Localization of the Coding Region for a 35000 Dalton Polypeptide on the Genome of Herpes Simplex Virus Type 2

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SUMMARY

The cloned Bg/II N fragment of herpes simplex virus type 2 (HSV-2) DNA has been shown to code for a 35K polypeptide. Subfragments made by cleavage with XhoI, BamHI, SstII and XorII were then cloned and used with RNA extracted from HSV-2-infected cells for mRNA selection and in vitro protein synthesis. We found that the major translation product of such hybrid-selected mRNA has a molecular weight of 35000. By further mapping, DNA coding sequences for this mRNA were located within the region of Bg/II N, at approximately 0.585 to 0.596 genome map units. DNA sequences complementary to mRNA encoding a 56K polypeptide were located between 0.607 and 0.612 map units.

Inactivated herpes simplex virus type 2 (HSV-2) (Duff & Rapp, 1971) and DNA or cloned DNA fragments from HSV-2 (Wilkie et al., 1974; Galloway & McDougall, 1981; Jariwalla et al., 1980; Macnab, 1974) have been used for oncogenic transformation of rodent fibroblasts in culture. In a number of laboratories, the presence of HSV-specific antigens has been detected in transformed cells, including membrane glycoproteins and the non-structural proteins VPI43 and ICP10 (Camacho & Spear, 1978; Duff & Rapp, 1973; Lewis et al., 1982; Reed et al., 1975; Flannery et al., 1977; Jariwalla et al., 1980). We have recently demonstrated that nine polypeptides with molecular masses of 110, 84, 77, 47, 41.5, 38, 37.5, 35 and 32 kilodaltons are precipitated from transformed or tumour-induced hamster cells by anti-HSV serum but not by control serum (Suh et al., 1980). The 35K polypeptide from HSV-2-transformed and HSV-2-infected cells had almost identical cleavage maps after partial proteolysis, indicating that they are probably the same or very similar polypeptides (Suh, 1982).

Two different regions of the HSV-2 genome have been reported to have transforming potential; neither is homologous to the transforming region of the HSV-1 genome (Camacho & Spear, 1978). Using focus formation assays, the Bg/II N restriction fragment (0.582 to 0.628 map units) has been shown to transform BALB/3T3 (Reyes et al., 1979), primary rat, and NIH/3T3 (Galloway & McDougall, 1981) cells. Jariwalla et al. (1980) selected transformed cells which grew out of continuous passage after exposure to fragments of HSV-2 DNA. These experiments identified a fragment with transforming activity mapping between map coordinates 0.43 and 0.58.

Docherty et al. (1981) examined the Bg/II N fragment of HSV-2 DNA by hybrid-arrested translation and mRNA selection. They demonstrated that the information for one polypeptide, VI 37.8K, is contained within this DNA fragment and is encoded by the major translatable mRNA from this region of the genome. Another report (Galloway et al., 1982) revealed that mRNAs homologous to the Bg/II fragment N direct the synthesis of five polypeptides of

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approximate mol. wt. 140K, 61K, 56K, 35K and 23.5K. Of these, the 35K protein is the only species detected before the onset of viral DNA replication, and accumulates to become a fairly prominent product late in infection (Galloway et al., 1982). We also found that the 35K polypeptide is the major product of a subfragment of BglII N we call XhoI3, and coding sequences for its mRNA can be further refined to be between 0.585 and 0.596 genome map units. The 35K protein may be the same protein that we found in HSV-2-transformed cells by immunoprecipitation (Suh et al., 1980) which may be involved in transformation by HSV-2.

BHK-21 clone 13 cells were grown in a 5% CO₂ atmosphere in α medium (Gibco) containing 10% foetal calf serum in plastic tissue culture flasks (Corning). Plaque-purified HSV-2 strain 333 was used for all infections. [³⁵S]Methionine-labelled viral polypeptides were prepared as previously described (Suh et al., 1980).

RNA preparations were made from BHK-21 cells infected with HSV-2 at a multiplicity of 20 p.f.u./cell. The cells were inoculated with virus for 60 min in α medium containing 2% foetal calf serum and washed twice with medium before incubation in medium containing 2% foetal calf serum. Total cytoplasmic RNA was extracted 6 h after infection as described previously (Shore & Tata, 1977).

Plasmid DNA (25 µg) was prepared for hybrid selection of mRNA by sonicating it in 10 mM-Tris–HCl pH 7.4, 1 mM-EDTA to yield fragments of 1 to 3 kilobases which were then denatured at 50 °C for 1 h in 50% dimethyl sulphoxide, 1 M-glyoxal, 0.1 M-phosphate buffer pH 6.0, cooled on ice, and freshly prepared diazobenzoylxyzomethyl paper (DBM) circles (Stark & Williams, 1979) were added. They were incubated on ice for 2 h and rotated overnight at 4 °C. The filters were rinsed, incubated for 2 h in 4% glutamine, 10 mM-phosphate buffer pH 6-0 at 37 °C, and were then washed four times for 15 min each at 37 °C with 0.4 M-NaOH, three times in distilled water, once with 2 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-sodium citrate) and stored at 4 °C in 2 × SSC, 0-1% SDS. For hybrid selection, cytoplasmic RNA (2 to 4 mg/ml) from infected cells was incubated with DBM circles for 6 h at 57 °C in hybridization medium [50% (v/v) recrystallized formamide, 0.8 M-NaC1, 0-1% SDS, 10 mM-PIPES pH 6-5, 2 mM-EDTA]; DBM circles had been pretreated with tRNA at 0.5 mg/ml in hybridization buffer for 1 h at 57 °C. After hybridization, the circles were rinsed three times at 25 °C with 2 × SSC containing 0-2% SDS followed by two washes at 65 °C for 15 min with 0-1 × SSC containing 0.2% SDS. RNA was eluted by three 5 min incubations at 70 °C with 0.8 ml of 99% recrystallized formamide/10 mM-PIPES, pH 6-5. Eluted RNA and 10 µg calf thymus DNA carrier were precipitated with ethanol. The RNA was resuspended in 20 µl of water and 10 µl portions were used for translation in vitro in a micrococcal nuclease-treated fractionated reticulocyte cell-free system as described previously (Shore & Tata, 1977).

Protein samples were resolved on 5 to 12.5% polyacrylamide gradient gels containing SDS according to the procedure of Marsden et al. (1976). The fixed gels were treated with En³Hance (New England Nuclear), dried, and exposed to Kodak X-Omat RP X-ray film.

Hyperimmune rabbit antiserum to HSV-2 was prepared as described by Esparza et al. (1974) with a minor modification as described previously (Suh, 1982). The Staphylococcus aureus Cowan I strain (The Enzyme Center, Boston, Mass., U.S.A.) was used as an immunoadsorbent to isolate antigen–antibody complexes of radiolabelled viral proteins and rabbit anti-HSV sera (Kessler, 1975; Suh et al., 1980). The immune complexes were dissociated in solubilization buffer (0-05 M-Tris–HCl pH 7.0, 2% SDS, 5% 2-mercaptoethanol, 0-005% bromphenol blue) at 100 °C for 3 min before analysis by SDS-polyacrylamide gel electrophoresis in 5 to 12.5% gradient slab gels.

All restriction digestions were incubated 2 h at 37 °C at 2 units of enzyme per µg of DNA in 20 mM-Tris–HCl pH 7-4, 10 mM-MgCl₂, 3 mM-2-mercaptoethanol, 100 µg bovine serum albumin/µl, except for Smal, BglI and BglII which were used at pH 8-6 and Smal which was incubated at 32 °C.

Cloning in phosphatase-treated plasmids pTK16 and pHCF79 was performed by standard procedures (Wu, 1979). Initially, the BglII N fragment from the 333 strain of HSV-2 was cloned in pHCF79. After digestion with XhoI, three fragments were generated. The fragment containing a portion of the BglII N sequence and all of pHCF79 was circularized, cloned, and called pXho2.
Cell-free translation of cytoplasmic RNA from HSV-2-infected cells directed incorporation of $[^{35}S]$methionine into many polypeptides which show sizes, but not necessarily the molar ratios, similar to those observed after labelling of infected cells in vivo [compare lanes 4 and 7, Fig. 1 (immunoprecipitated gel)]. Selection of infected-cell RNA by the BgII N-containing plasmid followed by translation yielded one predominant band at 35K (Fig. 2); faintly visible bands can also be seen at 56, 61 and 140K (Fig. 2), as were also observed by Galloway et al. (1982). When subfragments of BgII N were used, that in pXho3 was the only fragment which selected mRNA encoding 35K, and pXhol contained the only fragment which selected mRNA encoding the 56K polypeptide (Fig. 2). The 56K message could also be selected by pCla. The fainter bands seen in Fig. 2 (61K and 140K, see lane 7) cannot be assigned conclusively to any of the three subfragments. The pBam, pXor and pSst plasmids were also used to select RNA, but translation of the selected RNA did not reveal any labelled viral species (data not shown).

The 35K polypeptide was the only labelled species immunoprecipitated by anti-HSV serum from the translation products of the RNA selected by pBglII IN or pXho3 (see Fig. 1); no radioactive product was precipitated from the translation products from RNA selected by pXhol and pXho2. The faint bands seen in the direct analysis were probably not present in sufficient amounts to be visible after immunoprecipitation.

Hybrid-arrested translation (HART; Paterson et al., 1977; Preston & McGeoch, 1981) was used to confirm these results. The pXho3, but not pXhol or pXho2 plasmids (data not shown), blocked translation of the 35K polypeptide. The pXor and pBam plasmid DNAs did not block the synthesis of the 35K protein but the pSst plasmid DNA did. Denaturation of the pSst DNA–RNA hybrid allowed translation of the 35K polypeptide (data not shown).

In order to map proteins encoded by the BgII N fragment of HSV-2, we used cell-free translation of mRNA selected with specific cloned fragments of HSV-2 DNA and also blocked translation of these messages with the same DNAs. The sequences coding for the 35K polypeptide have already been situated in the BgII N fragment of HSV-2 (Docherty et al., 1981; Galloway et al., 1982). Plasmids carrying the sequences between the BgII site at 0-582 and the BamHI site at 0-585 (530 base pairs away) did not select the 35K message nor did they hybridize with it in the HART procedure. However, when the plasmid contained the first 790 base pairs to the right of the BgII site (up to the SstII site at 0-587) homology to the message could be detected by the HART procedure, but not by hybridization selection. It is possible that a short sequence of homology between pSst and the 35K message does not allow its retention during the stringent washing of the filters practised in hybridization selection, but is sufficient in the HART assay. This result indicates that one end of the 35K message (or its first or last splice point in BgII N) occurs in the 260 base pairs between the BamHI and SstII sites at 0-585 and 0-587 map units respectively. Because the 35K message was selected and its transcription was blocked by the pXho3 but not by the pXhol or the pXho2 plasmids, we have been able to locate the other end (or first or last splice point in BgII N) of the 35K message to the left of the XhoI site at 0-596.

We have also been able to refine the positioning of the 56K message initially reported by Galloway et al. (1982) to be to the right of position 0-607 in BgII N. Among the pXho series of plasmids it was selected solely by the pXho1 plasmid; thus, its 3' end must be to the left of 0-612. It is also selected by pCla, situating its 5' end to the left of 0-606. Thus, its 5' end (or first splice point) must be in the 120 base pairs between the ClaI site at 0-608 and the PstI site at 0-607.

We were unable to study and to locate precisely the other polypeptides reported by Galloway et al. (1982) because under our conditions they were present only in minimal amounts, in contrast to the large amounts (equal to the 35K polypeptide) they reported. We ascribe this to
Fig. 1. Analysis of polypeptides translated in vitro and immunoprecipitated with rabbit anti-HSV-2 serum. Proteins were immunoprecipitated and recovered with staphylococcal Protein A, resolved by polyacrylamide gel electrophoresis, and visualized by fluorography. Lanes 4 and 5 are immunoprecipitates of translation products of total cytoplasmic RNA from HSV-2-infected BHK cells (anti-HSV-2 serum, lane 4; anti-HSV-1 serum, lane 5). Lane 6, immunoprecipitate of translation products using total cytoplasmic RNA extracted from mock-infected cells. Lane 7, polyadenylated RNA from HSV-2-infected cells used for translation in vitro. Lanes 8 to 11 are immunoprecipitates of translation products from RNAs selected by hybridization to the following plasmids containing cloned HSV-2 DNA fragments: 8, plasmid with BglII N; 9, pXho1; 10, pXho2; 11, pXho3. Control translation products (without RNA) were also immunoprecipitated (lane 3). Lanes 2 and 12 are immunoprecipitates from HSV-2-infected cells. HSV-2 antiserum was used except in lanes 5 and 12 (HSV-1 antiserum). Lanes 1 and 13 are HSV-2 marker polypeptides labelled with [35S]methionine, but not immunoprecipitated.
Fig. 2. Analysis of polypeptides translated in vitro from RNA selected by hybridization to HSV-2 DNA fragments. Cell-free protein synthesis was programmed using RNA complementary to either cloned BglII N (lane 7), pXhol (lane 8), pXho2 (lane 9), or pXho3 (lane 10). Lanes 4 and 6 show translation products of total cytoplasmic RNA from HSV-2-infected cells before (lane 4) or after (lane 6) selection of polyadenylated RNA. Lane 5 contains translation products of total cytoplasmic RNA extracted from mock-infected cells. No RNA was added to the control translation mixture (lane 3). Polypeptides immunoprecipitated with rabbit anti-HSV-2 serum from HSV-2-infected BHK cells are in lane 2. Lanes 1 and 11 are HSV-2 marker polypeptides, which were not immunoprecipitated. The products of cell-free translation were analysed by SDS-polyacrylamide gel electrophoresis, using 5 to 12.5% gradient gels. The numbers on the right are the molecular masses of polypeptides in kilodaltons.
our earlier time of harvesting of RNA (6 h post-infection) as opposed to their later time (10 h post-infection). The 35K message is in the β transcription class whereas the other messages are transcribed later (γ class; Galloway et al., 1982).

In this study we have precisely located the 5' ends of messages for two polypeptides coded by the BglII N fragment. One of these polypeptides (35K) has been found in mouse and hamster cell lines transformed by HSV-2 (Suh et al., 1980; Suh, 1982; M. Suh & E. Frost, unpublished results). The BglII N fragment has the capacity to transform cells (Galloway & McDougall, 1981; Reyes et al., 1979) and has been used to detect HSV-like sequences in transformed cells. Whether or not the 35K polypeptide may play a role in transformation remains to be determined (Galloway & McDougall, 1983; Huszar & Bachetti, 1983). Although the functions of this polypeptide in viral replication are unknown, like the transforming proteins of Rous sarcoma virus, simian virus 40, polyoma and adenoviruses, it is phosphorylated (Baumann & Hand, 1979; Branton et al., 1981; Eckhart et al., 1979; Erikson et al., 1979). Clearly, elucidation of the role of this polypeptide in transformation will require temperature-sensitive mutants. It is hoped that the localization of this polypeptide will help in the construction of such mutants.

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