitated efficiently (Fig. 6, lanes 2 and 6). When HSV-1 antigens were reacted with HSV-2 antibody or HSV-2 antigens were reacted with HSV-1 antibody, extensive cross-reactivity was observed with many of the antigens precipitated by heterologous serum (Fig. 6, lanes 3 and 7). The most notable difference in these results was that anti-HSV-1 serum did not precipitate the 37,800-dalton polypeptide (Fig. 6, lane 3), which is consistent with the lack of precipitation seen when this antiserum was reacted with pGZ59-selected mRNA products (Fig. 6, lane 4). However, the 37,800-dalton polypeptide, plus others, was effectively precipitated by anti-HSV-2 serum (Fig. 6, lane 6). Proteins synthesized from cytoplasmic RNA of mock-infected cells were not precipitated by either anti-HSV-1 or anti-HSV-2 sera (Fig. 6, lanes 9 and 10). These studies were repeated with anti-HSV-1 sera from two additional rabbits and anti-HSV-2 sera from two additional rabbits with the same results.

DISCUSSION

In this study, the *Bgl*II N fragment of HSV-2 DNA was examined for encoded polypeptides by using the techniques of hybrid-arrested translation and mRNA selection. Our results demonstrated that the information for one polypeptide, VI 37.8, is contained within this DNA fragment and under our experimental conditions represented the major translatable mRNA from this region of the genome. A second polypeptide, VI 15, was observed in mRNA selection studies

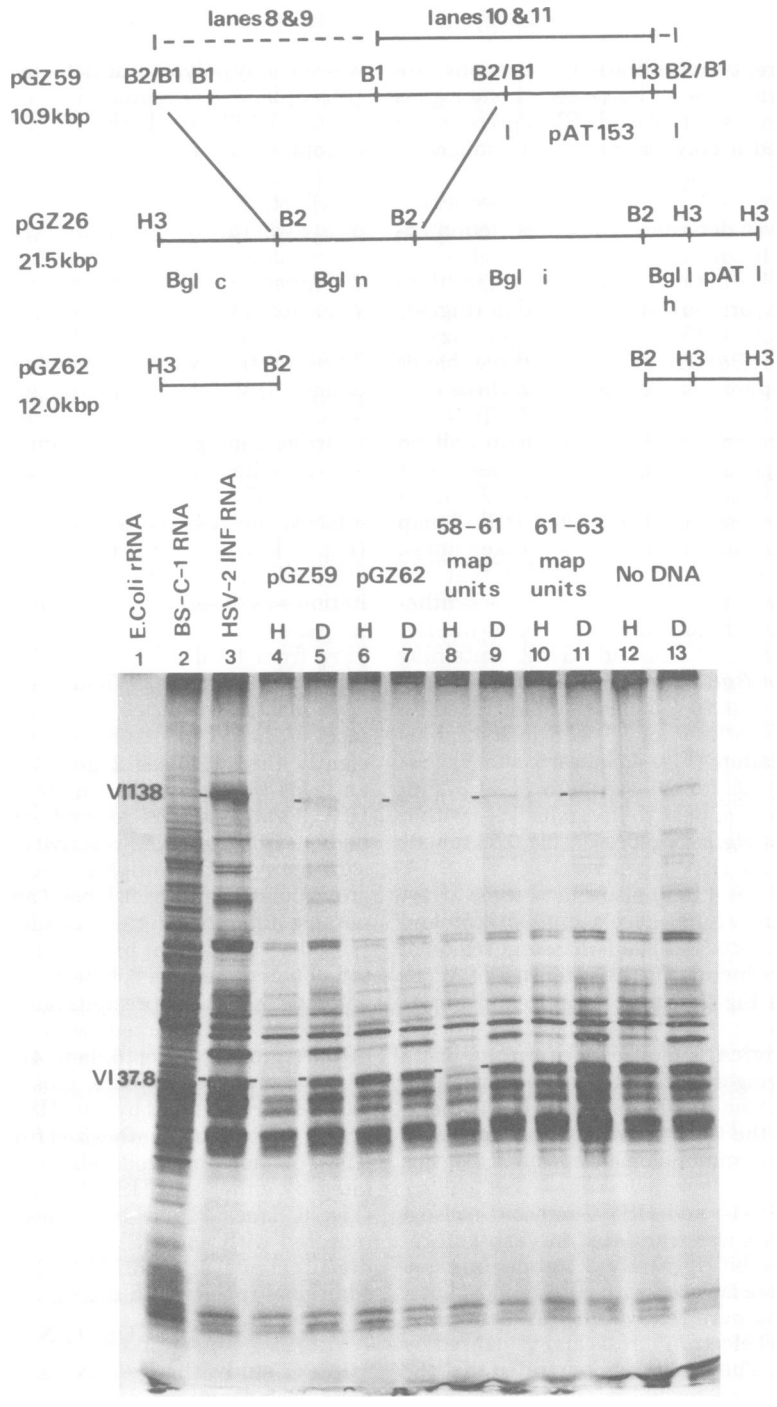

FIG. 4. *Mapping of VI 37.8. Hybrid-arrested translation was carried out with pGZ59 DNA (lanes 4 and 5), pGZ62 DNA (lanes 6 and 7), the lower pair of bands (lanes 8 and 9) or the upper band (lanes 10 and 11) of a double digest of pGZ59 with BamHI plus HindIII, or no DNA (lanes 12 and 13). H, Hybridized RNA; D, hybridized RNA that was denatured before translation. Polypeptides synthesized in response to E. coli rRNA (lane 1), BS-C-1 cell RNA (lane 2), or HSV-2-infected BS-C-1 cell RNA (lane 3) are also shown. The map above the autoradiograph depicts the DNA fragments used in hybrid-arrested translation, with restriction sites abbreviated as follows: B2, BglII; B1, BamHI; H3, HindIII; B2/B1, BglII-BamHI hybrid site.*

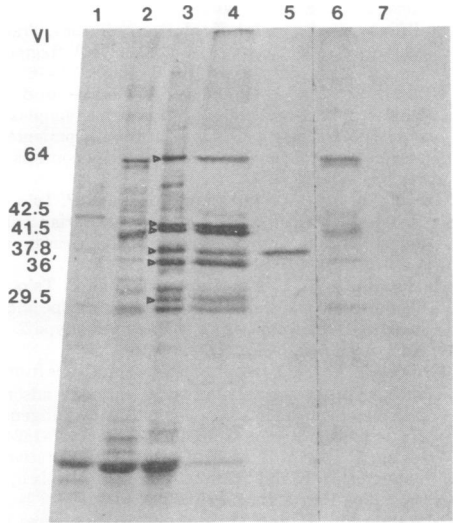

FIG. 5. *Immunoprecipitation of VI 37.8 by HSV-2 antiserum. Proteins were precipitated and recovered with staphylococcal protein A, resolved by polyacrylamide gel electrophoresis, and visualized by fluorography. (1) Mock-infected cell proteins synthesized in vitro; (2) HSV-1-infected cell proteins synthesized in vitro; (3) HSV-2-infected cell proteins synthesized in vitro; (4) HSV-2 proteins synthesized in vitro plus anti-HSV-2 serum; (5) VI 37.8 synthesized from pGZ59-selected mRNA plus anti-HSV-2 serum; (6) HSV-1 proteins synthesized in vitro plus anti-HSV-2 serum; (7) mock-infected cell proteins synthesized in vitro plus anti-HSV-2 serum. Numbers on the left are apparent molecular weights* $\times 10^{-3}$.

but was not detected in hybrid arrest or immunoprecipitation experiments. Likewise, polypeptide VI 138 was observed in some, but not all, hybrid-arrested translation studies, was not observed in mRNA selection studies, and was not immunoprecipitated. Immunoprecipitation of the in vitro translation products confirmed the viral origin of VI 37.8 and demonstrated its HSV-2 antigenicity.

Mapping studies to position VI 37.8 suggest that the coding sequences for the polypeptide lie entirely within the *Bgl*II N fragment of HSV-2 DNA. The limits of these experiments can be assessed, to some extent, by reference to previous studies designed to map the thymidine kinase mRNA encoded by the HSV-1 BamHI P fragment (15). In this case, it was found that efficient arrest of translation can be achieved by fragments which overlap the coding sequences at the 3′ terminus by only 20 base pairs. Fragments which hybridize to the 5′ untranslated region also arrest translation, although a modification of the reaction conditions is necessary. Furthermore, efficient selection was achieved by a plasmid which contains only the 5′-terminal 54 base pairs of the thymidine kinase mRNA. Plasmid pGZ62 fails to arrest the synthesis of VI 37.8 or select its mRNA under a variety of hybridization conditions (data not shown); therefore, it is probable that all coding sequences for VI 37.8 lie within *Bgl*II-N, although some untranslated regions may extend into the adjacent *Bgl*II C fragment. Analysis of the structure of VI 37.8 mRNA will be necessary to test this possibility.

The assignment of VI 37.8 to approximately 0.58 to 0.61 map unit on the HSV-2 genome appears to conflict with the map position of 0.44 to 0.57 obtained from an analysis of HSV-1 × HSV-2 intertypic recombinants (11). A re-examination of those data reveals that although recombinant B × 1 (24) clearly failed to induce Vmw 37.8, it was erroneously recorded as doing so. The corrected location by this analysis is 0.57 to 0.64 map unit (H. S. Marsden, personal communication; V. Preston, Ph.D. thesis, University of Glasgow, Glasgow, Scotland, 1980).

Whether any of the polypeptides encoded by *Bgl*II-N is tumor related is uncertain at present. However, in a recent study, it was demonstrated

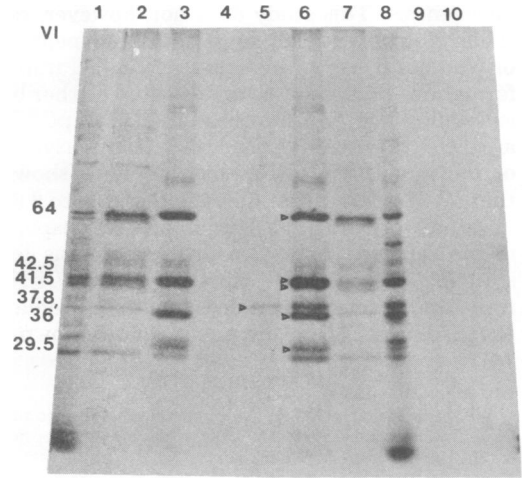

FIG. 6. *Type specificity of VI 37.8 as determined by precipitation with anti-HSV-1 and anti-HSV-2 sera. Proteins were precipitated and recovered with staphylococcal protein A, resolved by polyacrylamide gel electrophoresis, and visualized by fluorography. (1) HSV-1-infected cell proteins synthesized in vitro; (2) HSV-1 proteins synthesized in vitro plus anti-HSV-1 serum; (3) HSV-2 proteins synthesized in vitro plus anti-HSV-1 serum; (4) VI 37.8 synthesized in vitro from pGZ59-selected mRNA plus anti-HSV-1 serum; (5) as (4) but anti-HSV-2 serum; (6) as (3) but anti-HSV-2 serum; (7) as (2) but anti-HSV-2 serum; (8) HSV-2-infected cell proteins synthesized in vitro; (9) mock-infected BS-C-1 cell proteins synthesized in vitro plus anti-HSV-1 serum; (10) as (9) but anti-HSV-2 serum.*

that two HSV-2 polypeptides with molecular weights of 38,000 and 118,000 were immunoprecipitated more frequently by sera from uterine cervical cancer patients than by sera from control patients (5). Both polypeptides appeared to be HSV-2 specific, and their immunological reactivity was correlated with the presence of HSV-2-specific antibodies in patient sera and a variety of social and economic factors. In the present study, the VI 37.8 polypeptide was similar in molecular weight and immunological specificity to one of these tumor-associated proteins. Additionally, when Suh et al. (18) performed immunoprecipitation studies with proteins from HSV-2-transformed hamster cells, 11 polypeptides were precipitated by anti-HSV-2 sera. Notably, three of these polypeptides have molecular weights of 38,000, 37,500, and 35,000, which are similar in molecular weight to VI 37.8.

The finding that VI 37.8 is a major product encoded by the *Bgl*II-N region of HSV-2 DNA and that it has a molecular weight similar to that of a viral polypeptide found in HSV-2-transformed hamster cells (18) and may be precipitated by human sera from cervical cancer patients (5) is of considerable interest to HSV-2 oncogenesis. This study does not, however, establish the tumor relatedness of this polypeptide or whether it has a functional role in cell transformation. This could be investigated further by examining the transforming ability of pGZ59 after cleavage with various restriction enzymes or mutagenesis in vitro, since we have shown that VI 37.8 lies within the region of 0.58 to 0.61 map unit on the HSV-2 genome. The experiments reported here also provide a basis for studying HSV-2 gene expression in *Bgl*II-N-transformed cells, since at least one gene product encoded by this DNA fragment is now known.

ACKNOWLEDGMENTS

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