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Mapping of polypeptides encoded by the Epstein–Barr virus genome in productive infection

(hybrid selection/*in vitro* translation/proteins/antigens)

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ABSTRACT **Over 30 viral-specified polypeptides are translated *in vitro* from RNA of cells productively infected with Epstein–Barr virus (EBV). The polypeptides map to sites in EBV DNA by hybrid selection. Almost all of the polypeptides are reactive with EBV immune human serum. Several of the polypeptides are part of the early antigen complex. Two others are likely to be major structural components of the virus. Genes encoding persistent early and late polypeptides are intermixed through most of the EBV genome.**

The host range of Epstein–Barr virus (EBV) *in vitro* is limited to primate B lymphocytes, which are unique in the possession of virus receptors. Infection is usually latent and results in stimulation of lymphocyte growth (for reviews, see ref. 1). Only three relatively abundant and two minor viral mRNAs and two small nonpolyadenylylated RNAs are expressed in latently infected, growth-transformed cells (2–4). Two new intranuclear antigenic polypeptides and a new plasma membrane antigen (MA) have been detected in these cells (1, 5–7).

Some cultures of latently infected lymphocytes, such as Raji cells, can be induced to express additional viral RNAs and proteins by exposure to chemical inducers or by superinfection with the P3HR-1 EBV strain (8, 9). The predominant polypeptides synthesized in chemically induced Raji cells and in superinfected or productively infected cells treated with inhibitors of viral DNA synthesis to block "late" gene expression are components of the virus early antigen (EA) complex (10–16). EA antibody in immune human sera is usually associated with early primary EBV infection, with persistent active infection, or with the development of EBV-associated malignancies (17).

In cultures that are partially permissive of EBV replication, the prevalence of cells permissive for virus infection can be increased to 20–30% or more by chemical induction (18). The virus structural polypeptides, many of the polypeptides that are synthesized early and late in productive virus infection, and several of the components of EA, virus capsid antigen (VCA), and MA have been identified in extracts of cultures of productively infected cells (11–16, 19–25). Many of the cytoplasmic polyadenylylated RNAs encoded by viral DNA in productive infection also have been identified and mapped to specific restriction endonuclease fragments of EBV DNA (26). Persistent early RNAs (and polypeptides) have been distinguished from late RNAs (and polypeptides) by the greater sensitivity of late gene expression to inhibition of viral DNA synthesis (11–16, 26). We now report the mapping of persistent early and late polypeptides to specific fragments of viral DNA by hybrid selection and *in vitro* translation (27). The translation products can be correlated with the persistent early and late RNAs encoded by these fragments (26) and with the polypeptides and antigens previously identified in permissively infected cells.

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MATERIALS AND METHODS

Infected Cell RNA and Viral DNAs. B95-8 cells were induced (15–20% VCA induction) with 12-*O*-tetradecanoylphorbal 13-acetate (phorbal acetate; ref. 18). For the preparation of persistent early RNA, cultures also were treated with phosphonoacetic acid, which resulted in induction of EA in 10% of the cells (26). VCA was not detected in phosphonoacetic acid-treated cultures. Virus was prepared from phorbal acetate-treated B95-8 cells. B95-8 DNA was extracted from virions purified from the extracellular fluid of phorbal acetate-induced cells (23). [^{35}S]Methionine-labeled virus was purified from cultures incubated with [^{35}S]methionine from 72 to 96 hr after induction (23). Polyadenylylated cytoplasmic RNA and phage, plasmid, and cosmid recombinant EBV DNAs were purified as described (26, 28, 29).

Selection of DNA Fragment-Specific mRNA. Fifty micrograms of plasmid, phage, or viral DNA was bound to a 1-cm diazobenzyloxymethyl paper circle (30). Cytoplasmic polyadenylylated RNA (1 mg/ml) was hybridized in a single reaction vessel to several different paper circles for 4 hr at 50°C (31). Unbound RNA was removed by washing the circles individually five times for 5 min at 50°C in 5 ml of 50% recrystallized formamide/0.5% $NaDodSO_4$/40 mM Pipes, pH 6.5/15 mM NaCl. The selected RNA was eluted (31) and ethanol-precipitated with 20 μg of tRNA as a carrier. The size of unbound and bound RNAs was monitored on RNA blots (26) and was not affected by hybridization and elution.

***In Vitro* Translation, Immunoprecipitation, and Gel Electrophoresis.** Half of the RNA that was eluted from a filter was used for a 25-μl *in vitro* translation reaction with reticulocyte lysate (New England Nuclear) and [^{35}S]methionine (32). After preabsorption with EBV-negative serum and staphylococcal protein A-Sepharose (Pharmacia), EBV antigens were immunoprecipitated from the *in vitro* translation in buffer without $NaDodSO_4$ (33) by using 1 μl of human serum with high titers to EBV antigens [VCA, 1:40,960; EA, 1:10,240; EBV nuclear antigen (EBNA), 1:160]. EBV-specific antigens were eluted by resuspending the protein A-Sepharose in 50 μl of $NaDodSO_4$ gel buffer. *In vitro* translation products and immunoprecipitates were separated on 8.5%, 10%, or 12% gels (34). Gels were stained with Coomassie brilliant blue, destained, impregnated with scintillant, dried, and subjected to fluorography (23). Molecular weights were determined by mobility relative to standards (Bio-Rad) [myosin, 200 kilodaltons (kDal); β-galactosidase, 116.25 kDal; phosphorylase B, 92.5 kDal; bovine serum albumin, 66.2 kDal; ovalbumin, 45 kDal; soybean trypsin inhibitor, 21.5 kDal; and lysozyme, 14.4 kDal].

Abbreviations: EBV, Epstein–Barr virus; EA, early antigen; VCA, virus capsid antigen; MA, membrane antigen; phorbal acetate, 12-*O*-tetradecanoylphorbal 13-acetate; EBNA, EBV nuclear antigen; kDal, kilodaltons.

RESULTS

Mapping of Persistent Early and Late Polypeptides. To identify the polypeptides encoded by the most abundant viral RNAs, RNAs were selected by hybridization to viral DNA or to large fragments from *Eco*RI digestion of viral DNA and translated *in vitro*. Comparison of the polypeptides translated from cytoplasmic polyadenylylated RNA with the polypeptides translated from RNA that was eluted from viral DNA filters (Fig. 1 *A* and *B*) revealed 10- to 100-fold enrichment for specific polypeptides as a result of hybridization. Viral DNA selected for polypeptides of 150, 145, 135, 88, and 32 kDal. Several of these are similar in size to major viral structural polypeptides (19, 22–24). The *Bam*HI fragments H, F, and Q, which constitute part of the *Eco*RI fragment A, selected for RNAs encoding polypeptides of 39, 38, and 34 kDal. *Eco*RI fragment B selected for RNAs encoding polypeptides of 80, 78, 50, and 46 kDal; *Eco*RI fragment C, a polypeptide of 90 kDal; and *Eco*RI fragment Dhet, a polypeptide of 135 kDal. The translation of large polypeptides from the hybrid-selected RNAs indicates that the RNA is not degraded during hybridization and elution. RNA blot analysis for specific viral RNAs confirmed that the RNA remains intact and indicates that 1–2% of the RNA encoded by a DNA fragment is selected by DNA filter hybridization.

Some polypeptides were translated from all reactions whether RNA was added (Fig. 1 *A* and *B*, lanes b–f) or not (Fig. 1*B*, lane a). These polypeptides are products of RNAs endogenous to the reticulocyte lysate. Other polypeptides translated from reactions containing selected or nonselected RNA are polypeptides encoded by abundant infected-cell RNAs. These polypeptides were more abundant in early hybrid-selection experiments (e.g., Fig. 1) in which filters were washed less extensively prior to elution than in later selection experiments (e.g., see Fig. 2 or 3) done as described in *Materials and Methods*. The reticulocyte polypeptides and most of the background infected-cell polypeptides were not immunoprecipitated with hyperimmune human sera (Figs. 2 and 3). Polypeptides were considered to be viral-specific only if they were specifically translated from RNA selected by a viral DNA fragment.

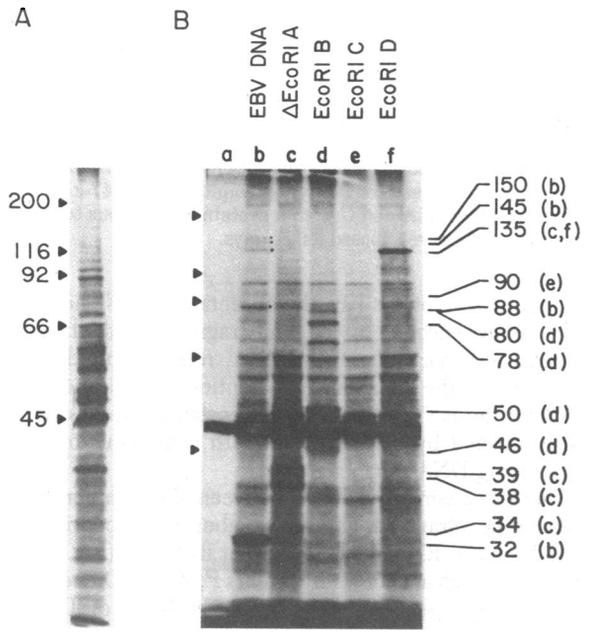

FIG. 1. Comparison of the *in vitro* translation products of RNA from B95-8 cells before (*A*) and after (*B*) selection with viral DNA or cloned *Eco*RI fragments of viral DNA. Lanes: a, product without added RNA; b, with RNA selected by viral DNA; c, with RNA selected by the portion of *Eco*RI fragment A containing the *Bam*HI H, F, and Q fragments; d, e, and f, with RNAs selected by *Eco*RI B, C, and Dhet fragments, respectively. Size markers in *A* are standards shown in kilodaltons. Size markers in *B* are selected polypeptides shown in kilodaltons; letters in parentheses indicate the lanes in which they are present.

Each of the fragments of viral DNA that are known to encode RNAs in productive infection (26) were used to select the RNAs for *in vitro* translation. The polypeptides translated from each selected RNA were denatured and separated by size on 8.5% or 12% polyacrylamide gels (Fig. 2 *A* and *B* or *C* and *D*, respectively). The hybrid-selected polypeptides (Fig. 2, lanes d–q) were compared with the structural polypeptides of the virus (lane c) and with the polypeptides translated from total infected-cell cytoplasmic polyadenylylated RNAs that were immunoprecipitated with high-titer immune polyvalent human antiserum (lane b) but not with nonimmune serum (lane a). The hybrid-selected polypeptides were also immunoprecipitated with immune human serum to distinguish viral proteins from those endogenous to the reticulocyte lysate and to identify the antigenically important polypeptides (Fig. 2 *B* and *D*); the polypeptides evident in the immunoprecipitate of polypeptides translated from infected-cell RNA (lane b) included a 150-kDal polypeptide similar in size to the major 150-kDal nucleocapsid polypeptide (lane c), a 145-kDal polypeptide similar in size to the major nonglycosylated viral membrane polypeptide (lane c), and polypeptides of 135, 94, 90, 85, 80, 78, 68, 47, 44, 39, 36, 34, 32, 22, and 18 kDal. Each polypeptide was tentatively mapped to a specific fragment of viral DNA by demonstrating that the fragment specifically selects for the RNA which translates that polypeptide and that the selected polypeptide is specifically immunoprecipitated by EBV-positive serum (Fig. 2, lanes d–p). The abundance of each polypeptide in translation products of selected RNAs and in their immunoprecipitates correlated roughly with the abundance of the polypeptide in the total infected-cell RNA translation product. This was particularly evident in comparison with the polypeptides smaller than 45 kDal (Fig. 2 *C* and *D*). Several polypeptides, such as the 220-kDal polypeptide (lane f), the 72-kDal polypeptide (lane k), the 63-kDal polypeptide (lane j), the 58-kDal polypeptide (lane n), the 52-kDal polypeptide (lane f), the 51-kDal polypeptide (lane n), the 50-kDal polypeptide (lanes j and l), etc., were evident in translation products of selected RNA or in immunoprecipitates of hybrid-selected translation products, or both, and were not evident in the immunoprecipitate of the total cell RNA product. The larger number of polypeptides evident in translation products of hybrid-selected RNA and their immunoprecipitates is probably due to the lower background and fewer specific translation products relative to the total cell RNA immunoprecipitate. Most of the polypeptides indicated in the figure were observed in several hybrid selection experiments. Those that are less certain are indicated by parentheses in the summary (see Fig. 4).

Differentiation of Persistent Early and Late Polypeptides. Persistent early polypeptides were differentiated from late polypeptides by the greater sensitivity of late mRNA synthesis to inhibition of viral DNA synthesis with phosphonoacetic acid (Fig. 3). Most of the polypeptides evident in the immunoprecipitate of productively infected-cell RNA translation product (Fig. 3, lane d) were reduced or absent from the immunoprecipitate of the translation product of RNA from phosphonoacetic acid-treated cells (Fig. 3, lane c). The 32-kDal polypeptide was relatively spared from phosphonoacetic acid inhibition (as compared with the 31-kDal or 22-kDal polypeptides); 135-, 50-, and 36-kDal polypeptides were also evident in the inhibitor-treated immunoprecipitate. Two 135-kDal polypeptides are identified in Fig. 2. One was encoded by *Bam*HI fragment L. The second was encoded by *Bam*HI fragment A and the partially overlapping *Eco*RI Dhet fragment. Because there is no homology be-

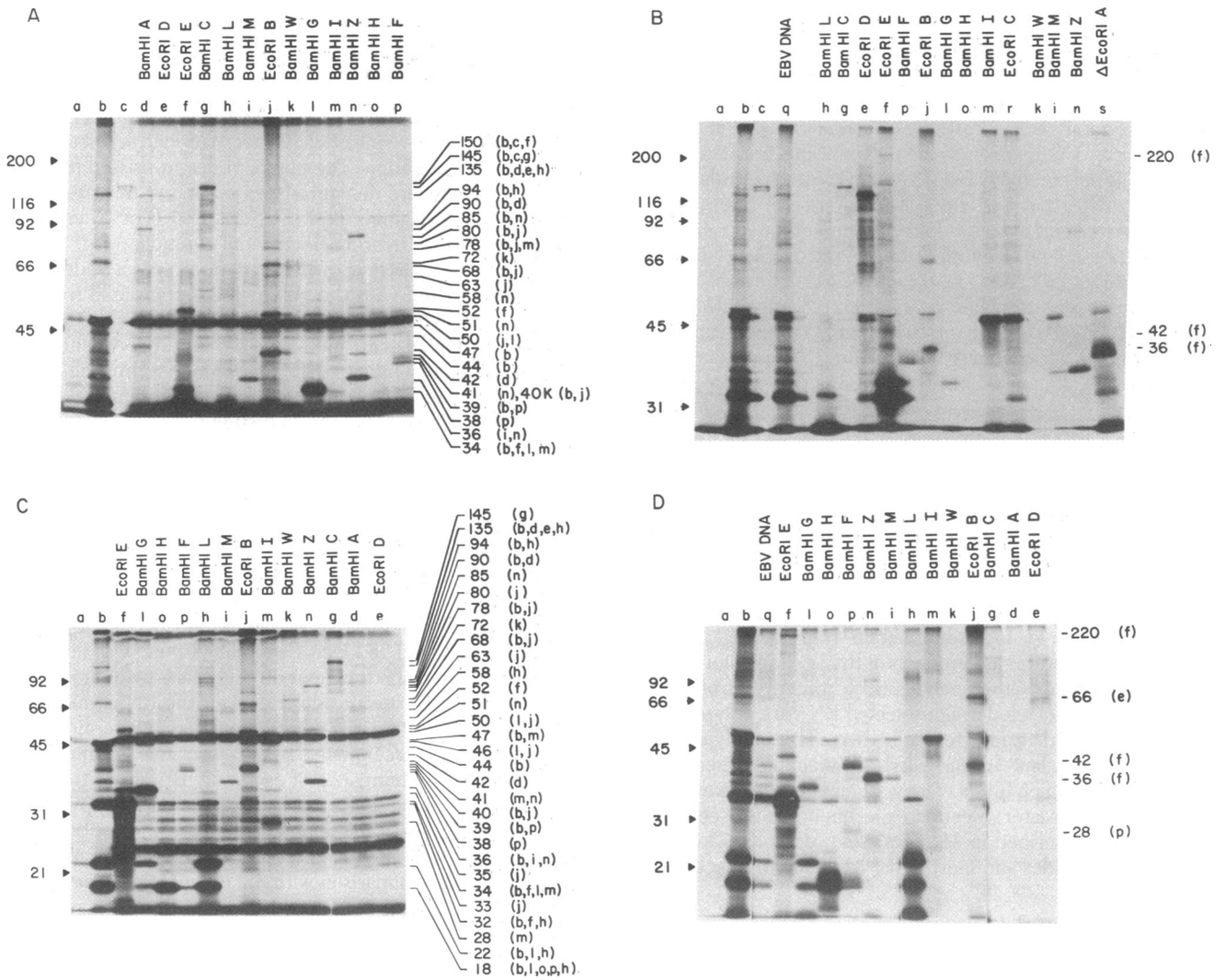

FIG. 2. Polyacrylamide gels [8.5% (*A* and *B*) and 12% (*C* and *D*)] of translation products of B95-8 RNA (lanes a and b) or of RNA hybrid-selected with cloned EBV DNA fragments (indicated above each lane) shown in kilodaltons. Letters in parentheses indicate the lanes in which the products are present. The translation product of B95-8 RNA in lane a was immunoprecipitated with EBV-negative sera and that in lane b, with EBV-positive sera; B95-8 virus in lane c (*A* and *B*) is labeled *in vivo* with [^{35}S]methionine. (*B* and *D*) Hybrid-selected immunoprecipitates. The 47-kDal protein in lane b is a viral protein that maps in *Bam*HI fragment I (*B*, lanes m and r). It comigrates in *A* and *C* with a protein endogenous to the lysate (lanes d–p). The fluorograms in *A* and *C* were exposed for 2 days, whereas those in *B* and *D* were exposed for 14 days.

tween the *Bam*HI L and A fragments, which could account for those fragments selecting the same RNAs, the RNAs and the polypeptides selected by *Bam*HI L and A are probably different. This was confirmed by the observation that the *Bam*HI L polypeptide is sensitive to phosphonoacetic acid treatment and is, therefore, a late gene product, whereas the *Bam*HI A polypeptide is a persistent early polypeptide and is resistant to the same treatment (Fig. 3, lanes f and o). The 78-, 50-, 39- to 40-, and 35-kDal polypeptides encoded by *Eco*RI fragment B (Fig. 3, lane h), the 58- and 36-kDal polypeptides encoded by *Bam*HI fragment Z (Fig. 3, lane g), the 32-kDal polypeptide encoded by *Bam*HI fragment L (Fig. 3, lane f), the 18-kDal polypeptide encoded by *Bam*HI fragment H (Fig. 3, lane j), and the 50-kDal polypeptide in the immunoprecipitate of *Bam*HI G-selected RNA (Fig. 3*C*, lane k) appeared to be differentially resistant to phosphonoacetic acid treatment. Therefore, these polypeptides also are classed tentatively as persistent early.

DISCUSSION

The maps of polypeptides synthesized *in vitro* and of RNA from productively infected cells (26) are summarized in Fig. 4 and Table 1. Polypeptides have been tentatively assigned to individual RNAs encoded by the same fragment on the basis of the minimal necessary size of the RNA, relative abundance, and sensitivity of the RNA and polypeptide to inhibition of viral DNA synthesis (Table 1). In some instances, the assignments are confirmed by mapping data with contiguous or partially overlapping DNA fragments.

There is a correspondence between the number of RNAs encoded by a fragment and the number of polypeptides translated from the RNA selected by that fragment (Fig. 4). Some fragments, such as *Bam*HI fragments F, Z, and D encode more RNAs and polypeptides than is expected based on DNA complexity. Although differences in transcript initiation could account for this phenomenon, most instances of multiple gene products from a single DNA region in papovaviruses or adenoviruses result from differences in RNA splicing (35, 36). A family of differentially spliced herpes simplex virus type 1 RNAs also has been described recently (37). Two of the persistent early RNAs and polypeptides encoded by *Bam*HI fragment M may result from differential splicing (unpublished observations). In contrast to *Bam*HI fragment M, for which the same number of

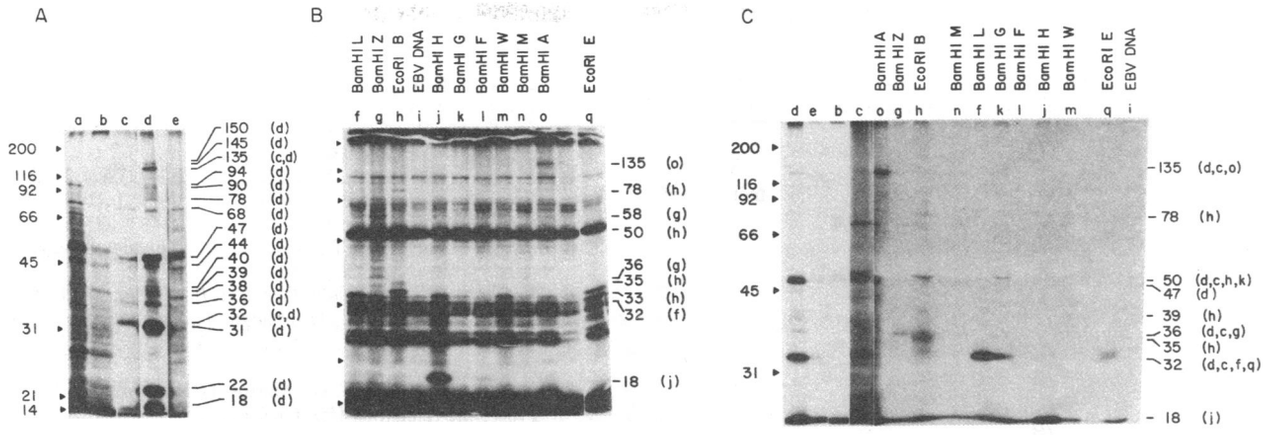

FIG. 3. Mapping of persistent early polypeptides and components of EA. Translation products are shown in kilodaltons; letters in parentheses indicate the lanes in which the products are present. (*A*) Translation products of B95-8 persistent early RNA before (lane a) or after immunoprecipitation with EBV-negative (lane b) or EBV-positive (lane c) sera are compared with translation products of persistent early and late RNA immunoprecipitated with positive (lane d) or negative (lane e) sera on a 10% gel. (*B* and *C*) Polypeptides translated from hybrid-selected persistent early RNAs are separated on a 12% gel (*B*) or immunoprecipitated and separated on a 10% gel (*C*).

RNAs and polypeptides have been detected, *Bam*HI fragment F encodes seven relatively abundant RNAs but only three polypeptides. Some of the *Bam*HI F RNAs may encode the same protein but differ in transcription initiation or termination as described for the RNAs encoded by the herpes simplex virus type 1 *Hin*dIII K fragment (38). Conversely, seven RNAs and eight polypeptides have mapped to *Eco*RI fragment E. This discrepancy could be due to inadequate resolution of RNAs of similar size or to premature termination of *in vitro* translation. An alternative explanation, based on an adenovirus example, is that two polypeptides could be translated from the same RNA in different reading frames (39).

Comparison of the immunoprecipitated polypeptides translated *in vitro* with the *in vivo* polypeptides immunoprecipitated with EA- and VCA-positive sera (11–16, 19, 21, 22, 25) reveals similarity in sizes of many of the polypeptides, although larger polypeptides are less abundant in *in vitro* translations. In several instances, the *in vitro* polypeptides could be correlated with polypeptides identified *in vivo* and with structural polypeptides of the virus on the basis of size, relative abundance, antigenicity, and dependence on DNA synthesis (Table 1). Thus, the 150- and 145-kDal polypeptides, previously correlated with the major capsid and membrane matrix proteins, respectively (11, 19, 22–25), now have been mapped to *Eco*RI E and *Bam*HI C fragments, respectively. Similarly, several of the *in vivo* polypeptides that were previously identified as components of EA have been mapped to specific viral DNA fragments (Table 1). Several other persistent early polypeptides that map in *Eco*RI fragment B are similar in size to polypeptides previously designated as components of EA (11, 12–14, 16). The *Eco*RI B-encoded polypeptides are designated as *probable* EA components (Table 1) because they are not abundant in immunoprecipitates of *in vitro* translation products, whereas the analogous *in vivo* polypeptides are major EA components. Additional evidence that these may be important components of EA comes from transfection studies in which *Eco*RI B fragment induced the synthesis of EA-like fluoresence (40).

The polypeptide mapping data and those previously reported

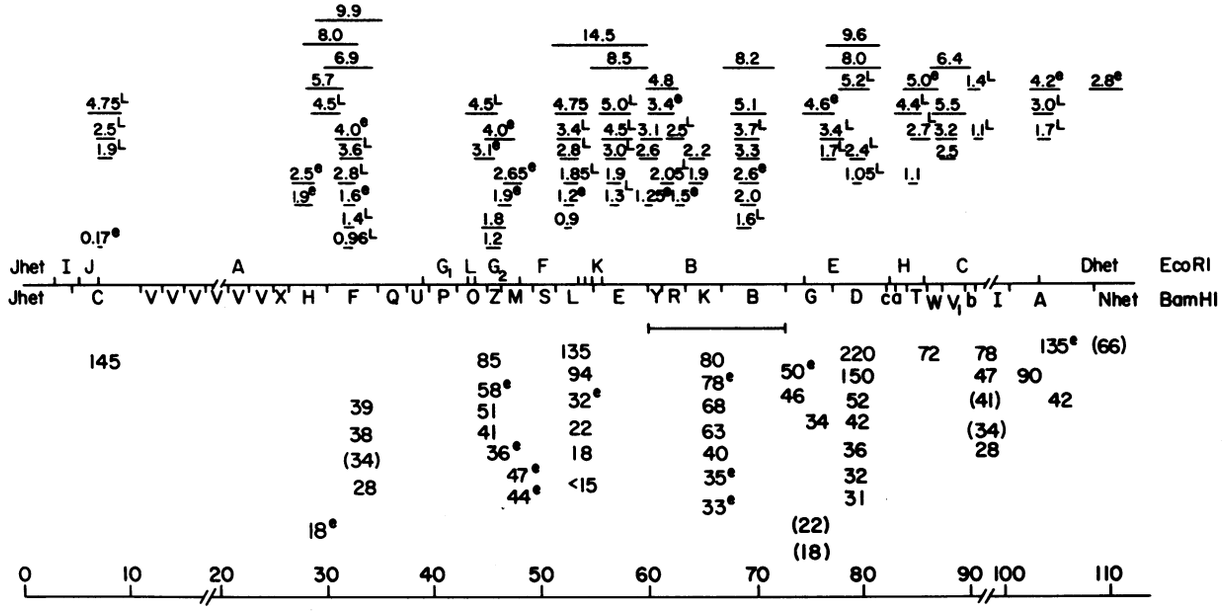

FIG. 4. Summary of the map locations of EBV RNAs (26) and polypeptides translated after hybrid selection and immunoprecipitation. Superscript e indicates proteins detected by translation of persistent early RNA. The 22- and 18-kDal proteins shown in parentheses are translated from RNA selected by *Bam*HI G fragment but not from RNA selected by *Eco*RI B or E fragments. The scale is shown in megadaltons.

Table 1. Criteria for assignment of a RNA to a polypeptide

In vitro protein, kDal/class*	DNA fragment, *Bam*HI/*Eco*RI	RNA, kb/criteria†	*In vivo* correlate
220/	/E	9.6,8.0/s,a	
150/L	/E	5.2/s,c,a	VCA‡
145/L	C/	4.75/s,a	membrane‡
135/L	L/	4.75,3.4/s,c,a	
135/E	A/D	4.2/s,c,a,o	EA§
94/L	L/	3.4,4.75/s,c,a,	
90/L	A/C	3.0/s,c,o	
85/L	O,Z/	4.5/c,o	
80	/B		
78/E	/B	2.6/s,c	(EA)¶
72	W/		
68	/B		
63	/B		
58/E	O,Z/	3.1/c,o	
52	/E		
51	Z/	1.8/s,c,a	
50/E	G/B	4.6/c	EA§
47/L	I/C	1.4/a,s	
47/E	M/	2.65,1.9/c,o	EA§
44/E	M/	2.65,1.9/c,o	EA§
46	G/B		
42/L	A/	1.7/s,c	
41/L	Z/	1.2/s,o,c	
40/L	/B		
39	F/		
38	F/		
36/E	Z,M/	4.0/c,a,o	EA§
35/E	/B	1.5,1.25/s,c,a	(EA)¶
33/E	/B	1.5,1.25/s,c,a	(EA)¶
34/L	G/E	1.7/c,a,o	
32/E	L/	1.2/c	EA§
32/L	/E		
31/L	/E		
28	F/		
28/L	I/	1.1/s,a	
22/L	L/	2.8,1.8,0.9/s,c,a	
18/L	L/	2.8,1.8,0.9/s,c,a	
15/L	L/	2.8,1.8,0.9/s,c,a	
18/E	H/	2.5/c,a	EA§

kb, Kilobase.

* E, early; L, late.

† Criteria: s, minimum RNA size necessary to encode the polypeptide; c, correspondence between the E or L class assignment of the RNA and polypeptide; a, correspondence in relative abundance of the RNA and polypeptide; and o, selection of the RNA and polypeptide by an overlapping or adjacent fragment.

‡ Refs. 11–16, 19, 20, 22, and 24.

§ Refs. 11, 13–16, 19, and 20.

¶ Refs. 11, 13, 14, 19, and 40.

for persistent early and late RNAs from productively infected B95-8 cells (26) indicate that early and late functions are intermixed and distributed throughout the viral genome.

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