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Detection by RNA Blot Hybridization of RNA Sequences Homologous to the *Bgl*II-N Fragment of Herpes Simplex Virus Type 2 DNA

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RNA species, extracted at the time of peak synthesis of the α, β, and γ classes of herpes simplex virus polypeptides from lytically infected Vero cells, were examined for homology to the *Bgl*II-N fragment (map units 0.58 to 0.63) of herpes simplex virus type 2 DNA. By using northern blot analysis, two major and several minor polyadenylated RNA species showed homology to the *Bgl*II-N fragment at times corresponding to the maximum synthesis of the β (7 h postinfection) and γ (12 h postinfection) herpes simplex virus polypeptides. No α RNA homologous to the *Bgl*II-N fragment was detected.

Herpes simplex virus type 2 (HSV-2) was first shown to induce morphological cell transformation more than a decade ago (8, 9). Recent efforts have focused on the direct identification of the region(s) of the HSV-2 genome responsible for induction and maintenance of morphological transformation (14, 16, 19). Reyes and co-workers (19) reported morphological transformation of BALB/c/3T3 cells with the 4.6 × 10^6-dalton *Bgl*II-N fragment of HSV-2 strain 333, which maps between 0.58 and 0.63 on the virus genome. Galloway and McDougall (14) later extended these findings by transforming primary rat embryo cells and NIH/3T3 cells with a recombinant DNA containing the *Bgl*II-N fragment cloned into pBR322.

Docherty and co-workers (7) identified one protein (molecular weight, 37,800 [37.8K]) coded by the *Bgl*II-N region of HSV-2 DNA with hybrid-arrested translation experiments. Galloway and co-workers (13) recently reported that RNA selected by hybridization to a cloned *Bgl*II-N fragment of HSV-2 DNA could direct synthesis of five polypeptides with molecular weights of 140K, 61K, 56K, 35K, and 23.5K. The RNA for one of these (35K) is apparently synthesized in small amounts in the absence of DNA synthesis. This protein probably corresponds to the abundant 37.8K protein reported by Docherty et al. (7). Because of the potential significance of the *Bgl*II-N region of HSV-2 DNA in transformation, we were interested in the number and type of RNAs homologous to the *Bgl*II-N fragment in lytically infected Vero cells.

Monolayers of Vero cells were grown at 37°C in medium 199 supplemented with 5% fetal calf serum, 5% donor calf serum, 0.075% $NaHCO_3$, and 20 μg of kanamycin per ml. HSV-2 strain 333 stocks were routinely grown in Vero cells at 37°C at 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 (9). HSV-2 DNA was extracted from virus particles concentrated by zinc acetate precipitation and partially purified by pelleting onto a 1.4-g/ml CsCl cushion (unpublished data). Recombinant plasmid p29, containing the HSV-2 strain 333 *Bgl*II-N fragment, was kindly provided by N. Frenkel, University of Chicago, Chicago, Ill. The plasmid was amplified in *Escherichia coli* HB101, and the DNA was isolated as previously described (5). Recombinant lambda bacteriophage FJ518 was constructed by shotgun cloning of *Bgl*II-digested HSV-2 strain 333 DNA into the *Bam*HI site of the lambda cloning vector Charon 27. The resulting recombinant clones were screened with whole HSV-2 DNA probe by the in situ hybridization procedure of Benton and Davis (3) for virus DNA-containing recombinants. FJ518 DNA was grown and purified as described previously (11). Insertion of the *Bgl*II-N fragment was confirmed by restriction enzyme analyses of FJ518 on agarose gels and Southern blot analyses with FJ518 probe against *Bgl*II-N-cleaved HSV-2 DNA (data not shown).

Cytoplasmic RNA was extracted from lytically infected Vero cells at three separate times. Monolayers of Vero cells were infected with HSV-2 strain 333 at a multiplicity of infection of 10 to 20 PFU per cell. Cytoplasmic RNA, rou-

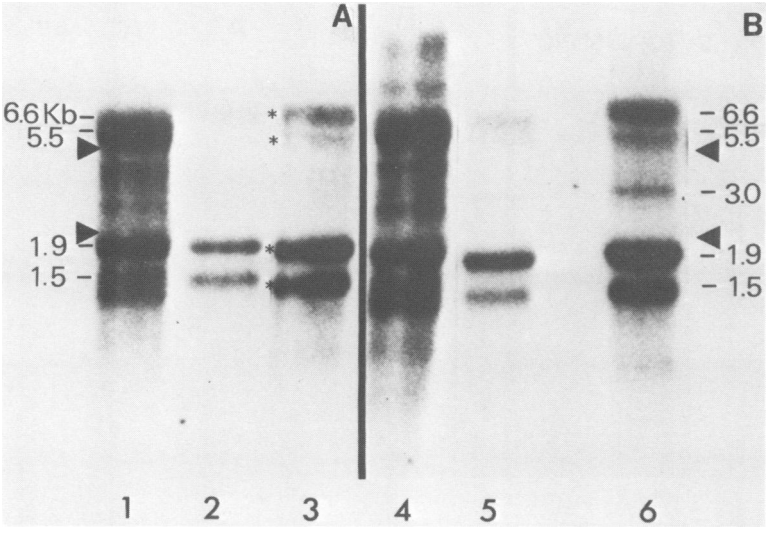

FIG. 1. Northern blot analysis of β (A) and γ (B) cytoplasmic RNA from HSV-2-infected Vero cells. RNA samples were electrophoresed in 1.4% agarose–10% Formalin horizontal slab gels at 30 V overnight (17). After ethidium bromide staining, the gels were soaked in 20× SSC (1× SSC = 0.15 M NaCl plus 0.015 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. Nitrocellulose strips containing transferred RNA were prehybridized in 5 ml of buffer containing 50% deionized formamide, 5× SSC, 5× Denhart solution (1× Denhart solution = 0.02% bovine serum albumin–0.02% Ficoll–0.02% polyvinyl pyrrolidone [6]), 50 mM $NaPO_4$ (pH 6.5), 1% glycine, and 200 μg of sonicated, denatured calf thymus DNA for 16 to 20 h at 42°C. Hybridization was carried out at 42°C for 20 to 24 h in 5 ml of hybridization buffer containing 50% deionized formamide, 5× SSC, 1× Denhart solution, 20 mM $NaPO_4$ (pH 6.5), 10% sodium dextran sulfate, 100 μg of sonicated denatured calf thymus DNA, and 5×10^5 cpm of radioactive probe DNA per ml, denatured by boiling. After hybridization, the nitrocellulose strips were washed four times for 5 min in 2× SSC and 0.1% sodium dodecyl sulfate at room temperature, followed by two washes in 0.1× SSC and 0.1% sodium dodecyl sulfate at 50°C for 15 min. The strips were air dried and exposed to X-ray film (Cronex 4; E. I. Du Pont de Nemours, Wilmington, Del.) at −70°C. Cronex intensifying screens were used to amplify the radioactive signals. ▶, Position of 28S and 18S rRNA markers; lanes 1 and 4, probed with ^{32}P-labeled HSV DNA; lanes 2 and 5, probed with ^{32}P-labeled FJ518 DNA; lanes 3 and 6, probed with ^{32}P-labeled p29 DNA. Each lane contains 25 μg of cytoplasmic RNA.

tinely labeled in vitro by adding 5 μCi of [^{3}H]uridine per ml of culture medium 3 h before RNA isolation, served as a marker during RNA purification. RNA was extracted from Nonidet P-40-treated cells as described previously (10). For the first time point, Vero cells were pretreated for 1 h with 50 μg of cycloheximide per ml before infection and then continuously during and after virus infection. Cytoplasmic RNA was extracted 6 h postinfection, corresponding to the α RNA class of HSV polypeptide synthesis (15). For the second time point, cytoplasmic RNA was extracted from normally infected Vero cells at 7 h postinfection, corresponding to the time of maximum synthesis of virus DNA and β HSV polypeptides. For the third time point, cytoplasmic RNA was extracted from normally infected Vero cells at 12 h postinfection, corresponding to the time of maximum synthesis of structural (γ) HSV polypeptides. In this paper, we refer to the RNAs extracted from infected cells treated with cycloheximide as α RNA and those extracted at 7 and 12 h postinfection as β and γ RNAs, respectively.

RNA blot hybridization analysis of cytoplasmic RNAs (representing the β and γ classes) from HSV-2-infected Vero cells is shown in Fig. 1. When [^{32}P]dCTP-labeled (2×10^8 cpm/μg) p29 DNA was used as a probe, this analysis revealed two major and several minor RNA bands that hybridized to *Bgl*II-N DNA. Analysis of β RNA revealed two major size classes of approximately 1.9 and 1.5 kilobases (kb) that were homologous to *Bgl*II-N (Fig. 1, lane 3). The sizes of these and all RNA species detected were based on their migration patterns relative to the 28S and 18S ribosomal bands (5.2 and 2.0 kb, respectively). This sizing method has been used by other investigators (2).

In addition to the two major RNAs, two minor species were detected during the β phase. These RNAs had approximate sizes of 6.6 and 5.5 kb.

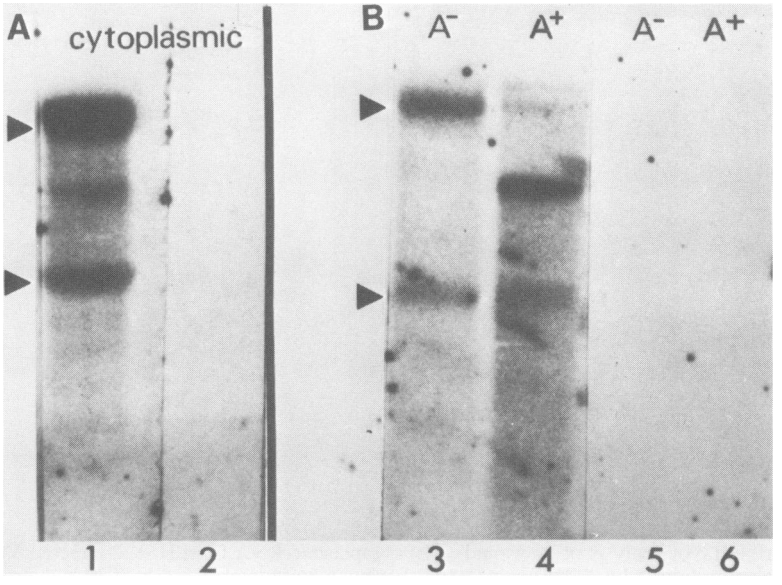

FIG. 2. Northern blot analysis of α RNA from HSV-2-infected Vero cells. (A) Cytoplasmic RNA; (B) non-polyadenylated RNA. ▶, Position of 28S and 18S rRNA markers; A^-, non-polyadenylated RNA; A^+, polyadenylated RNA; lanes 1, 3, and 4, probed with ^{32}P-labeled HSV virion DNA; lanes 2, 5, and 6, probed with ^{32}P-labeled p29 DNA. Lanes 1 and 2 contain 25 μg of cytoplasmic RNA; lanes 3 and 5 contain 15 μg of non-polyadenylated RNA; lanes 4 and 6 contain 5 μg of polyadenylated RNA.

Hybridization of β cytoplasmic RNAs to whole HSV DNA probe gave the expected pattern of heterogeneous virus RNAs (Fig. 1, lane 1). It was, however, still possible to visualize the two major bands that comigrated with the 1.9- and 1.5-kb bands detected with the p29 DNA probe. Analysis of γ RNA revealed several *Bgl*II-N-specific bands (first detected during the β phase) and one unique γ band (Fig. 1, lane 6). The prominent 1.9- and 1.5-kb β RNA species were usually present in greater abundance during the γ phase and were the most prominent γ RNA species detected with the p29 DNA probe. Based on the intensity of short-exposure autoradiography, these two species appeared to increase in their relative abundance during the shift from β to γ RNA synthesis (data not shown). A unique γ RNA species of approximately 3.0 kb was also detected. No α RNA homologous to the *Bgl*II-N fragment was detected (Fig. 2, lane 2), even though labeled probe representative of the entire HSV genome demonstrated several nonabundant cytoplasmic RNA species synthesized by this time (Fig. 2, lane 1); this observation agrees with previously published reports (21).

The preceding results with a plasmid *Bgl*II-N recombinant (60% HSV-specific DNA) were confirmed with FJ518, a lambda bacteriophage recombinant constructed in this laboratory. Because FJ518 DNA contains only 14% HSV-2-specific DNA, an FJ518 probe labeled to 2×10^8 cpm/μg is not as sensitive as a comparable p29 plasmid probe. However, even with the less sensitive probe, it was still possible to detect the two major (1.9- and 1.5-kb) *Bgl*II-N-specific RNA bands (Fig. 1, lanes 2 and 5). Minor bands were not detected as readily with the FJ518 probe as with the plasmid probe.

The *Bgl*II-N-specific RNA species detected were present only in infected cells. Normal Vero cell cytoplasmic RNA failed to hybridize with the *Bgl*II-N DNA probe (data not shown) under conditions identical to those shown in Fig. 1. In these control experiments, the specific activity of the p29 probe was identical to that of the p29 probe used for the blot hybridizations of infected cell RNAs.

To more closely define the homology of the three cytoplasmic RNA preparations to *Bgl*II-N, the preparations were further separated into polyadenylated and non-polyadenylated species by oligodeoxythymidylate [oligo(dT)]-cellulose chromatography as described previously (10). Analyses of polyadenylated and non-polyadenylated species of the α class (Fig. 2) again showed no homology to the *Bgl*II-N DNA probe (Fig. 2, lanes 5 and 6), whereas whole virus DNA probe detected several nonabundant species (Fig. 2, lanes 3 and 4).

Analyses of β and γ RNA polyadenylated and non-polyadenylated RNA species (Fig. 3) re-

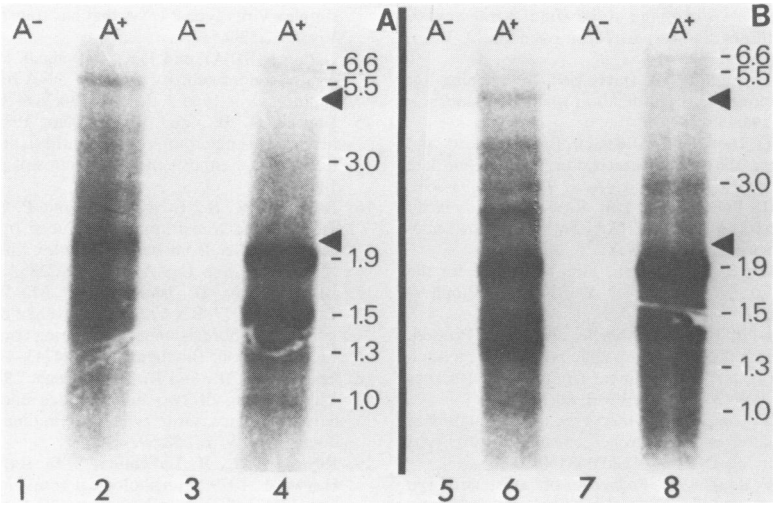

FIG. 3. Northern blot analysis of β (A) and γ (B) RNA from HSV-infected Vero cells. ▶, Position of 28S and 18S rRNA markers; A^-, non-polyadenylated RNA; A^+, polyadenylated RNA; lanes 1, 2, 5, and 6, probed with ^{32}P-labeled HSV DNA; lanes 3, 4, 7, and 8, probed with ^{32}P-labeled p29 DNA. Lanes 1, 3, 5, and 7 contain 15 μg of non-polyadenylated RNA; lanes 2, 4, 6, and 8 contain 5 μg of polyadenylated RNA.

vealed that all of the *Bgl*II-N-specific RNA detected in cytoplasmic RNA was polyadenylated. In addition to the RNA species detected with whole cytoplasmic β RNA (6.6, 5.5, 1.9, and 1.5 kb), three additional minor RNA species were detected with approximate sizes of 3.0, 1.3, and 1.0 kb (Fig. 3, lane 4). To detect these minor species, overexposure of the blot was necessary, which obscured the region of the 1.9- and 1.5-kb RNAs. In these experiments, the abundance of the 6.6- and 5.5-kb RNAs relative to that of the 1.9- and 1.5-kb RNAs was diminished, probably owing to partial degradation of the large RNA during separation of polyadenylated and non-polyadenylated RNAs. Although none of these additional minor RNA species was detected in whole cytoplasmic β RNA extracts, the 3.0-kb species was detected in whole γ cytoplasmic RNA. Therefore, it was concluded that the 3.0-kb RNA species was present in very small amounts during the β phase and increased in copy number during the γ phase. Analysis of γ polyadenylated RNA also revealed small minor RNA species (1.3 and 1.0 kb) not detected in whole cytoplasmic extracts (Fig. 3, lane 8).

Assuming that the two major and the three to five minor RNAs detected can each code for a separate polypeptide, one would expect that five to seven polypeptides are coded at least partially by *Bgl*II-N. Galloway et al. (13) detected five polypeptides (140K, 61K, 56K, 35K, and 23.5K) that can be translated in vitro after the addition of *Bgl*II-N-selected RNA to a rabbit reticulocyte lysate translation system. With the exception of two α RNAs (22) and one γ RNA (12), the HSV RNAs examined do not have any large introns or noncontiguous leader sequences (2, 4, 18). At present, we cannot directly relate the RNAs detected in this study to the polypeptides described by Galloway et al. (13).

Although our results indicate that the coding capacity and RNA transcription of the *Bgl*II-N fragment are complex, we cannot discern between RNA species that are fully encoded for by the *Bgl*II-N fragment and those that contain only 5′ or 3′ sequences in the fragment. Our results simply indicate that the RNA species detected contain some homology to the *Bgl*II-N fragment.

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ADDENDUM IN PROOF

These RNA studies were repeated with the addition of the DNA synthesis inhibitor arabinosylcytosine (20 μg/ml) for 7 h after infection, instead of using a 7-h postinfection time point, to define the β class. In the presence of this inhibitor, the 6.6-, 1.9- and 1.5-kb species appeared during the β phase, suggesting that the 5.5- and 3.0-kb species are early γ RNA transcripts.

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