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An Epstein-Barr Virus DNA Fragment Encodes Messages for the Two Major Envelope Glycoproteins (gp350/300 and gp220/200)

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The genes encoding the two major Epstein-Barr virus glycoproteins (gp350/300 and gp220/200) have been mapped to a 5-kilobase fragment of the viral genome (*Bam*HI-L). This fragment encodes 3.4- and 2.8-kilobase RNAs which translate proteins of 135 and 100 kilodaltons, respectively. Both proteins react with antiserum specific for gp350/300 and gp220/200. The 135-kilodalton protein is identical in size to the nascent polypeptide precursor to gp350/300, and the 100-kilodalton protein is the expected size of the polypeptide precursor to gp220/200.

Epstein-Barr virus (EBV) causes infectious mononucleosis and has been implicated in the etiology of human cancers, including Burkitt's lymphoma, polyclonal B cell lymphoma, and nasopharyngeal carcinoma (8). The virus is coated by a membrane composed of lipid and four major virus-specified proteins, gp350/300, gp220/200, p140, and gp85 (4–6, 15, 28, 31). These proteins are also found in the plasma membrane of productively infected cells (6, 10, 15, 16, 18, 19, 23, 28–30). The expression of gp350/300, gp220/200, p140, and gp85 is inhibited by treatment of cells with inhibitors of viral DNA synthesis, indicating that these are late proteins (18, 23, 28). Antibodies against gp350/300 and gp220/200 neutralize virus and comprise much of the neutralizing activity in human sera (10, 30, 31). These proteins are therefore important viral antigens for assaying human immunity to viral infection and for development of an effective immunogen to prevent EBV infection.

In cells infected with the B95-8 virus strain, gp350/300 is more abundant than gp220/200, whereas in P3HR-1-infected cells the reverse is true (6, 7, 19, 30). Although B95-8 virus is grown in marmoset cells and P3HR-1 virus is grown in human cells, the ratio of gp350/300 to gp220/200 is not a function of cell type (6). gp350/300 and gp220/200 are equally abundant in cells infected with other EBV isolates (6). Removal of high-mannose oligosacharrides from gp350/300 and gp220/200 with endo-β-*N*-acetylglucosaminidase H (endo H) (26) reduces gp350/300 to 160 kilodaltons (kd) and gp220/200 to 120 kd, indicating that gp350/300 and gp220/200 are both extensively glycosylated (7). gp350 and gp220 are also antigenically related since both react with the same monoclonal antibodies (10, 24, 30). The cross-reactive epitopes may be protein determinants since digestion of the 160 and 120 kd precursors with V8 protease yields several large polypeptides of identical size (7).

Although many EBV-encoded polypeptides and antigens have been mapped, their assignment to specific functions has been hampered by lack of monospecific antisera (11, 12, 17). Recently, however, a polyvalent anti-gp350/300 and -gp220/200 serum (anti-gp350/gp220) has been raised in rabbits with gp350/300 and gp220/200 purified by lectin affinity and monoclonal antibody chromatography. This serum specifically precipitates only gp350/300 and gp220/200 from EBV-infected cells (31). We have used hybrid selection, in vitro translation, and immunoprecipitation with the rabbit antiserum specific for gp350/300 and gp220/200 to map these proteins on the EBV genome. The data suggest that these two glycoproteins are translated from mRNAs encoded by the same EBV DNA restriction endonuclease fragment. Since gp350/300 and gp220/200 share common peptides and map to the same fragment, they are likely to be encoded by overlapping DNA segments.

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MATERIALS AND METHODS

Preparation of RNA. B95-8 and P3HR-1 cytoplasmic polyadenylated RNAs were purified from cells induced with 12-*O*-tetradecanoyl-phorbol-13-acetate (20 ng/ml) or 12-*O*-tetradecanoyl-phorbol-13-acetate and butyrate (3 mM) as previously described (11). When the cells were harvested 3 days after induction, ca. 25% of the cells were positive for viral capsid antigen (11).

Hybrid-selection, in vitro translation, and immunoprecipitation. The RNAs encoded by specific EBV DNA fragments were selected by hybridization to DNA fragments (3) covalently bound to paper (21) and translated in a rabbit reticulocyte lysate (NEN) as previously described (12, 17). After preabsorption with normal rabbit serum, the polypeptide products were immunoprecipitated (12, 17) with anti-gp350/220, a rabbit antiserum specific for the 350- and 220-kd EBV membrane proteins (31) and staphylococcal protein A-Sepharose (Pharmacia Fine Chemicals).

Preparation of unglycosylated gp350/300 precursor. 12-*O*-tetradecanoyl-phorbol-13-acetate-treated B95-8 cells (100 ml) were incubated with tunicamycin, an inhibitor of *N*-linked glycosylation (13, 25), at a concentration of 2 μg/ml for 3 h. The cells were then pulse-labeled for 15 min with 10 mCi of [^{35}S]methionine (>1,000 Ci/mmol, NEN) as described previously (7). The cells were lysed and prepared for immunoprecipitation with anti-gp350/220 serum as described previously (7).

Assignment of the 135- and 100-kd polypeptides to RNAs encoded by *Bam*HI-L. B95-8 polyadenylated RNA was size fractionated by continuous electroelution through a 0.8% agarose gel containing 10 mM methylmercury (1). B95-8 polyadenylated RNA (150 μg) was loaded onto a tube gel (0.8% HGT-P agarose; SeaKem) and electrophoresed at 75 V for 10 h. Agarose, gel buffer, and tubing were treated with diethylpyrocarbonate before use. Fractions were collected from the bottom of the gel (Bethesda Research Laboratories,

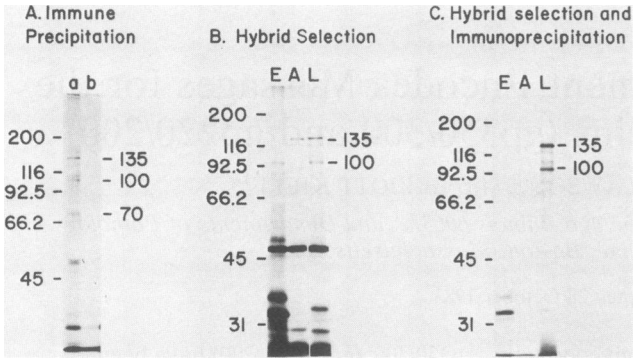

FIG. 1. Mapping the precursors to gp350/300 and gp220/200. (A) A 5-μg amount of B95-8 cytoplasmic polyadenylated RNA was translated in vitro and immunoprecipitated with rabbit anti-gp350/220 (a) or normal rabbit serum (b). (B) In vitro translation products of B95-8 RNA selected by hybridization to B95-8 DNA fragments *Eco*RI-E (E), *Bam*HI-A (A), or *Bam*HI-L (L). (C) Immunoprecipitation of the polypeptides shown in (B) with anti-gp350/220. Proteins were separated on an 8.5% polyacrylamide gel. Numbers on the left indicate the sizes (in kd) of protein standards; numbers on the right indicate the sizes of polypeptides specifically immunoprecipitated by anti-gp350/220. The doublet at 100 kd may be an artifact of in vitro translation but has been detected in three different experiments.

Bethesda, Md.). β-Mercaptoethanol was added to each 0.5-ml fraction to a concentration of 50 mM. Part of every third fraction was precipitated with ethanol and analyzed for the presence of *Bam*HI-L RNAs by Northern blot hybridization. The appropriate fractions were pooled and adjusted to a concentration of 0.4 M NaCl–20 mM Tris (pH 7.4)–0.2% sodium dodecyl sulfate–2 mM EDTA. The polyadenylated RNA was repurified by chromatography on 300 mg of oligodeoxythymidylic acid-cellulose (Collaborative Research). The RNAs were precipitated with 20 μg of tRNA and ethanol and translated in vitro in a 125-μl reaction. After preabsorption with normal rabbit serum, the polypeptide products were immunoprecipitated with anti-gp350/220.

Northern blot analysis. B95-8 or P3HR-1 polyadenylated RNA or fractionated B95-8 RNAs were electrophoretically separated on 0.8% agarose gels containing 2.2 M formaldehyde (14, 27), transferred to nitrocellulose, and hybridized to nick-translated *Bam*HI-L probe as previously described (11).

RESULTS

The precursor to gp350/300 is 135 kd. To determine the size of the protein precursor to gp350/300 (and possibly also to gp220/200), polyadenylated RNA from B95-8 cells was translated in vitro, and the resultant polypeptide antigens were immunoprecipitated with the rabbit anti-gp350/220 serum. A polypeptide of 135 kd was specifically immunoprecipitated (Fig. 1A). Other translated polypeptides of 100 and 70 kd were also immunoprecipitated by this serum and not by normal rabbit serum. A 32-kd polypeptide was evident in immunoprecipitates with the rabbit anti-gp350/220 and to a lesser extent in immunoprecipitates with normal rabbit serum (Fig. 1A). Unlike the 135-, 100-, and 70-kd proteins, which are barely discernible in translations of total B95-8 RNA without immunoprecipitation, the 32-kd protein is one of the most abundant polypeptides translated from B95-8 RNA. Since the 32-kd protein is frequently seen in immunoprecipitates with both immune and nonimmune serum, we assume that it is not specifically recognized by the anti-gp350/220 serum.

The 135-kd polypeptide was significantly smaller than the smallest precursor to gp350/300 previously identified, which has a size of 160 kd and was derived by endo H digestion of pulse-labeled gp350/300. However, endo H digestion leaves the initial *N*-linked *N*-acetylglucosamine moiety on the glycoprotein precursor (26), and this could affect its apparent molecular size. We therefore attempted to derive a completely unglycosylated precursor by performing immunoprecipitation with anti-gp350/220 on extracts of B95-8 cells pulse-labeled for 15 min in the presence of tunicamycin, an inhibitor of *N*-linked glycosylation (13, 25). Pulse-labeling in the absence of tunicamycin gives rise to a glycosylated precursor for gp350/300 of 190 kd which, in the presence of increasing amounts of tunicamycin, shifts to a size of 135 kd (7; Fig. 2). This polypeptide was specifically immunoprecipitated with anti-gp350/220 serum and was identical in size to the 135-kd in vitro translation product (Fig. 2). The 100-kd polypeptide, which is probably the precursor to gp220/200 (see below), is not detected in immunoprecipitates of B95-8 cells pulse-labeled in the presence of tunicamycin.

***Bam*HI-L encodes the 135- and 100-kd proteins.** Many of the early and late EBV polypeptides have been mapped by hybrid selection and in vitro translation (12). Two fragments, *Bam*HI-A and *Bam*HI-L, select RNAs which translate 135-kd polypeptides. Other high-molecular-weight polypeptides are encoded by *Eco*RI-E (150 kd) and *Bam*HI-C (145 kd). To identify the EBV DNA fragment which encodes the 135-kd gp350/220 precursor, DNA fragments *Bam*HI-A, C, and L and *Eco*RI-E were each used to hybrid select RNA from productively infected B95-8 cells. The selected RNAs were translated in vitro (Fig. 1B), and the polypeptide products were immunoprecipitated with anti-gp350/220 (Fig. 1C; data from *Bam*HI-C selections were run on other gels which are not shown). The 135-kd polypeptide encoded by *Bam*HI-L was specifically immunoprecipitated by anti-gp350/220 (Fig. 1) and is identical in size to the nascent protein synthesized in vivo (Fig. 2). The 100-kd polypeptide was also translated from *Bam*HI-L-selected RNA and was specifically recognized by the anti-gp350/220 serum. It is likely that this is the precursor to gp220/200, since it is about the expected size

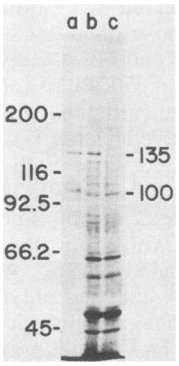

FIG. 2. Comparison of the *Bam*HI-L 135-kd in vitro translation product with the precursor to gp350/300 made in vivo. Lane a, *Bam*HI-L-specific RNA was selected by hybridization and translated in vitro, and the polypeptides were immunoprecipitated with anti-gp350/220. Lanes b and c, B95-8 cells were pulse-labeled with [^{35}S]methionine for 15 min in the presence of tunicamycin, lysed, and immunoprecipitated with rabbit anti-gp350/220 (b) or normal rabbit serum (c). Numbers are as in Fig. 1. Proteins were separated on a 7.5% polyacrylamide gel.

(see below), and gp350/300 and gp220/200 have some common component peptides, consistent with their being encoded by the same DNA fragment. The 70-kd protein which was specifically immunoprecipitated by the anti-gp350/220 serum was translated from RNA selected by *Bam*HI-R and K (data not shown), two adjacent fragments in EBV DNA which have no homology or contiguity to *Bam*HI-L (3, 9). RNA selected by *Bam*HI-R and K also translated proteins of 50 and 46 kd which appeared to be specifically immunoprecipated by anti-gp350/220. *Bam*HI-R and K do not select RNAs encoding the 135- and 100-kd polypeptides (12; data not shown). The abundant 32-kd polypeptide which is nonspecifically immunoprecipitated is encoded by *Eco*RI-E (Fig. 1C).

Identification of the mRNAs for the 135- and 100-kd proteins further links the 100-kd protein to gp220. If the 100-kd polypeptide is the precursor to gp220/200, it would be expected to be the product of a different mRNA than that which encodes the 135-kd gp350/300 precursor. *Bam*HI-L is known to encode late RNAs of 3.4 and 2.8 kilobases (kb) (11). These are barely large enough to encode the 135- and 100-kd proteins. To establish that the 3.4- and 2.8-kb RNAs encode the 135- and 100-kd proteins, B95-8 RNA was fractionated on a methylmercury agarose gel. The fractions containing RNAs encoded by *Bam*HI-L were identified by Northern blots (Fig. 3). The fractions were pooled as shown in Fig. 3, translated, and immunoprecipitated with anti-gp350/220. The 3.4-kb *Bam*HI-L RNA encodes the 135-kd polypeptide (Fig. 3, lane 5), whereas the 2.8-kb *Bam*HI-L RNA encodes the 100-kd polypeptide (Fig. 3, lane 4). Since P3HR-1 virus expresses more gp220/200 than gp350/300, there should be a difference in the ratio of the 3.4- to 2.8-kb RNAs between P3HR-1 cells and B95-8 cells. Consistent with this prediction, the 3.4- to 2.8-kb RNA ratio (Fig. 4) was higher in B95-8 cells (ca. 10:1) than in P3HR-1 cells (ca. 2:1).

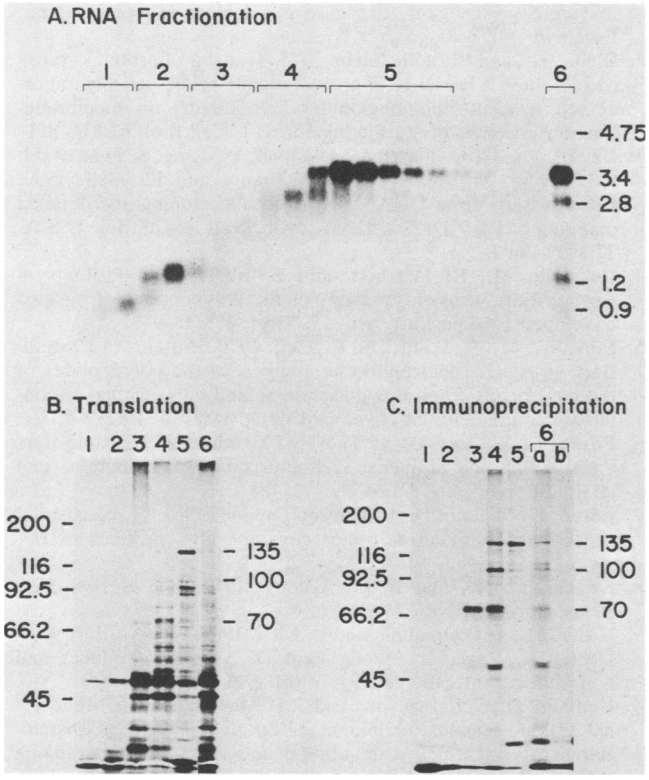

FIG. 3. Size fractionation of B95-8 RNA, identification of the fractions containing the *Bam*HI-L RNAs, and RNAs encoding the 135- and 100-kd *Bam*HI-L polypeptides. (A) B95-8 polyadenylated RNA was fractionated on a 0.8% methylmercury agarose gel as described in the text. Part of every third fraction was precipitated with ethanol, electrophoretically separated on a formaldehyde agarose gel, transferred to nitrocellulose, and hybridized to *Bam*HI-L probe. The fractions were pooled as indicated by the brackets. Lane 6, unfractionated B95-8 polyadenylated RNA. The numbers at right indicate the sizes (in kb) of *Bam*HI-L RNAs (11). (B) In vitro translation of size-fractionated B95-8 RNA. The numbers above the lanes correspond to the fractions shown in (A). The numbers at left and right are as described in the legend to Fig. 1. (C) Immunoprecipitation of the in vitro translation products shown in (B) with anti-gp350/220. Numbers are as described in (B). Polypeptides in lane 6 were immunoprecipitated with anti-gp350/220 (a) or normal rabbit serum (b).

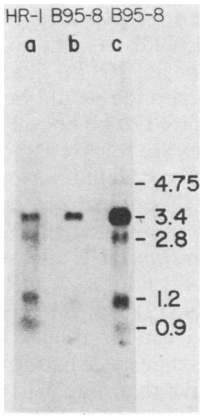

FIG. 4. Comparison of the *Bam*HI-L RNAs in cells infected with B95-8 or P3HR-1 virus. P3HR-1 polyadenylated RNA (5 μg) (a) was coelectrophoresed with 1 μg (b) and 5 μg (c) of B95-8 RNA on a 0.8% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose, and hybridized to nick-translated *Bam*HI-L probe. Lane a, 1-week exposure; lanes b and c, 1-day exposure. The numbers at right indicate the sizes (in kb) of RNAs encoded by B95-8 *Bam*HI-L (17). These RNAs probably correspond to the four major *Bam*HI-L RNAs previously reported in P3HR-1 cells (32).

DISCUSSION

In this report, we show that the major structural glycoproteins of EBV, gp350/300 and gp220/200, map to *Bam*HI-L of the genome. The main evidence for this is that *Bam*HI-L hybridizes to a late-occurring 3.4-kb RNA that encodes a 135-kd polypeptide which is identical in size to the nascent gp350/300 polypeptide and is immunoprecipitated by an antiserum which specifically precipitates only gp350/300 and gp220/200. Furthermore, the 135-kd polypeptide is too large to be the precursor to gp220/200. Including the proximal *N*-acetylglucosamine residues remaining after endo H digestion, the gp220/200 glycopolypeptide is only 120 kd (7). The evidence indicating that *Bam*HI-L also encodes gp220/200 is less complete. First, the fragment hybridizes to a late-occurring 2.8-kb RNA that encodes a 100-kd polypeptide that is specifically immunoprecipitated with anti-gp350/220 serum. Second, the size of the protein translated from the 2.8-kb RNA in vitro (100 kd) agrees well with the predicted size (95 kd) of the gp220/200 polypeptide precursor. This prediction is based on the knowledge that gp350/300 and gp220/200 have similar amounts of *N*-linked sugars, since endo H digestion of their 190- and 160-kd pulse-labeled

precursors yields glycopolypeptides of 160 and 120 kd, respectively (7). The 135-kd nascent gp350/300 polypeptide is 25 kd smaller than its 160-kd glycopolypeptide. The nascent gp220/200 polypeptide would be ca. 95 kd if it were also 25 kd smaller than its 120-kd endo H-treated derivative. Third, common peptides are released upon proteolytic cleavage of the partially glycosylated forms of gp350/300 and gp220/200, suggesting that these proteins are encoded by overlapping or partially duplicated genes. There is no detectable homology between *Bam*HI-L and other regions of EBV DNA (9). Since the 3.4- and 2.8-kb mRNAs do not extend into adjacent fragments (11) and *Bam*HI-L is only 5 kb (3), the two RNAs must overlap for at least 1 kb. (The similarity in the partial protease digestion patterns suggests that the overlap is more extensive than 1 kb [7].) Since P3HR-1 cells, unlike B95-8, express more gp220/200 than gp350/300, direct demonstration that there is a 100-kd pulse-labeled precursor in tunicamycin-treated P3HR-1 cells would provide additional evidence that the 100-kd polypeptide is the precursor to gp220/200. However, at this point, we are unable to achieve adequate EBV replication in P3HR-1 cells to detect the pulse-labeled precursor in tunicamycin-treated cultures.

In B95-8 cells, the high abundance of the 3.4-kb RNA relative to the 2.8-kb RNA correlates with the abundance of gp350/300 relative to gp220/200. Because the amount of gp220/200 in B95-8 cells is so low, the failure to detect the 100-kd precursor to gp220/200 in tunicamycin-treated pulse-labeled B95-8 cells is not surprising. The relative amounts of the 135- and 100-kd proteins translated from the 3.4- and 2.8-kb B95-8 RNAs in vitro does not correlate with the relative abundance of their RNAs. Nor does the relative abundance of gp350/300 and gp220/200 in P3HR-1 cells correlate with the relative amounts of the 3.4- and 2.8-kb P3HR-1 RNAs. In vitro translations of B95-8 RNA yield approximately equal amounts of the 135- and 100-kd proteins despite the greater abundance of the 3.4-kb RNA, which translates only the 135-kd protein (Fig. 1 to 3). Thus, the enhanced translation in vitro of the 2.8-kb RNA relative to the 3.4-kb RNA must be an artifact of translation by the reticulocyte lysate. P3HR-1 cells have a lower ratio of 3.4- to 2.8-kb RNA than B95-8 cells but there is still more 3.4- than 2.8-kb RNA. The greater abundance of gp220/200 in P3HR-1 cells is therefore due only in part to differences in RNA abundance and must also be due to deficient translation of the 3.4-kb RNA or deficient processing of the 135-kd polypeptide. For example, a mutation in the signal sequence (2) of P3HR-1 gp350/300 might impair its insertion into the membrane and subsequent glycosylation. A small deletion which could have such an effect has been detected in *Bam*HI-L of P3HR-1 viral DNA (20).

Polypeptides of 70, 50, and 46 kd are also immunoprecipitated by the anti-gp350/220 serum. These polypeptides are all encoded by *Bam*HI-R and K and probably share common antigenic determinants encoded by common nucleotide sequences. There is no detectable nucleic acid homology between *Bam*HI-L and R or K (9), and these proteins are not found in immunoprecipitates of infected cell extracts with the anti-gp350/220 serum (7, 31; Fig. 2). Thus, this precipitation is probably due to a cross-reactive epitope(s) exposed only in the in vitro translation product and cryptic in native protein. Although a monoclonal antibody specific for gp85 identifies a 70-kd precursor in cells pulse-labeled in the presence of tunicamycin (22), the anti-gp350/220 serum has no reactivity to gp85 or its partially glycosylated precursor (7, 31).

Confirmation that gp350/300 and gp220/200 are encoded by overlapping *Bam*HI-L nucleotides will likely come from precise mapping and sequencing of the DNA encoding the 3.4- and 2.8-kb RNAs and from synthesis of the derivable peptides. Antisera to peptides translated from the nucleotides common to the 3.4- and 2.8-kb RNAs should react with both gp350/300 and gp220/200. These peptides will also be useful as diagnostic reagents and possibly as immunogens.

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