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Identification and mapping of polypeptides encoded by the P3HR-1 strain of Epstein–Barr virus

(hybrid selection/cell-free translation/DNA-binding proteins/immunoprecipitations)

LAWRENCE K. COHEN*, SAMUEL H. SPECK*, BRYAN E. ROBERTS†, AND JACK L. STROMINGER*

*Dana–Farber Cancer Institute and †Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115

Contributed by Jack L. Strominger, December 29, 1983

ABSTRACT **The Epstein–Barr virus (EBV)-specified polypeptides induced upon viral replication in the P3HR-1 cell line have been examined by immunoprecipitation with a high-titer human anti-EBV serum. Twenty-five predominant polypeptides were identified in cell extracts, whereas 18 polypeptides were precipitated from cell-free translation reactions directed by total mRNA. Hybrid selection of mRNA to the *Bam*HI DNA clones of the EBV genome and immunoprecipitation of the corresponding cell-free translation products revealed 98 EBV-specified polypeptides and their coding location along the viral genome. In addition, the viral polypeptides that bind reversibly to DNA-cellulose have been characterized and the deduced map locations of this functional group of EBV-specified polypeptides is presented.**

In most eukaryotic viral systems, identification of the polypeptides that are synthesized during viral replication facilitates detailed studies on the activities encoded by the viral genome and the mechanisms that control host cell–virus interactions. For Epstein–Barr virus (EBV), a B-lymphotrophic human herpesvirus, the lack of a cell culture system fully permissive for viral replication has hampered such an analysis. Virus infection of B lymphocytes *in vitro* results in growth transformation of the host cell with only limited transcription of viral DNA (reviewed in ref. 1).

In specific EBV-transformed lymphoblastoid cell lines, activation of the resident viral genome occurs spontaneously in a small number (1–5%) of the cells resulting in viral replication (2). Chemical inducers of viral gene expression have been described that expand this percentage 10- to 20-fold (3, 4). In addition, subclones of the P3HR-1 cell line are available that exhibit a greater sensitivity to one of these agents, phorbol 12-myristate 13-acetate (PMA; ref. 5).

This paper presents an examination of the EBV proteins detected upon induction of one such clone [clone 13 (Cl-13)] of the P3HR-1 line (5). The proteins have been identified by immunoprecipitation of extracts of metabolically labeled cells and compared to the polypeptides obtained from cell-free translation. Hybrid-selected translation was used to locate the coding sequences along the viral genome. The potential involvement of a number of these polypeptides in nucleic acid biosynthesis or regulation of gene expression was suggested by their affinity for DNA-cellulose. These results are compared to previous characterizations.

MATERIALS AND METHODS

Cell Culture. The Cl-13 cell line (5) was generously provided by G. Miller (New Haven, CT). EBV gene expression was induced by exposure of the cells to PMA for 96 hr as described (3). For induction of exclusively the early viral functions, phosphonoacetic acid (PAA) was added simultaneously with PMA to a final concentration of 200 μg/ml. Both the degree of induction and the selectivity of expression in the presence of PAA were monitored by indirect immunofluorescence (2) employing a high-titer human anti-EBV serum (6). By this criterion, an induced culture typically contained 35–45% EBV antigen-producing cells. Cellular proteins were metabolically labeled as described (6). DNA-free protein extracts were prepared from these cells by the procedure of Sugawara *et al.* (7).

DNA-Cellulose Fractionation. Denatured calf thymus DNA-cellulose was prepared as described by Alberts and Herrick (8). Protein extracts derived from labeled cells or cell-free translation products were diluted 1:10 in binding buffer (50 mM ammonium acetate/20 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM 2-mercaptoethanol/10% glycerol) prior to analysis. One-tenth volume of a 50% (wt/vol) suspension of DNA-cellulose was added and the mixture was incubated for 1 hr with agitation at 4°C to allow binding. Subsequently, the DNA-cellulose was collected by centrifugation and washed extensively in binding buffer. Those proteins specifically bound were eluted in 2 M ammonium acetate/20 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM 2-mercaptoethanol/10% glycerol and were lyophilized repeatedly to remove the volatile salt.

Isolation of RNA and Cell-Free Translation. Total cytoplasmic RNA was extracted as described (9). When required, polyadenylylated cytoplasmic RNA was isolated by two cycles of oligodeoxythymidilate-cellulose chromatography performed as recommended by the manufacturer (Collaborative Research, Waltham, MA). RNA was translated in the message-dependent rabbit reticulocyte system (New England Nuclear) as described by Paterson *et al.* (10).

Hybrid-Selected Translation. Fifty micrograms of each of the non-overlapping *Bam*HI clones of EBV viral genomic DNA, from the B95-8 (11) and FF41 (12) strains, were immobilized to 1-cm^2 pieces of diazobenzyloxymethyl-paper as described (13). Complementary RNA was selected by hybridization in 50% formamide (Fluka)/0.6 M NaCl/80 mM Tris·HCl, pH 7.8/4 mM EDTA/0.1% $NaDodSO_4$ and 0.5–1.5 mg of polyadenylylated RNA per ml (13).

Immunoprecipitations, Gel Electrophoresis, and Fluorography. Immunoprecipitations were performed essentially as described by Kessler (14) and modified by Edson *et al.* (6). Both immunoprecipitations and protein fractions were analyzed on $NaDodSO_4$/polyacrylamide gels as described by Laemmli and Favre (15). Fluorography was performed as described by Bonner and Laskey (16).

RESULTS

Identification by Immunoprecipitation of EBV-Specific Polypeptides and DNA-Binding Proteins (DBPs) in Extracts of Cells Induced for Viral Gene Expression. The superinducible EBV-producing line Cl-13 was induced for viral gene expression by exposure of the cells to the phorbol ester PMA, and

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Abbreviations: EBV, Epstein–Barr virus; Cl-13, clone 13; DBP, DNA-binding protein; PMA, phorbol 12-myristate 13-acetate; PAA, phosphonoacetic acid.

the proteins were metabolically labeled with [^{35}S]methionine. DNA-free protein extracts were prepared from the radioactively labeled cells and fractionated on denatured calf thymus DNA-cellulose. Six to 10% of the total radioactivity applied to the DNA-cellulose bound to the matrix and was recovered in the high-salt eluate. To determine which of the proteins were viral specific, both the total protein extracts and the eluted fractions were analyzed by immunoprecipitation employing a previously characterized high-titer human anti-EBV serum (6).

Protein extracts prepared from untreated cells were compared with extracts prepared from cells induced by PMA in the presence and absence of PAA, an inhibitor of the viral DNA polymerase (17). Those polypeptides induced by the phorbol ester and recognized by the antiserum were considered EBV specific and were kinetically classified as either early or late viral-specific polypeptides (18, 19). Accordingly, the immunoprecipitations of the total protein extracts (Fig. 1) showed that after induction with PMA, ≈35 polypeptides could be identified as viral specific and, of these, 25 were quantitatively predominant (Table 1).

Ten predominant polypeptides were immunoprecipitated from the DNA-cellulose eluates (Fig. 1 and Table 1). Polypeptides that appeared in a small amount and with a relative abundance much less than that in the immunoprecipitation of total cell extracts were considered nonspecific carryover and were excluded from the enumeration. Three of the DBPs

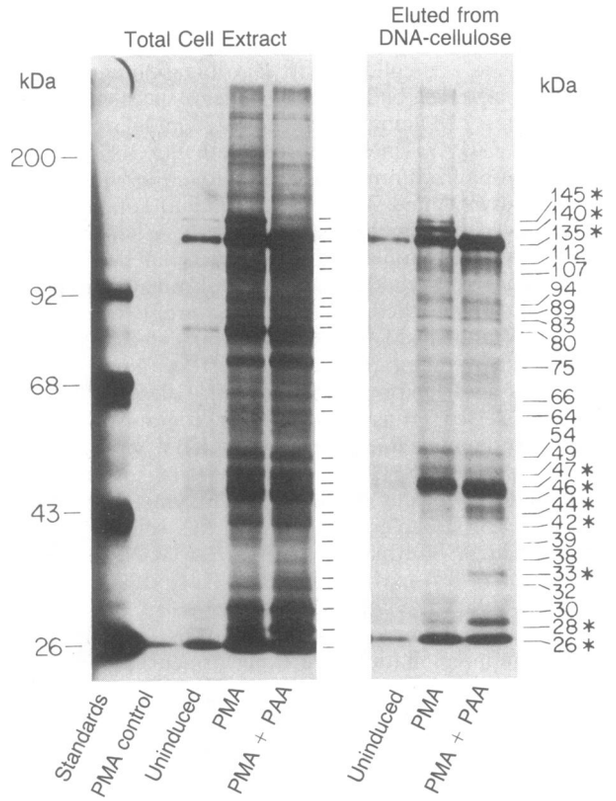

FIG. 1. Analysis of the immunoprecipitation products from extracts of metabolically labeled cells. Immunoprecipitates from extracts of uninduced cells (Uninduced), cells induced for late and early viral gene expression (PMA), and cells induced for exclusively early viral gene expression (PMA + PAA) were compared by electrophoresis on a $NaDodSO_4$/8% polyacrylamide gel. Both total cell extract and those proteins that reversibly bound to denatured calf thymus DNA-cellulose were analyzed. The lane labeled PMA control represents a control precipitation of extract from PMA-induced cells with an EBV-negative serum. The dashes denote those polypeptides judged to be quantitatively predominant and those that are starred are considered to be DBPs.

Table 1. Summary of the major EBV-specific polypeptides identified by immunoprecipitation

Molecular size, kDa			
Cell extract	Cell-free translation	Kinetic class	DBP
145	145	Late	+
140	140	Late	+
135	135	Early	−
112	—	Early	−
—	110	Late	−
107	—	Early	−
—	105	Late	−
—	95	Late	−
94	—	Early	−
89	88	Early	−
83	—	Early	−
80	80	Early	−
75	—	Early	−
66	—	Early	−
61	—	Early	−
54	—	Early	−
49	—	Early	−
47	—	Late	+
46	—	Early	+
—	45	Late	−
44	44	Early	+
42	43	Early	+
—	40	Late	+
39	—	Early	−
35	—	Early	−
33	—	Early	+
32	—	Early	−
30	—	Early	−
28	—	Early	+
26	—	Early	+
—	25	Late	−
—	24	Early	+
—	23	Late	+
—	22	Late	−
—	15	Late	+
—	14	Late	+

with molecular sizes of 145, 140, and 47 kDa were not synthesized in the presence of PAA and were classified as late viral-specific products, whereas the synthesis of the remaining 7 DBPs with molecular sizes of 135, 46, 44, 42, 33, 28, and 26 kDa was unaffected by the presence of PAA and they were therefore classified as early viral-specific polypeptides. All of these polypeptides that bound to denatured DNA-cellulose also bound to native DNA-cellulose, but exhibited no affinity for unsubstituted cellulose (data not shown).

Identification of EBV-Specific Polypeptides and DBPs Synthesized in Cell-Free Translations. The rabbit reticulocyte translation system directed by mRNA isolated from Cl-13 cultures induced by PMA with and without PAA, as well as mRNA from uninduced cultures, was analyzed by immunoprecipitation before and after DNA-cellulose fractionation. Approximately 10% of the radioactivity applied to the DNA-cellulose was bound and recovered in the high-salt elution, whereas in parallel experiments typically <1% of the radioactivity demonstrated any affinity for the cellulose itself (data not shown).

Immunoprecipitation with the anti-EBV sera of total translations directed by mRNA from induced Cl-13 cells (Fig. 2) revealed that 18 polypeptides were recognized by this serum. The species that ranged in molecular size from 145 to 14 kDa consisted of 12 late and 6 early polypeptides (Table 1). Immunoprecipitation from the DNA-cellulose elu-

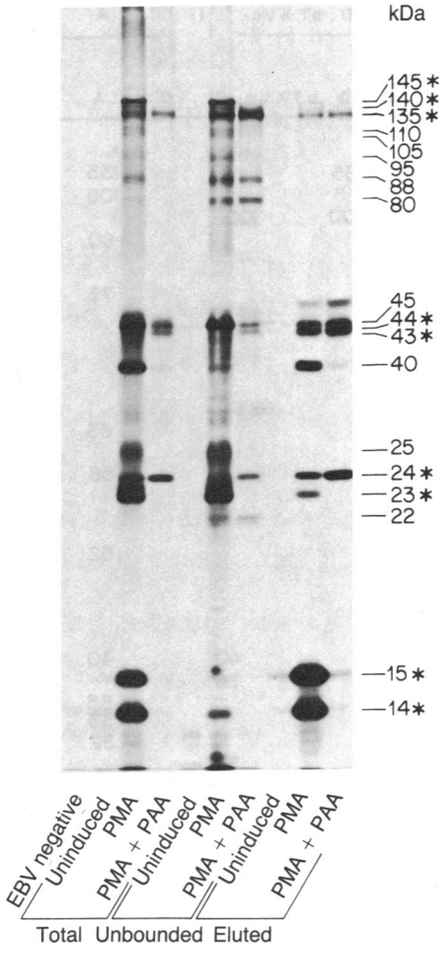

FIG. 2. Analysis of cell-free translation products synthesized from mRNA isolated from the Cl-13 line. mRNA isolated from cells treated as described in the legend for Fig. 1 was used to direct the reticulocyte cell-free translation system, and the products were analyzed on a $NaDodSO_4$/10% polyacrylamide gel after immunoprecipitation. The dashes denote those proteins judged to be quantitatively predominant and those that are starred are considered to be DBPs.

ates indicated that 10 of these polypeptides demonstrated an affinity for DNA-cellulose (Fig. 2). Of these, 3 of the polypeptides with molecular sizes of 135, 44, and 43 kDa were early polypeptides and 7 polypeptides with molecular sizes of 145, 140, 40, 25, 23, 15, and 14 kDa were classified as late polypeptides (summarized in Table 1).

Genomic Mapping of EBV-Specified Polypeptides by Hybrid-Selected Translation. The *Bam*HI restriction map of P3HR-1 and B95-8 EBV DNA is shown in Fig. 4 below. Each of the *Bam*HI cloned DNA sequences from the B95-8 viral genome (11), with the exception of the I fragment (which contains a deletion relative to P3HR-1 DNA), was used in conjunction with the cloned *Bam*HI B′, W′, and I′ fragments from the FF41 viral genome (12) (which span the deletion in B95-8 DNA) to hybrid select corresponding mRNAs from a total mRNA preparation isolated from PMA-induced Cl-13 cells. These cloned EBV DNA sequences represent without overlap the entire viral genome with the exception of the terminal repeat sequences. The hybrid-selected mRNAs were translated in the rabbit reticulocyte cell-free translation system and subsequently analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis after immunoprecipitation with the anti-EBV sera (Fig. 3). Immunoprecipitations of the hybrid-selected translations allowed those polypeptides obscured by polypeptides synthesized from mRNAs endogenous to the cell-free translation system to be readily visualized.

Fig. 3 shows a $NaDodSO_4$/polyacrylamide gel analysis of the immunoprecipitates resulting from one such experiment; these data compiled together with three other total genomic hybrid selections are summarized in Fig. 4. Surprisingly, close examination of the immunoprecipitations revealed that 98 polypeptides were synthesized from hybrid-selected mRNAs. Control experiments conducted by hybrid selecting RNA from PMA-induced Cl-13 with pBR322 DNA revealed, after cell-free translation, only those polypeptides synthesized from mRNA endogenous to the reticulocyte lysate translation, indicating adequate stringency of the hybridization and washing conditions for the mRNA selection (Fig. 3). The polypeptides map to ≈80% of the unique genomic sequences. The remaining regions, represented by the *Bam*HI W, Q, U, P, b, T, X, B′, W′ and I′ cloned fragments, either do not encode polypeptides or are being transcribed at levels that are too low to be detected or immunoprecipitated with the antiserum used.

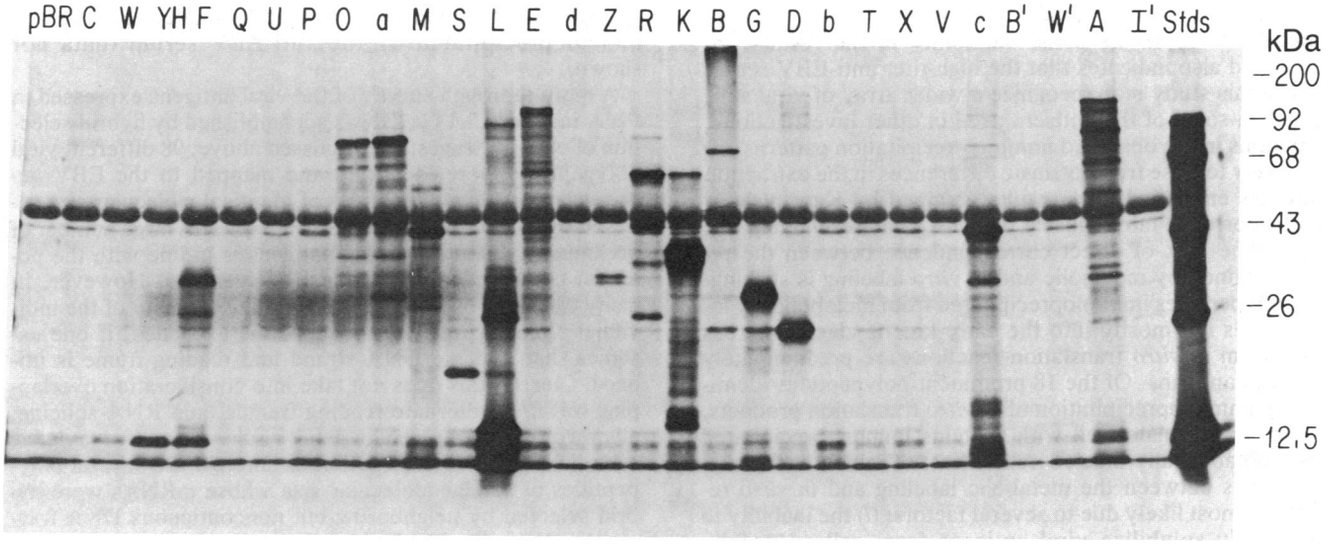

FIG. 3. Analysis of the cell-free translation of mRNA hybrid selected to the *Bam*HI DNA clones of the EBV viral genome. Cell-free translation products were immunoprecipitated and resolved in genomic order on a $NaDodSO_4$/10% polyacrylamide gel. Stds, standards; pBR, pBR322.

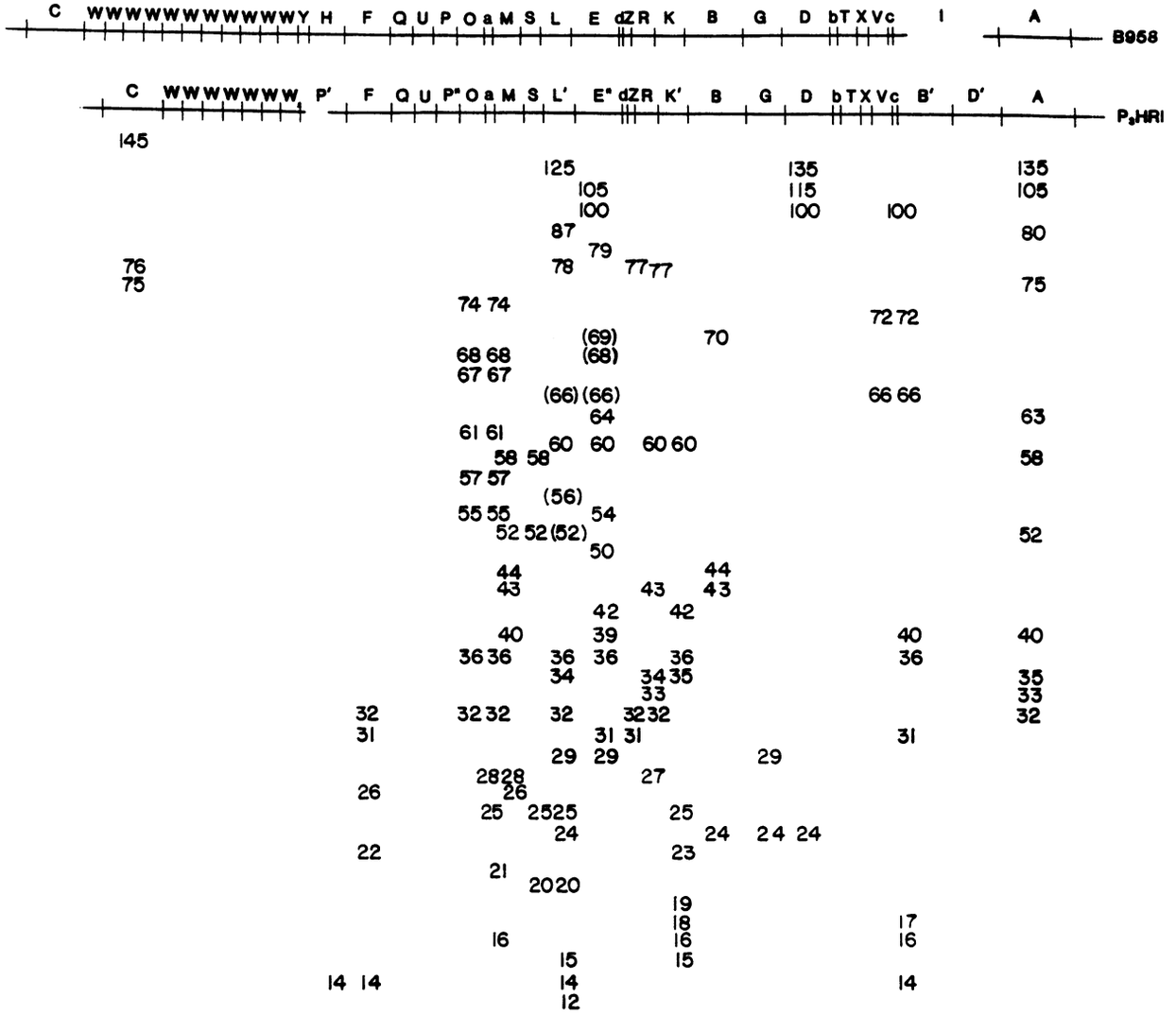

FIG. 4. Summary of the map locations of the EBV-encoded polypeptides. The polypeptides detected by hybrid-selected translation and immunoprecipitation appear underneath aligned *Bam*HI restriction maps of the B95-8 and P3HR-1 viral DNAs. The DNA fragment labeled D' in the P3HR-1 genomic map represents a fusion of the W' and I' fragments. Those polypeptides in parentheses were consistently visualized but in reduced amounts.

DISCUSSION

Comparison of the metabolic labeling data with previous studies (20–23) shows some variability in the results obtained and also indicates that the high-titer anti-EBV serum used in this study may recognize a wider array of viral antigens than some of the antisera used in other investigations. Variations in the observed immunoprecipitation patterns are also likely to arise from intrinsic differences in the extraction protocols employed. The results obtained by Kawanishi *et al.* (20) correlate fairly closely with the data presented in this paper. The lack of direct correspondence between the results obtained by metabolic and *in vitro* labeling is striking. The polypeptides immunoprecipitated from metabolically labeled cells fall mostly into the early kinetic class, whereas those from *in vitro* translation reactions are predominately late viral antigens. Of the 18 prominent polypeptides identified by immunoprecipitation of *in vitro* translation products, 7 seem to correlate well with proteins immunoprecipitated from metabolically labeled cell extracts (Table 1). The discrepancies between the metabolic labeling and *in vitro* results are most likely due to several factors: (*i*) the inability to adequately solubilize viral antigens from cells; (*ii*) posttranslational modification of the metabolically labeled polypeptides; and (*iii*) the relative paucity of viral messages and proteins in induced cells. The latter point is crucial to an understanding of the limitations of the system. Indeed, only about 2% of the total protein from PMA-induced cells can be immunoprecipitated by the anti-EBV serum (data not shown).

A more thorough survey of the viral antigens expressed in PMA-induced Cl-13 cells was accomplished by hybrid selection of viral messages. As discussed above, 98 different viral polypeptides were identified and mapped to the EBV genome (*excluding* polypeptides of identical molecular size selected by adjacent DNA clones, Fig. 4). This large number of preliminarily identified viral antigens is in line with the potential coding capacity of the EBV genome. However, in many cases (10 of 29 clones) the coding capacity of the individual cloned viral DNA sequence is exceeded, if one assumes that a single DNA strand and reading frame is utilized. Clearly, this does not take into consideration overlapping mRNAs, alternate reading frames, and RNA splicing, all of which are prevalent in many viral systems (for reviews, see refs. 24 and 25). Of particular interest are several polypeptides of similar molecular size whose mRNAs were hybrid selected by neighboring but noncontiguous DNA fragments (i.e., the 25-kDa species hybrid selected by the *Bam*HI A, S, and L clones or the 60-kDa polypeptide hybrid selected by the L, E, R, and K *Bam*HI fragments), perhaps indicative of mRNA splicing events. Additionally, the

*Bam*HI c fragment, which has been shown to contain a strong RNA polymerase II promoter in *in vitro* transcription assays (26), hybrid selected messages that together exceeded by >17-fold the coding capacity of the 0.5-kilobase fragment, suggesting the presence of a common 5′ leader sequence as described for the late adenovirus mRNAs (27, 28) and similarly for *Bam*HI a, L′, E* and, to a lesser extent, O, Z, R, K, D, and A.

A previous study of Hummel and Kieff (29) employing a similar approach, using PMA-induced B95-8 cells (a marmoset cell line which, in contrast to P3HR-1 cells, produces a transforming strain of EBV), identified and mapped 48 polypeptides. Of these, 30 correlate well with the antigens mapped in this paper, based on size and genome location. The large number of polypeptides that are not correlated in these two surveys may reflect strain variation, fundamental differences in the productive infection in marmoset versus human cells, and/or differences in experimental protocol. Notwithstanding the differences between these two surveys, it seems clear that the EBV genome codes for a large number of polypeptides. A recent sequence analysis of the EBV genome has identified >60 major open reading frames, many of which are flanked by putative promoter sequences and polyadenylylation signals (ref. 26 and B. Barrell, personal communication). Moreover, 54 mRNAs have been mapped to the genome following induction of P3HR-1 cells with PMA (30).

DNA-cellulose fractionation has been used to operationally define a subset of the polypeptides that reversibly bind to DNA. Of the 35 polypeptides immunoprecipitated with the anti-EBV serum from extracts of PMA-induced cells, 10 demonstrated an affinity for DNA. Proportionally, this is in agreement with herpes simplex virus, for which 16 or 17 polypeptides were identified as DBPs from a total of 50 polypeptides recognized as viral specific at that time (31, 32). More specifically, the DBPs identified are in partial agreement with those characterized in two previous studies identifying the DBPs induced upon EBV replication (7, 33). Seven of the 10 DBPs identified in this study correlate with DBPs detected in the two previous determinations. Only the 140-kDa late polypeptide and the 28- and 26-kDa early polypeptides have not been reported. The demonstration that cell-free translation products can also be fractionated based on affinity for DNA-cellulose is not surprising in light of reports of the measurement of enzymatic activities from cell-free translation products (34, 35). It should be noted that the specificity of the binding to DNA was demonstrated by the lack of affinity these polypeptides exhibited to cellulose alone and was supported by the finding that 6 of the 10 DBPs identified from cell-free translation reactions appear to have cognates, based on size and kinetic class, with the DBPs identified from cell extracts.

Attempts to analyze hybrid-selected translation products directly by DNA binding and immunoprecipitation, as conducted on translation products from total mRNA, were unsuccessful, presumably due to the very small amount of protein synthesized under these conditions. Nevertheless, based on relative molecular size and abundance, the coding regions for 7 of the DBPs could be tentatively assigned as follows: The 145-kDa DBP maps to *Bam*HI C, the 44- and 43-kDa DBPs map to *Bam*HI M, the 24-, 15-, and 14-kDa DBPs map to *Bam*HI L, and the 135-kDa DBP maps to *Bam*HI A. It is interesting to note that the assignment of the coding sequence for the 135-kDa early DBP to the *Bam*HI A DNA fragment correlates with previous suggestions that this region encodes a polypeptide intimately associated with viral DNA replication (36).

The experimental approach employed in this paper has facilitated the genomic mapping of a large number of EBV-specific polypeptides. By identifying those polypeptides that demonstrate an affinity for DNA, we have attempted to provide a functional focus to the study of the viral polypeptides. Clearly, as an initial survey it provides a starting point for detailed transcriptional mapping of the EBV genome and functional studies of the proteins synthesized during viral replication.

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