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# Characterization of mRNAs That Map in the *Bgl*II N Fragment of the Herpes Simplex Virus Type 2 Genome

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**The *Bgl*II N DNA fragment (0.580 to 0.620 map units) of herpes simplex virus type 2 strain (333) is of interest because of its transforming potential. This fragment contains either partial or the complete coding sequences for nine mRNA transcripts that can be detected during a lytic infection. Subclones of the *Bgl*II N DNA fragment were generated in plasmid vectors, and the approximate locations of the mRNA transcripts were mapped by RNA blot hybridization technology. Precise 5′ or 3′ ends (or both) of these mRNA species were determined by S1 nuclease mapping, using the *Bgl*II N subclones as DNA probes. At least four mRNA transcripts are fully encoded in the *Bgl*II N fragment. The coding regions for all of the mRNA transcripts are densely packaged along the *Bgl*II N fragment with less than 150 base pairs between neighboring mRNA ends. Analysis of both neutral and alkaline gels failed to reveal the presence of any detectable introns. This manuscript reports a detailed transcription map for this region.**

Herpes simplex virus type 2 (HSV-2) was first shown to induce morphological cell transformation more than a decade ago (5, 6). Since these initial reports, two regions of the HSV-2 DNA have been shown to induce morphological transformation (10, 13, 18). One of these regions, the *Bgl*II N DNA fragment, maps between positions 0.580 and 0.620 on the virus DNA genome.

Recent efforts have focused on identifying the proteins encoded by the *Bgl*II N DNA fragment. Docherty and coworkers (4) identified a single abundant protein (molecular weight, 37,800 [37.8K]) by hybrid-arrested translation experiments. Galloway et al. (9) reported that RNA selected by hybridization to cloned *Bgl*II N DNA could direct synthesis of five polypeptides with molecular weights of 140K, 61K, 56K, 35K, and 23.5K. RNA for the 35K polypeptide is apparently synthesized in small amounts in the absence of DNA synthesis and probably corresponds to the abundant 37.8K protein reported by Docherty et al. (4). Suh and coworkers (19) mapped the coding sequences for the 35K protein at ca. 0.585 to 0.596 map units and the coding sequences for the 56K protein at ca. 0.607 to 0.612 map units. Recently, McLauchlan and Clements (17) mapped the location of two early, unspliced mRNAs of 4.5 and 1.2 kilobases (kb) in length whose 3′ ends map in the left end of the *Bgl*II N DNA fragment and are coterminal.

Because of the potential significance of the *Bgl*II N region of HSV-2 DNA in transformation, we were interested in the transcriptional activity of the *Bgl*II N DNA fragment in lytically infected cells. We previously reported the identification of several polyadenylated [poly(A)$^+$] mRNA species that showed homology to the *Bgl*II N fragment at times corresponding to the maximum synthesis of the β (7 h postinfection [p.i.]) and γ (12 h p.i.) HSV polypeptides (14). We were unable to detect any mRNA transcribed during the α phase of RNA transcription.

This paper reports the map location of nine mRNA transcripts that are either fully or partially encoded by the HSV-2 *Bgl*II N DNA fragment. To accomplish this work, subclones of the *Bgl*II N DNA fragment were generated, and approximate locations of the mRNA transcripts were mapped by RNA blot hybridization. The precise 5′ or 3′ end (or both ends) of these mRNA species were determined by S1 nuclease mapping techniques, using the various *Bgl*II N subclones labeled at either the 5′ or the 3′ end.

## MATERIALS AND METHODS

**Cells and viruses.** Monolayers of TC7 (green monkey kidney) cells were grown at 37°C in Dulbecco medium supplemented with 5% fetal calf serum, 0.075% $NaHCO_3$, and 20 μg of kanamycin per ml. HSV-2 strain 333 stocks were routinely grown in an additional monkey cell line (Vero) at 37°C, using a multiplicity of infection of 0.01 PFU per cell. Virus titers were determined by plaque assay on primary rabbit kidney cells as previously described (6).

**Enzymes.** All enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, or Boehringer Mannheim and were used according to the suppliers' recommendations.

**Production of recombinant DNA molecules.** The recombinant plasmid p29 containing the HSV-2 (333) *Bgl*II N DNA fragment, cloned into the plasmid vector pKC7, was kindly provided by N. Frenkel, University of Chicago, Chicago, Ill. Subclones of the *Bgl*II N DNA fragment were produced by digesting p29 with the appropriate restriction enzyme and ligating the cleaved DNA products with similarly digested pACYC184 or pBR322 plasmid vector DNA. Recombinant molecules were initially selected by antibiotic resistance. The herpesvirus DNA inserts were identified by restriction enzyme analysis of the subclonal recombinant DNAs on agarose gels and DNA blot analyses utilizing plasmid DNA probes from the subclones against p29-cleaved DNA (data not shown). The recombinant plasmids were amplified in *Escherichia coli* HB101, and the DNA was isolated as previously described (3).

**Isolation of cytoplasmic and poly(A)$^+$ RNA.** To establish an HSV-2 infection for isolation of infected cell RNA, monolayers of TC7 cells were infected with HSV-2 (333) at a multiplicity of infection of 10 to 20 PFU per cell. For β RNA

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isolation, TC7 cells were infected and maintained in the presence of 100 μg of arabinosylcytosine per ml, and the cytoplasmic RNA was extracted at 6 to 7 h p.i. γ cytoplasmic RNA was extracted from normally infected TC7 cells at 12 to 14 h p.i. Cytoplasmic RNA was purified from Nonidet P-40 cytoplasmic extracts as described previously (7). Briefly, cell monolayers were washed twice with phosphate-buffered saline, once with isotonic buffer (150 mM NaCl, 10 mM Tris-hydrochloride, 1.5 mM $MgCl_2$ [pH 7.4]), and scraped gently into a small volume of isotonic buffer. Nonidet P-40 was added to a final concentration of 0.1%, and the cell suspension was aspirated gently to allow the cytoplasm to solubilize away from the cells without disruption of the nuclear membranes. The Nonidet P-40 extracts were centrifuged at low speed to remove cell debris and then at high speed (17,300 × *g*) to remove any remaining nuclei and mitochondria. Supernatants containing cytoplasmic RNA were adjusted to a final concentration of 0.2% sodium dodecyl sulfate and 2 mM EDTA and extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (50:50). After addition of sodium acetate to a concentration of 0.2 M, RNA was precipitated with 2.5 volumes of ice-cold ethanol.

Poly(A)$^+$ RNA was separated from cytoplasmic RNA by oligodeoxythymidylic acid-cellulose chromatography as described previously (7). Precipitated cytoplasmic RNA was dissolved in binding buffer (10 mM Tris-hydrochloride, 500 mM KCl [pH 7.5]) and loaded on a small column of oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc., Waltham, Mass.). The eluant from the cytoplasmic RNA was passed through the column six times to ensure binding of the poly(A)$^+$ RNA. The column was then washed four times with 5 ml of binding buffer. Poly(A)$^+$ RNA was eluted with 3 ml of elution buffer (10 mM Tris, pH 7.4) and the resulting RNA fractions were precipitated with ethanol.

**RNA blot analysis.** RNA samples were electrophoresed in 1.4% agarose–10% formalin horizontal slab gels at 125 V for 4 h (15). After ethidium bromide staining, the gels were soaked in a solution containing 10 mM NaOH and 50 mM NaCl for 10 min to nick the RNA. The gels were rinsed in distilled water and soaked in 20× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) for 30 min, and the RNA was transferred by blotting to nitrocellulose in 10× SSC overnight (1, 20). The nitrocellulose strips were rinsed in 5× SSC and baked in vacuo for 2 h.

Radiolabeled DNA used as probes in hybridization reactions was prepared by labeling 0.5 μg of DNA with [$^{32}$P]dCTP to high specific activity (1 × $10^8$ to 2 × $10^8$ cpm/μg) with a nick translation kit (Amersham Corp., Arlington Heights, Ill.). Hybridization of radioactive DNA probes to nitrocellulose strips containing transferred RNA and subsequent autoradiography were performed as previously described (14).

**Specific end labeling of DNA probes.** DNA fragments were generated by restriction enzyme cleavage of recombinant plasmid DNA. Isolated DNA fragments were prepared by restriction enzyme digestion followed by fractionation in agarose gels. DNA bands were visualized by ethidium bromide staining, and the DNA band(s) of interest was removed and eluted by electroelution as described previously (16). To label the 5′ end, dephosphorylated DNA fragments were labeled with polynucleotide kinase and [γ-$^{32}$P]ATP (>5,000 Ci/mmol; Amersham Corp.) as described previously (16). DNA fragments were labeled at the 3′ end with the Klenow fragment of *E. coli* DNA polymerase and 125 μCi of appropriate [α-$^{32}$P]dNTP as described previously (16). The DNA fragments were routinely end labeled to specific activities of $10^5$ to $10^6$ cpm/μg. It is important to remember that 5′ end labeling of DNA can result in internal incorporation of label into probe as a consequence of internal DNA nicks. This phenomenon results in high background levels when using S1 nuclease technology. In this work, true protection of 5′ end-labeled probe by RNA was only assumed if that protection was reproducible and compatible with RNA sizes determined by other criteria.

**Nuclease mapping of HSV-2 RNA.** End-labeled DNA (0.1 μg) was added to 150 μg of infected cell cytoplasmic RNA and precipitated with 2 volumes of cold ethanol. The pellets were dissolved in 20 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid] [pH 6.4]), denatured by incubation at 70°C for 10 min, and hybridized overnight at 61°C. Hybridization was terminated by the addition of 10 volumes of S1 buffer (250 mM NaCl, 1 mM $ZnSO_4$, 30 mM sodium acetate, 5% glycerol [pH 4.6]). S1 nuclease (50 U) was added, and digestion was carried out for 30 min at 45°C. After digestion, the samples were extracted first with phenol-$CHCl_3$ and then with $CHCl_3$. Carrier tRNA (10 μg) was added to each sample, followed by ethanol precipitation.

Samples were fractionated by electrophoresis in horizontal 1.4% agarose gels run in either a neutral (50 mM Tris, 50 mM boric acid, 1 mM EDTA [pH 8.3]) or an alkaline (30 mM NaOH, 2 mM EDTA) buffer at 90 V for 9 and 24 h, respectively. A 123-base-pair (bp) DNA ladder was purchased from Bethesda Research Laboratories and run on these gels as a molecular weight marker. The presence of the marker provided an extremely accurate method for sizing the RNA-DNA hybrids on neutral gels and the single-stranded DNAs on alkaline gels. The precision of resolution was within 0.01 kb for fragments less than 1 kb in size and to within 0.1 kb for larger fragments. After electrophoresis, the gels were dried in vacuo, and bands were visualized by autoradiography.

## RESULTS

**General characterization of mRNA species sharing homology to the *Bgl*II N DNA fragment.** Restriction endonuclease sites of the *Bgl*II N DNA fragment (p29) and the DNA inserts of the recombinant plasmid subclones used in this study are shown in Fig. 1. The position in map units of the restriction endonuclease sites and the location of the mRNA transcripts were based on the map location of the *Bgl*II N DNA fragment on the HSV-2 virus genome (0.580 to 0.620 map units) and the length of the *Bgl*II N DNA fragment (7.3 kb). Although most of the *Bgl*II N DNA restriction enzyme sites agreed with previously published reports (10, 18), we identified a *Bst*EII site 60 bp to the right of the left *Bgl*II site (0.580 map units) that has not been reported previously. In addition, the position of the *Bst*EII site located between the *Sal*I (0.614 map units) and the *Bgl*II site (0.620 map units) was identified 100 bp to the right of the *Sal*I site and not 480 bp to the right of the *Sal*I site, as previously reported (18). We were unable to find the *Hin*dII site reported between 0.605 and 0.614 map units by Galloway and McDougall (10). All of these mapping results were duplicated with a separate, cloned *Bgl*II N DNA fragment kindly provided by R. Hyman, The Pennsylvania State Univeristy, Hershey, Pa. (data not shown). Because the locations of the two *Bst*EII sites were identical on two separately cloned *Bgl*II N DNA fragments, and because both clones lacked the *Hin*dII site in question, we believe these sites to be accurately mapped. Since previous workers (10, 18) also mapped restriction

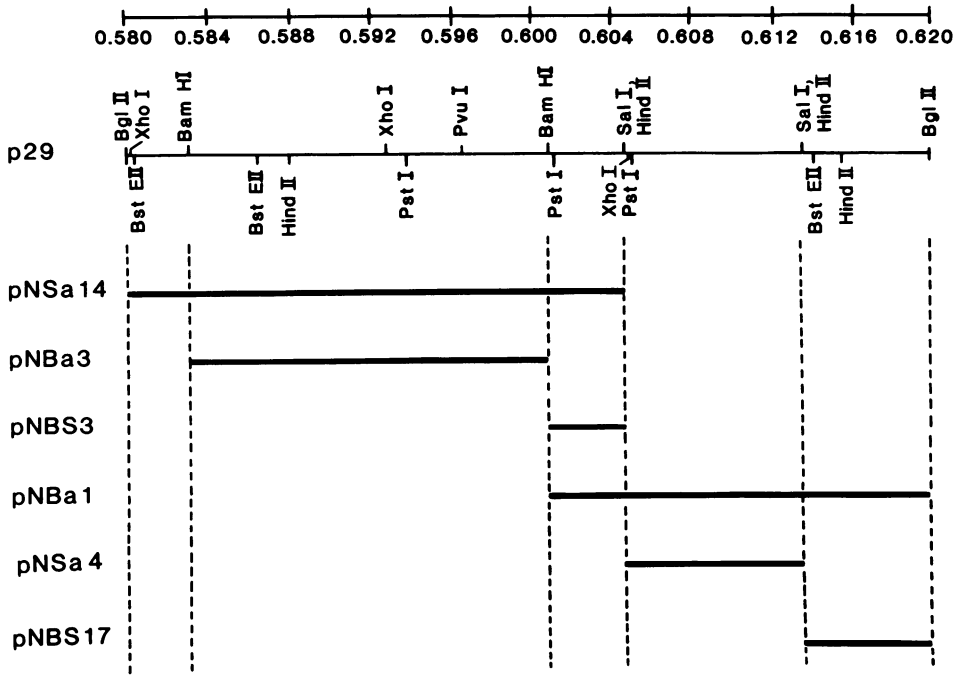


FIG. 1. Restriction endonuclease cleavage sites in the region 0.580 to 0.620 map units of HSV-2 (333). The *Bgl*II N DNA fragment was digested with *Bam*HI, *Sal*I, or both and subcloned into plasmid vectors. Heavy lines represent the portion of the *Bgl*II N DNA fragment contained in each subclonal recombinant plasmid.

endonuclease sites on the 333 strain of HSV-2, we can only assume they are mistaken in regard to these specific sites.

The plasmid DNAs (Fig. 1) represent *Bgl*II N subclones that were constructed and used as DNA probes in RNA blot hybridization experiments against either β or γ HSV-2-specific poly(A)$^+$ mRNA (Fig. 2–4). Hybridization of *Bgl*II N DNA against poly(A)$^+$ β mRNA revealed three major bands of 5.2, 1.9, and 1.5 kb in length (Fig. 2). In addition to hybridization to the whole N fragment, the 5.2- and 1.5-kb mRNA species hybridized to subclones representing the

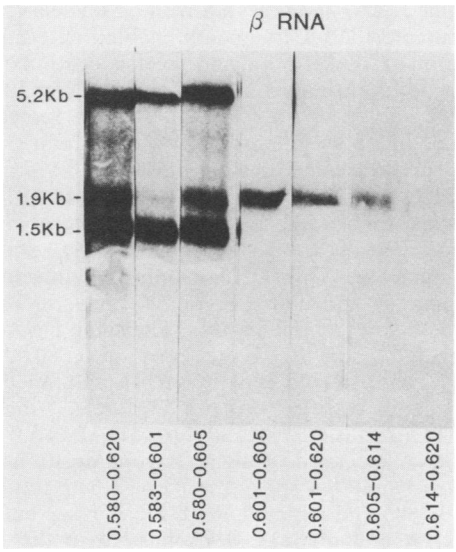


FIG. 2. Northern blot analysis of poly(A)$^+$ β mRNA from HSV-2-infected TC7 cells. Each lane contains 5 μg of poly(A)$^+$ RNA. Recombinant plasmid DNA containing the region of the *Bgl*II N DNA fragment shown at the bottom of each lane was labeled with $^{32}$P to 2 × 10$^8$ cpm/μg and hybridized to the individual lanes. Sizes of mRNA were based on the migration of 28S and 18S rRNA markers (5.2 and 2.0 kb).

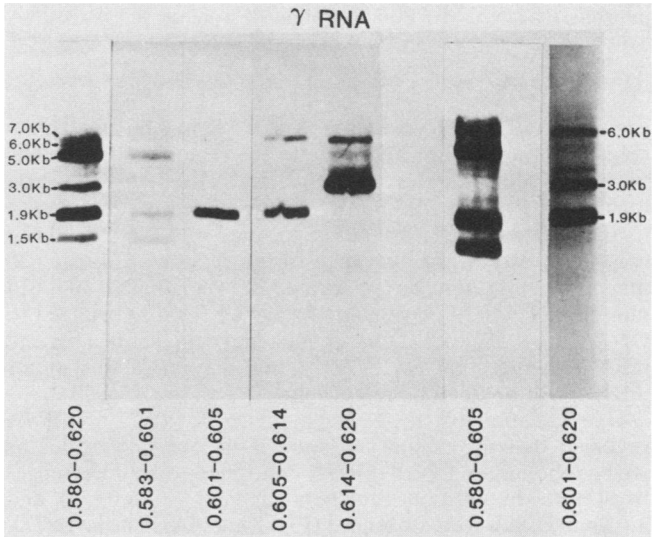


FIG. 3. Northern blot analysis of poly(A)$^+$ γ mRNA from HSV-2-infected TC7 cells. Each lane contains 5 μg of poly(A)$^+$ RNA. Recombinant plasmid DNA containing the region of the *Bgl*II N DNA fragment shown at the bottom of each lane was labeled with $^{32}$P to 2 × 10$^8$ cpm/μg and hybridized to the individual lanes. Sizes of mRNA were based on the migration of 28S and 18S rRNA markers (5.2 and 2.0 kb).

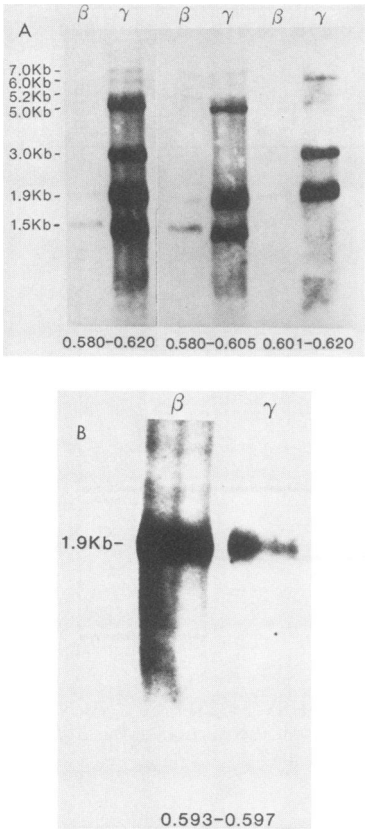


FIG. 4. Northern blot analysis of poly(A)$^+$ β and γ mRNA from HSV-2-infected TC7 cells. Each lane contains 5 μg of poly(A)$^+$ RNA. Recombinant plasmid DNA containing the region of the *Bgl*II N fragment shown at the bottom of each lane was labeled with $^{32}$P to 2 × 10$^8$ cpm/μg and hybridized to the individual lanes. Sizes of mRNA were based on migration patterns of 28S and 18S rRNA markers. (A) Demonstration of RNAs transcribed from the left (0.580 to 0.605 map units) or right (0.601 to 0.620 map units) half of the *Bgl*II N DNA. (B) Demonstration of a minor β 1.9-kb RNA hybridizing to probe from the 0.593 to 0.597 region.

regions 0.580 to 0.605 map units and 0.583 to 0.601 map units (Fig. 2). They did not hybridize to the region 0.601 to 0.605 map units and, therefore, are located to the left of position 0.601 (Fig. 2). The 1.9-kb mRNA hybridized predominantly to the regions 0.601 to 0.605 map units and 0.605 to 0.614 map units. It did not hybridize to the region 0.614 to 0.620 map units and, therefore, is located between 0.601 and 0.614 map units (Fig. 2). A very minor 1.9-kb band hybridized to DNA from 0.583 to 0.601 map units and represents a second mRNA comigrating at 1.9 kb. Confirmation of this minor mRNA will be demonstrated below.

Hybridization of the N fragment to poly(A)$^+$ γ mRNA revealed that the major 1.9- and 1.5-kb mRNA species as well as the minor 1.9-kb mRNA continued to be detected 12 to 14 h p.i. In addition, four new bands of 7.0, 6.0, 5.0, and 3.0 kb in length were observed (Fig. 3 and 4A). The large 7.0- and 5.0-kb species were detected with the regions 0.580 to 0.605 map units and 0.583 to 0.601 map units but were not detected with the region 0.601 to 0.605 map units and, therefore, are located to the left of position 0.601. The 6.0-kb mRNA was detected to the right of position 0.601 (Fig. 4A) and did not hybridize to the region 0.583 to 0.601 map units (Fig. 3). The 3.0-kb mRNA is located to the right of position 0.614 since it hybridized to the region 0.614 to 0.620 map units and did not hybridize to the region 0.605 to 0.614 map units (Fig. 3). For the purposes of this paper, we will refer to mRNA transcripts detected before the onset of DNA synthesis as β mRNA, and those detected only after the onset of viral DNA synthesis as γ mRNA. Transcripts detected as β and continually after the onset of viral DNA synthesis will still be referred to as β mRNA. Nomenclature established by Frink and co-workers (8) designates this class of RNA as "β,γ."

To ensure that the faint 1.9-kb band detected to the left of position 0.601 was not due to the major 1.9-kb band located to the right of position 0.601, pNBa3 DNA (0.583 to 0.601 map units) was digested with *Xho*I and *Pvu*I, producing a 700-bp fragment (0.593 to 0.597 map units) (Fig. 1). Hybridization of this fragment to β and γ poly(A)$^+$ mRNA revealed the presence of a 1.9-kb mRNA (Fig. 4B). The abundance was less in the γ cytoplasmic RNA population. Later data will demonstrate that we fail to detect this mRNA by S1 nuclease analysis of γ cytoplasmic RNA under conditions in which we do detect it in the β cytoplasmic RNA. We can only speculate that Northern analysis with a highly specific probe (0.593 to 0.597 map units) for RNA is highly sensitive for the presence of this RNA.

**S1 nuclease mapping of mRNA species sharing homology to the *Bgl*II N DNA fragment.** The precise locations of the 5′ or 3′ end (or both) of mRNA species sharing homology to the *Bgl*II N DNA fragment were determined by S1 nuclease mapping techniques. Having determined the approximate locations of the mRNA species by RNA blot hybridization, we were able to end label DNA fragments at restriction endonuclease sites that were known to cut the DNA at regions encoding the different mRNAs. The DNA probes used for these studies were either recombinant plasmid DNAs linearized at the junction of plasmid DNA and the viral DNA insert or restricted DNA fragments isolated from agarose gels by electroelution. The DNA probes were labeled at the 5′ or 3′ end, hybridized to either β or γ cytoplasmic RNA, and digested with S1 nuclease. Analyses of the protected DNA fragments enabled determination of the direction of transcription and precise location of the 5′ or 3′ end (or both) of the mRNA (Table 1 and Fig. 10, with the actual data shown in Fig. 5 to 9). Results for each DNA region encoding a specific mRNA were as follows.

**mRNA species located between 0.580 and 0.589 map units.** Both total cytoplasmic β and γ RNA protected a 1.6-kb DNA fragment when hybridized to linearized pNSa14 DNA 3′ end labeled at the *Bgl*II site (position 0.580) and digested with S1 nuclease. This DNA band was present in both neutral (Fig. 5) and alkaline (Fig. 6) gels, indicating the absence of any detectable introns. The same DNA fragment 5′ end labeled at the *Bgl*II site (position 0.580) detected a minor DNA band of 0.30 kb with β RNA, but failed to detect any DNA bands with γ RNA (Fig. 7). Because the length of the pNSa14 insert is 4.53 kb and the β 5.2-kb, γ 5.0-kb, and γ 7.0-kb mRNA species do not map beyond position 0.601, the 3′ end of these mRNA species must be 1.6 kb to the right of position 0.580. The minor 0.30-kb DNA fragment detected with β RNA and pNSa14 DNA labeled at the 5′ end at position 0.580 indicates the 5′ end of a minor mRNA species of unknown length not previously detected by our RNA blot hybridization techniques. The 5′ end of this mRNA would be located 0.30 kb to the right of position 0.580.

The β 1.5-kb mRNA overlaps with the β 5.2-kb, γ 5.0-kb, and γ 7.0-kb mRNA species and shares its 3′ end with these mRNA species. These results were determined with linear-

TABLE 1. Summary of S1 nuclease mapping results

| DNA fragment (map units) | Position of label | Gel pH[a] | Protected DNA (kb)[b] | mRNA responsible (kb) |
|---|---|---|---|---|
| pNSa14 (0.580–0.605) | 3′ at 0.580 | N and A | 1.6 | β 5.2, γ 5.0, γ 7.0 |
| | 5′ at 0.580 | N | 0.30 | β ND[c] |
| | 3′ at 0.605 | N and A | None found | |
| | 5′ at 0.605 | N | 0.984 | β 1.9, γ 6.0 |
| | 5′ at 0.586 and 0.5803 | N | 0.75<br>1.1 | β 1.5<br>β 5.2, γ 5.0, γ 7.0 |
| pNBa3 (0.583–0.601) | 3′ at 0.586 | N | 0.43, 1.1 | β 1.5, β 5.2, γ 5.0, γ 7.0 |
| | | A | 0.43 | β 1.5, β 5.2, γ 5.0<br>γ 7.0 |
| | 3′ at 0.593 | N | 0.615 | Minor β 1.9 |
| | 5′ at 0.593 | N | 1.1 | Minor β 1.9 |
| pNBa1 (0.601–0.620) | 5′ at 0.620 | N and A | 0.55 | γ 3.0 |
| | 3′ at 0.620 | N and A | None found | |
| | 5′ at 0.601 | N and A | None found | |
| | 3′ at 0.601 | N and A | 1.4 | β 1.9 |
| pNSa4 (0.605–0.614) | 5′ at 0.614 | N | 0.738 | Minor β 1.5 |
| pNBS17 (0.614–0.620) | 5′ at 0.614 | N and A | None found | |
| | 3′ at 0.614 | N and A | 0.615 | Minor β 1.5 |

[a] N, Neutral pH; A, alkaline pH.
[b] The precision of resolution was within 0.01 kb for fragments less than 1 kb in size and to within 0.1 kb for larger fragments (see text).
[c] ND, Not determined.

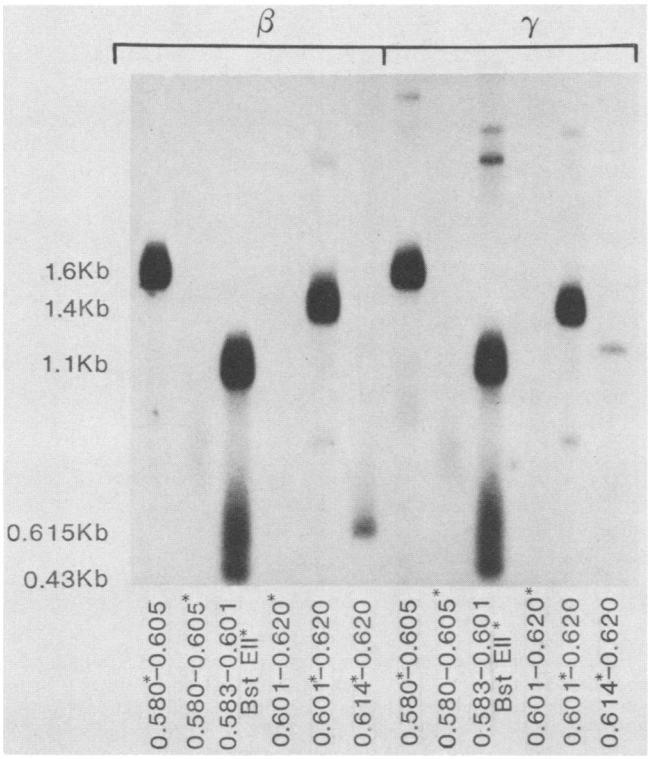


FIG. 5. Neutral gel of S1 nuclease mapping. DNA clones labeled at the 3′ ends indicated by the asterisk (*) were hybridized to either β or γ cytoplasmic RNA and digested with S1 nuclease, and the protected DNA fragments were fractionated by electrophoresis. Sizes of DNA were determined by migration patterns relative to a 123-bp DNA ladder (Bethesda Research Laboratories).

ized pNBa3 DNA (0.583 to 0.601 map units) 3′ end labeled at the *Bst*EII site (position 0.586). S1 digestion products of this DNA fragment with either β or γ cytoplasmic RNA (separated on a neutral gel) revealed two DNA bands of 0.43 and 1.1 kb in length (Fig. 5). Separation of duplicate samples on an alkaline gel (Fig. 6) showed the presence of the 0.43-kb DNA band only. The 0.43-kb DNA band supports the location of the 3′ end of the β 1.5-kb, β 5.2-kb, γ 5.0-kb, and γ 7.0-kb

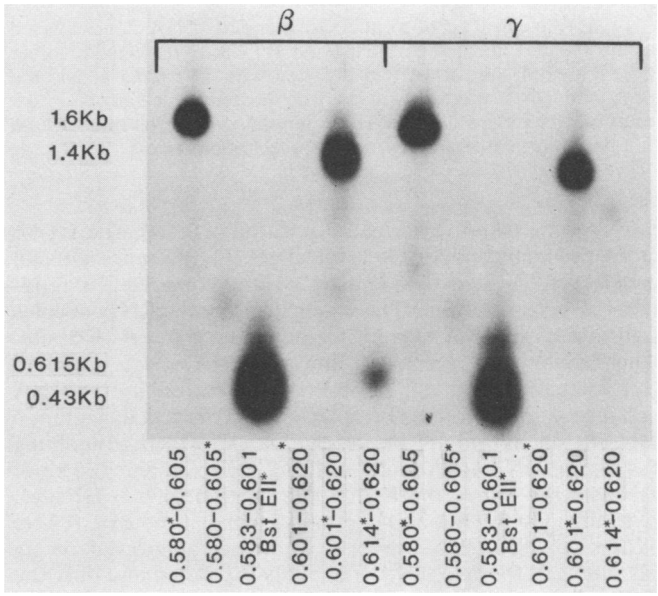


FIG. 6. Alkaline gel of S1 nuclease mapping. DNA clones labeled at the 3′ ends indicated by the asterisk (*) were hybridized by either β or γ cytoplasmic RNA and digested with S1 nuclease, and the protected DNA fragments were fractionated by electrophoresis. Sizes of DNA were determined by migration patterns relative to a 123-bp DNA ladder (Bethesda Research Laboratories).

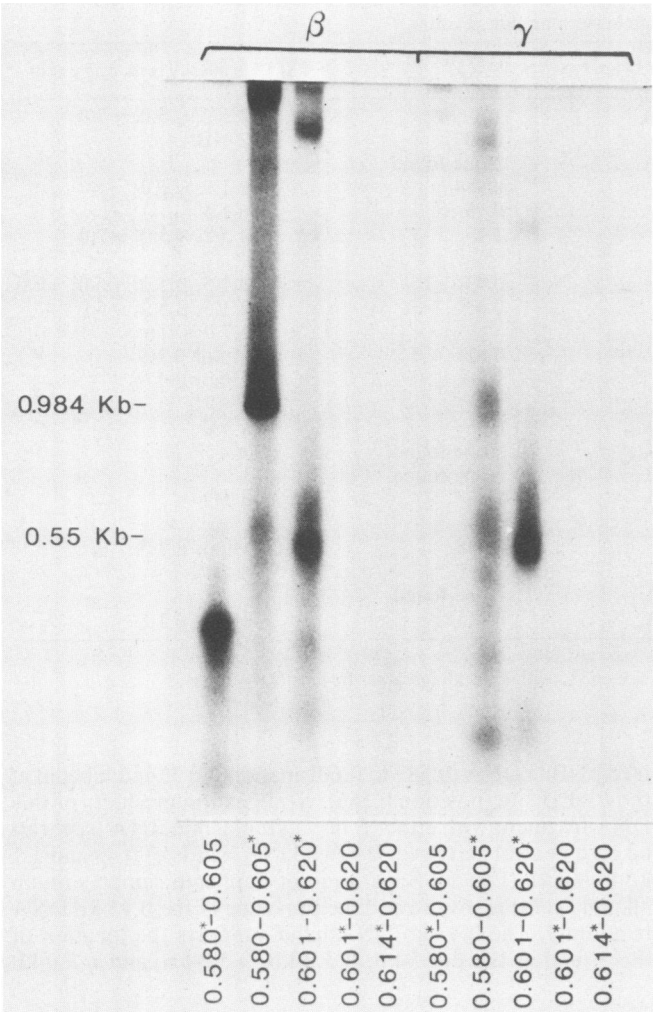


FIG. 7. Neutral gel of S1 nuclease mapping. DNA clones labeled at the 5′ ends indicated by the asterisk (*) were hybridized to either β or γ cytoplasmic RNA and digested with S1 nuclease, and the protected DNA fragments were fractionated by electrophoresis. Sites of DNA were determined by migration patterns relative to a 123-bp DNA ladder (Bethesda Research Laboratories).

mRNAs at 1.6 kb to the right of position of 0.580. The 1.1-kb DNA fragment seen on the neutral gel (Fig. 5) represents an artifact of S1 digestion caused by the linearized 3′ end-labeled pNBa3 probe. The 3′ end of these mRNA species hybridized to DNA located to the right of the *Bst*EII site. This leaves the 5′ ends of these mRNA species free to hybridize to the free end of pNBa3, which contains the DNA sequences to the left of the *Bst*EII site. After S1 digestion, a DNA-RNA hybrid 1.1 kb in length was formed, containing 0.43 kb of DNA to the right of the *Bst*EII site plus 0.6 kb of DNA to the left of the *Bst*EII site (*Bst*EII to *Bam*HI site), hybridized to mRNA and containing a gap at the *Bst*EII site. When this RNA-DNA hybrid was electrophoresed on an alkaline gel, the two DNA fragments separated and only the radiolabeled 0.43-kb band was detected.

The 5′ end of the β 1.5-kb mRNA was determined with pNSa14 DNA (0.580 to 0.605 map units) 5′ end labeled at the *Bst*EII sites (0.5803 and 0.586 map units). Under these labeling conditions, the 5′ protruding end of the 3′→5′ DNA strand of the internal *Bst*EII fragment will be protected by the mRNAs transcribed left to right between 0.580 and 0.588 map units. Hybridization with β or γ cytoplasmic RNA followed by S1 nuclease digestion protected two DNA bands of 0.75 and 1.1 kb in length (Fig. 8). Bands larger than 1.1 kb in these lanes represent self-annealing of probe. The 1.1-kb DNA fragment represents protection by the large mRNA species (β 5.2 kb, γ 5.0 kb, γ 7.0 kb). The 0.75-kb DNA fragment positions the 5′ end of the β 1.5-kb mRNA. Therefore, the β 1.5-kb RNA protects a DNA fragment 0.43 kb to the right of the *Bst*EII site at position 0.586 and 0.75 kb to the left of position 0.586. The total length of DNA protected by the β 1.5-kb RNA is therefore 1.18 kb.

**mRNA species located between 0.589 and 0.599 map units.** pNBa3 digested with the restriction endonucleases *Xho*I and *Sal*I produced two bands of 6.6 and 1.8 kb in length (data not shown). The 6.6-kb band represents 1.78 kb of viral DNA sequences from 0.583 to 0.593 map units plus 4.8 kb of plasmid DNA sequences. The 1.8-kb band spans the region from the *Xho*I site in the insert to the *Sal*I site in the plasmid vector and therefore represents 1.53 kb of viral DNA sequences from 0.593 to 0.601 map units plus 0.3 kb of plasmid DNA.

Total cytoplasmic β RNA protected a DNA band of 0.615 kb in length from S1 nuclease digestion when hybridized to the 6.6-kb *Xho*I-*Sal*I DNA fragment of pNBa3 labeled at the 3′ end (Fig. 8). This positions a 3′ end 0.615 kb to the left of the *Xho*I site (position 0.593). Hybridization and subsequent S1 digestion of β and γ RNA to the 1.8-kb *Xho*I-*Sal*I DNA fragment of pNBa3 labeled at the 5′ end protected a DNA

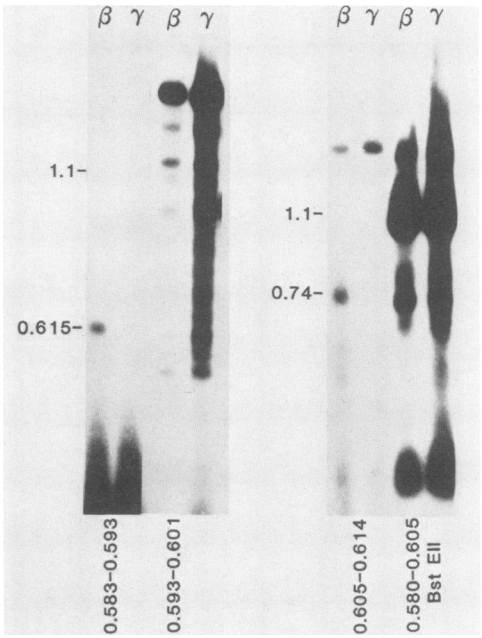


FIG. 8. Neutral gel of S1 nuclease mapping. DNA subclones labeled at either the 3′ or 5′ end were hybridized to either β or γ cytoplasmic RNA, digested with S1 nuclease, and fractionated by electrophoresis. DNA representing 0.583 to 0.593 map units was 3′ end labeled at position 0.593. DNA representing 0.593 to 0.601 map units was 5′ end labeled at position 0.593. DNA representing 0.605 to 0.614 map units was 5′ end labeled at position 0.614. DNA representing 0.580 to 0.605 map units was 5′ end labeled at the *Bst*EII sites (positions 0.5803 and 0.586). DNA sizes were determined by migration patterns relative to a 123-bp DNA ladder (Bethesda Research Laboratories).

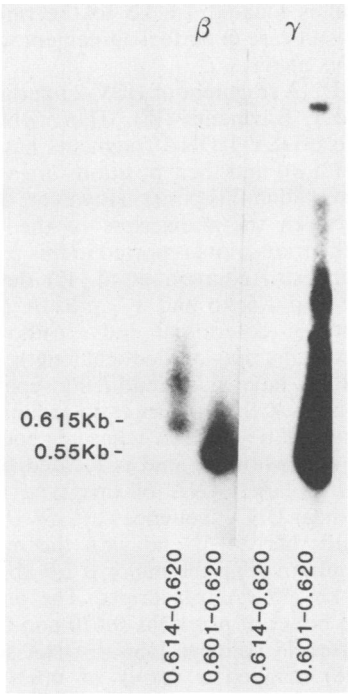


FIG. 9. Alkaline gel of S1 nuclease mapping. pNBS17 DNA (0.614 to 0.620 map units) 3′ end labeled at the *Sal*I site (position 0.614) and pNBa1 DNA (0.601 to 0.620 map units) 5′ end labeled at the *Bgl*II site (position 0.620) were hybridized to either β or γ cytoplasmic RNA, digested with S1 nuclease, and fractionated by electrophoresis. DNA sizes were determined by migration patterns relative to a 123-bp DNA ladder (Bethesda Research Laboratories).

band of 1.1 kb in length (Fig. 8). This positions the 5′ end of the minor β 1.9-kb mRNA 1.1 kb to the right of the *Xho*I site (position 0.593). The minor β 1.9-kb mRNA therefore protects a DNA fragment of 1.715 kb in length. It is not clear why the 0.615-kb DNA band was not protected by γ RNA. Because RNA blot hybridization experiments clearly detected the minor 1.9-kb mRNA in both β and γ RNA preparations, the absence of the 0.615 kb DNA band with γ RNA must be due to detection problems.

**mRNA species located between 0.600 and 0.609 map units.** RNA blot experiments indicated that the major β 1.9-kb mRNA was located approximately between 0.601 and 0.614 map units. Hybridization and S1 nuclease digestion of β and γ RNA to pNSa14 DNA (0.580 to 0.605 map units) 5′ end labeled at the *Sal*I site (position 0.605) protected a DNA band of 0.984 kb in length, positioning the 5′ end of the β 1.9 kb mRNA 0.984 kb to the left of position 0.605 (Fig. 7). The 3′ end of this mRNA was determined by hybridization and S1 nuclease digestion of β and γ cytoplasmic RNA to pNBa1 DNA (0.601 to 0.620 map units) 3′ end labeled at the *Bam*HI site (position 0.601). A protected DNA band of 1.4 kb in length was seen in both neutral and alkaline gels, indicating the absence of any detectable introns (Fig. 5 and 6). Therefore, the major β 1.9-kb mRNA protects a total of 1.7 kb of DNA.

**mRNA species located between 0.617 and 0.620 map units.** RNA blot hybridization experiments indicated that the γ 3.0-kb mRNA was located to the right of position 0.614. Hybridization and S1 nuclease digestion of β and γ cytoplasmic RNA to pNBa1 DNA (0.601 to 0.620 map units) 5′ end labeled at the *Bgl*II site (position 0.620) protected a DNA band of 0.55 kb in length which was more prominent with γ RNA than with β RNA (Fig. 7 and 9). This positions the 5′ end of the γ 3.0-kb mRNA 0.55 kb to the left of position 0.620. This band was detected in both neutral and alkaline gels, indicating the absence of any detectable introns.

The detection of this band with β RNA may be due to a difference in the limits of detection between RNA blot hybridization experiments and S1 nuclease mapping. In the presence of the DNA synthesis inhibitor arabinosylcytosine, the abundance of γ mRNA transcripts was greatly diminished, as evidenced by RNA blot hybridization experiments (Fig. 4A). A few copies of the γ 3.0-kb mRNA must still be transcribed in the presence of arabinosylcytosine. Detection under S1 mapping conditions appears more sensitive than RNA blot hybridization in this situation. Frink and coworkers (8) have described two minor overlapping species of mRNA (2.6 to 2.8 kb) encoded from the opposite strand from the 2.7-kb gC mRNA to the right (called 3 kb here). We specifically looked for such RNAs by probing with pNBS17 5′ end labeled at the *Sal*I site (Fig. 7, lanes 5 and 10) and were unable to detect them.

**Other mRNA species located between 0.601 and 0.620 map units.** RNA blot hybridization experiments revealed the presence of a large γ mRNA 6.0 kb in length which was located to the right of position 0.601. Since this mRNA hybridized to every region right of position 0.601, it must have either its 5′ or 3′ end located near positions 0.601 to 0.605. Hybridization and S1 nuclease digestion of β and γ RNA to either pNBa1 DNA (0.601 to 0.620 map units) 3′ end labeled at the *Bgl*II site (position 0.620) or pNSa14 DNA (0.580 to 0.605 map units) 3′ end labeled at the *Sal*I site (position 0.605) failed to protect any DNA sequences (Fig. 5 and 6). This indicates that the γ 6.0-kb mRNA must have its 5′ end near position 0.601. However, as discussed earlier, hybridization and S1 nuclease digestion of γ RNA to pNSa14 DNA (0.580 to 0.605 map units) 5′ end labeled at the *Sal*I site (position 0.605) protected only a single band of 0.984 kb in length (Fig. 7). Therefore, we hypothesize that the γ 6.0-kb mRNA shares a 5′ end with the major β 1.9-kb mRNA (located 0.984 kb to the left of the *Sal*I site) and that its 3′ end is located to the right of position 0.620. However, we cannot exclude the possibility that the protection we observe is due

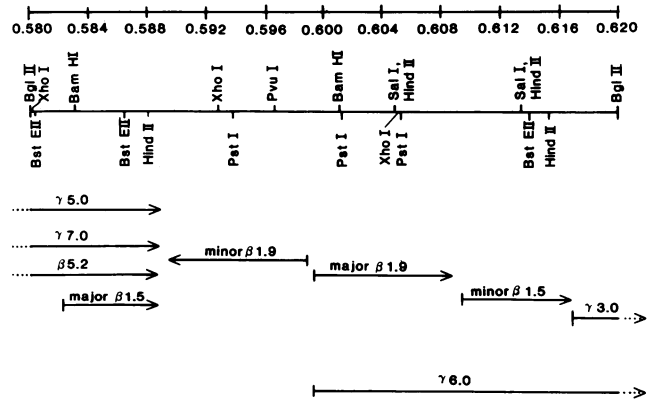


FIG. 10. Schematic localization of HSV-2 mRNA species mapping between positions 0.580 and 0.620 of the HSV-2 genome. This figure is based on data summarized in Table 1. The arrows indicate direction of transcription. The total length of the poly(A)$^+$ mRNAs and their time of appearance during infection are shown above them.

predominantly to hybridization of the major β 1.9-kb mRNA and that we have failed to detect shorter protected regions that would arise if the 5′ end of the γ 6.0-kb mRNA is located somewhere to the right of the 5′ end of the major β 1.9-kb mRNA.

The mRNAs mapped thus far are densely packaged throughout the *Bgl*II N DNA fragment with the exception of the region 0.610 to 0.617 map units. Besides the γ 6.0-kb mRNA, RNA blot hybridization experiments did not reveal any viral mRNA that would map in this region. To investigate this, β and γ RNA were hybridized to pNBS17 DNA (0.614 to 0.620 map units) 3′ end labeled at the *Sal*I site (position 0.614) and digested with S1 nuclease. Analysis of the digestion products on both neutral and alkaline gels revealed a DNA band protected by β RNA of 0.615 kb in length, whereas γ RNA failed to protect any DNA sequences (Fig. 6 and 9). This positions the 3′ end of a mRNA transcript 0.615 kb to the right of position 0.614. To locate the 5′ end, pNSa4 DNA (0.605 to 0.614 map units) was digested with *Sal*I and *Pst*I. *Sal*I releases the viral DNA insert, and *Pst*I cuts 60 bp to the right of position 0.605. This produces a DNA fragment containing a *Pst*I site at position 0.605 and a *Sal*I site at position 0.614. Since *Pst*I digestion produces 3′ protruding ends, the *Pst*I site is not labeled at the 5′ end by polynucleotide kinase. The pNSa4 *Sal*I-*Pst*I DNA fragment was isolated from an agarose gel and 5′ end labeled. Hybridization and S1 nuclease digestion of β and γ RNA to the 5′ end-labeled pNSa4 *Sal*I-*Pst*I DNA fragment revealed a DNA band protected by β RNA of 0.738 kb in length, whereas γ RNA failed to protect any DNA sequences (Fig. 8). Thus, we have characterized a β mRNA transcript whose 5′ end is 0.738 kb to the left of position 0.614 and whose 3′ end is 0.615 kb to the right of position 0.614. The total amount of protected DNA sequences is 1.35 kb, representing a minor β mRNA that we were unable to detect by RNA blot hybridization experiments. Assuming a poly(A)$^+$ tail of 200 to 300 bp, the length of this minor mRNA would be 1.5 to 1.6 kb. In this case, we feel the S1 analysis was more sensitive because in Northern blotting the only probes utilized were, first, much larger than the minor β 1.5-kb RNA itself and, second, also capable of binding to much more abundant mRNAs, such as the major β 1.9 kb or the γ 3.0 kb. Specific end labeling of the *Sal*I site at position 0.614 would produce probes that would only bind to the minor β 1.5-kb RNA when cytoplasmic β RNA was probed.

## DISCUSSION

We have precisely mapped the locations of nine mRNAs encoded by the *Bgl*II N DNA fragment of HSV-2. A summary of the mRNA maps is shown in Fig. 10. The mRNA transcripts are densely packaged throughout the *Bgl*II N DNA fragment with less than 150 bp located between the ends of neighboring mRNA transcripts and do not appear to have any detectable introns. McLauchlan and Clements (17) recently mapped two β mRNAs of 5.4 and 1.5 kb with 3′ coterminal ends 1.6 kb to the right of position 0.580. They located the 5′ end of the 1.5 kb mRNA 143 bp to the left of the *Bam*HI site at position 0.583. These results are in perfect agreement with the results presented in this paper. In addition, we have identified two γ mRNA transcripts of 5.0 and 7.0 kb that also overlap with the β 5.2-kb and β 1.5-kb mRNAs sharing coterminal 3′ ends. Recently, Galloway and Swain (12) reported the nucleotide sequence of the left-hand 1.9 kb of the *Bgl*II N fragment. Based on the nucleotide sequence, the 5′ end of the major β 1.5-kb mRNA is located 140 bp to the left of the *Bam*HI site at position 0.583, and the poly(A)$^+$ signal is located 1.6 kb to the right of position 0.580. These results are in perfect agreement with the results presented in this paper.

The *Bgl*II N DNA fragment of HSV-2 overlaps the HSV-1 *Hin*dIII K and L fragments (18). The mRNA transcripts encoded by these HSV-1 DNA fragments have been previously mapped (2, 8), and their positions are very similar to the results presented in this paper. However, there are a few differences between the transcripts of the HSV-1 DNA sequence and the transcripts reported in this paper for HSV-2 DNA sequences. Anderson et al. (2) detected two β mRNAs of 5.2 and 1.5 kb and a γ mRNA of 7.0 kb that overlapped with 3′ coterminal ends. Although we have mapped mRNA transcripts of identical length in the *Bgl*II N DNA fragment, we have also identified a separate, overlapping 5.0-kb γ mRNA that shares a coterminal 3′ end. We have mapped a 6.0-kb γ mRNA whose 5′ end is located at position 0.600 and whose 3′ end is located to the right of position 0.620. A large RNA of this type has not been reported in colinear DNA sequences of HSV-1. This mRNA transcript would share a 5′ end with the major β 1.9-kb mRNA and would overlap the major β 1.9-kb, minor β 1.5-kb, and γ 3.0-kb mRNA transcripts. The minor β 1.5-kb mRNA located between positions 0.610 and 0.617 has also not been reported in colinear HSV-1 DNA sequences (8). Frink et al. (8) mapped a family of mRNA transcripts (average length = 3.0 kb) encoded by the HSV-1 *Hin*dIII L DNA fragment, whereas we have detected only a single mRNA transcript, the γ 3.0-kb mRNA, encoded by the *Bgl*II N DNA fragment. However, since the 5′ end of this transcript is located only 0.55 kb to the left of position 0.620, we cannot rule out the presence of additional related transcripts whose 5′ ends may be located to the right of position 0.620. We specifically looked for the two minor overlapping species of mRNA (2.6 to 2.8 kb) that have been reported (8) as encoded from the opposite strand from the 2.7-kb gC mRNA to the right (called 3 kb here) and were not able to find them. Frink and co-workers (8) did not detect an mRNA that would correspond to our minor β 1.5 kb; however, they may not have specifically examined this issue. Based on these results, it is possible that HSV-1 and HSV-2 transcripts in this region may come from opposite strands.

In attempts to associate protein products with the transformation process, the coding capacity of the transforming *Bgl*II fragment has been examined. Galloway et al. (9) reported that RNA selected by hybridization to cloned *Bgl*II N DNA fragments could direct synthesis of five polypeptides with molecular weights of 140K, 61K, 56K, 35K, and 23.5K. The sizes of the selected RNAs were not characterized. Based on the approximate locations of the DNA coding sequences reported for four of these proteins, it is probable that the 56K protein is encoded by the major β 1.9-kb mRNA (0.600 to 0.609 map units), the 61K protein is encoded by the minor β 1.9-kb mRNA (0.599 to 0.590 map units), and the 35K protein is encoded by the major β 1.5-kb mRNA (0.582 to 0.589 map units). In vitro translation experiments of isolated mRNA transcripts are necessary to confirm these results. The 140K protein detected by Galloway and co-workers (9) would be encoded by either the γ 5.0-kb or the γ 7.0-kb mRNA since this protein was detected after the onset of viral DNA synthesis. Suh and co-workers (19) mapped the coding sequences for the 35K protein at ca. 0.585 to 0.596 map units and coding sequences for the 56K protein at 0.607 to 0.612 map units.

We cannot guarantee that every transcript reported here yields a distinct protein. One way to do so would require

each RNA species to be translated in vitro and the corresponding protein to be identified in infected cell lysates.

The results presented here demonstrate that at least four separate proteins are fully encoded by the *Bgl*II N DNA fragment. No single protein has been consistently identified to date in HSV-2-transformed cells. Galloway and McDougall (11) have published that the region mapping from positions 0.583 to 0.594 is sufficient to initiate transformation. We agree with these investigators that there is not an apparent complete mRNA mapping in this region. This would exclude the necessity for a complete protein product in the transformation process. Since the mechanism for such a transformation has not yet been defined, the coding capacity of this DNA fragment remains a focus of interest. The possibility remains that one or more of the protein products of the *Bgl*II-N region may initiate or regulate (or both) the transforming event. The results presented in this paper give a solid foundation for further research into the molecular biology of the *Bgl*II N DNA fragment.

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