Immunological Conservation between Epstein–Barr Virus and Herpes Simplex Virus

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SUMMARY

We have analysed Epstein–Barr virus (EBV)- and herpes simplex virus (HSV)-infected cells for evidence of antigenic conservation of virus-coded proteins. Immunofluorescence and Western blot analyses of EBV-transformed cell lines demonstrated the presence of proteins that are antigenically related to the HSV alkaline DNase, infected cell-specific protein 34/35, glycoprotein B, thymidine kinase and the major DNA-binding protein. These proteins were characterized on the basis of Mr, and possible kinetic class.

INTRODUCTION

The herpesviruses are characterized as a number of diverse viruses of eukaryotes which replicate in the nuclei of infected cells and have a single, linear, double-stranded DNA genome contained within an enveloped icosahedral nucleocapsid consisting of 162 capsomeres. Based largely upon viral pathogenicity and behaviour in tissue culture, the group has been subdivided into the alpha-, beta- and gammaherpesviruses (Honess & Watson, 1977; Roizman, 1982).

Analysis of viral DNAs, proteins and antigens has revealed a dearth of common properties within the herpesvirus group as a whole, except between those considered to be pathologically closely related (for reviews, see Honess & Watson, 1977; Honess, 1984). For example, a large variety of immunological and biochemical approaches have been used to compare the proteins of many different herpesviruses (Blue & Plummet, 1973; Killington et al., 1977; Littler et al., 1981; Sterz et al., 1974; Yeo et al., 1981) but have failed to provide good evidence for a group-specific antigen similar to those which characterize the adenoviruses (Norrby, 1966) or papillomaviruses (Jenson et al., 1980).

Epstein–Barr virus (EBV) is a human gammaherpesvirus which, because of its clinical and oncogenic importance, is the focus of much current investigation. However, due to the lack of a fully permissive cell culture system for the replication of EBV, analysis of viral gene expression has been severely hampered. Nevertheless, classically a number of antigen complexes have been identified in EBV-transformed cell lines. These have been termed EBNA (EBV nuclear antigen), EA (EBV early antigen) and VCA (EBV capsid antigen) (Henle et al., 1970; Pearson, 1980; Reedman & Klein, 1973). In an attempt to define EBV gene products more precisely, several groups have used hybrid selection of EBV mRNAs coupled with in vitro translation (Hummel & Kieff, 1982; Pearson et al., 1983; Seibl & Wolf, 1985). As yet, however, few functions have been assigned to EBV gene products.

Biochemical analysis of EBV-transformed cell lines has identified two novel virus-specified
enzymes, a DNA polymerase and an alkaline DNase (Allaudeen & Bertino, 1978; Allaudeen & Rani, 1982; Clough, 1979, 1980; Datta et al., 1980; Feighny et al., 1980; Miller et al., 1977; Ooka et al., 1979; Tan et al., 1982a, b). More recently, evidence in favour of an EBV-coded thymidine kinase (TK) has been established by efficient purification and characterization of a novel TK activity in EBV-infected cells (Stinchcombe & Clough, 1985) and by expression of the enzyme in heterologous eukaryotic and prokaryotic systems (Littler et al., 1986).

Analyses of EBV DNA and antigens suggested that the virus is closely linked only with the pathologically related primate gammaherpesviruses (Falk et al., 1976; Heller & Kieff, 1981; Lee et al., 1981; Prachova et al., 1983; Rabin et al., 1977) and this led to the general opinion that EBV is only distantly related to the other human herpesviruses (Kieff et al., 1983). This consensus is now challenged by the recent comparisons between the amino acid sequences of well characterized herpes simplex virus (HSV) genes and open reading frames (ORFs) found in the EBV genomic DNA sequence, which have led to the identification and mapping of several EBV genes; these include ORFs for a DNA polymerase, a major capsid protein, a ribonucleotide reductase, potential glycoproteins with significant homology to the HSV gB and gH genes (Gibson et al., 1984; Pellett et al., 1985; Quinn & McGeoch, 1985; McGeoch & Davison, 1986; Davison & Scott, 1986a, b), an alkaline DNase (McGeoch et al., 1986) and an EBV gene homologous to an HSV gene with a 2.7 kb spliced mRNA (Costa et al., 1985). However, as a complement to these predictive approaches it is important to demonstrate the physical existence of the homologous proteins expressed by EBV.

We report here an extension of comparative analysis of EBV and HSV genes in which we utilize immunological probes to reveal extensive homology between the viruses' gene functions. This work has been presented in preliminary form by Littler et al. at the 10th International Herpesvirus Workshop (Ann Arbor, 1985; Abstracts p. 109).

METHODS

**Cells.** The EBV-negative lymphoid cell line Ball-1 (Hiraki et al., 1977) and EBV-positive lymphoid cell lines Raji (Pulvertaft, 1965), B95-8 (Miller & Lipman, 1973), W91 (Miller et al., 1976), P3HR-1 (Hinuma et al., 1967) and BL18 (Bernheim et al., 1983) were grown as described previously (Arrand et al., 1983). Where indicated, the cells were chemically induced using 10 ng/ml 12-O-tetradecanoylphorbol 13-acetate (TPA) and 3 mM-sodium butyrate as described by Bauer (1983).

**Antisera.** Rabbit polyvalent antisera to the HSV alkaline DNase (Banks et al., 1983), infected cell-specific protein (ICSP) 34/35 (Vaughan et al., 1984), glycoprotein B (gB) (Snowden et al., 1985) and the major DNA-binding protein (DBP) (Powell et al., 1981) were prepared by immunization of rabbits with purified preparations of proteins. Mouse hyperimmune serum to HSV TK was prepared using purified HSV-1 TK as the immunogen (Banks et al., 1984). Antisera were not affinity-purified before use.

**Immunofluorescence tests.** Cells were washed with and resuspended in phosphate-buffered saline (PBS), spotted onto microscope slides and air-dried. After fixation of the cells in acetone they were reacted first with normal goat serum at a 1:50 dilution, and then with the primary rabbit antisera (dilutions shown in figure legends), for 1 h at 37 °C. The slides were washed in PBS and incubated with the appropriate fluorescein-linked secondary antibody (at a dilution of 1:100) for 1 h at 37 °C. The slides were washed as before and mounted for viewing. In some experiments a human EBV VCA-positive antiserum was included in the primary reaction. In this case rhodamine-labelled anti-human IgG (Dako, Copenhagen, Denmark) was included in the secondary reaction at a 1:100 dilution. In each experiment appropriate preimmune sera were used as controls.

**Western blot analysis of EBV-transformed cell lines.** The technique used was as described by Towbin et al. (1979) using high salt buffer extracts of EBV-transformed cell lines prepared as described by Purifoy & Powell (1976). Dilutions of antisera and the amount of protein loaded in each track are indicated in figure legends.

RESULTS

**Immunofluorescence analysis of EBV-transformed cells**

To determine whether polypeptides immunologically related to defined HSV proteins were present in EBV-transformed cell lines, indirect immunofluorescence assays were done on the EBV-negative cell line Ball-1 and the EBV-positive cell lines Raji, B95-8, P3HR-1 and W91. The antisera used were hyperimmune rabbit sera specific for the following HSV proteins: (i) the HSV alkaline DNase, obtained by purification of the DNase to homogeneity by chromato-
Table 1. Summary of homologous HSV and EBV gene products

<table>
<thead>
<tr>
<th>HSV protein designation</th>
<th>Reaction with EBV-infected cells</th>
<th>Western blot</th>
</tr>
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<tbody>
<tr>
<td>DNase</td>
<td>+ (late)</td>
<td>+ (64K, late)</td>
</tr>
<tr>
<td>ICSP 34/35</td>
<td>+ (early)</td>
<td>+ (50K, early)</td>
</tr>
<tr>
<td>TK</td>
<td>+ (early)</td>
<td>–</td>
</tr>
<tr>
<td>DBP</td>
<td>+/−*</td>
<td>+ (140K, late)†</td>
</tr>
<tr>
<td>gB</td>
<td>+ (late)</td>
<td>+ (70K to 75K, late)</td>
</tr>
<tr>
<td>gC</td>
<td>–</td>
<td>ND‡</td>
</tr>
<tr>
<td>gD</td>
<td>–</td>
<td>ND‡</td>
</tr>
</tbody>
</table>

* Reactivity also observed with Ball-I EBV-negative cell line so the result is not interpretable.
† Reactivity seen only with EBV producer cell lines but the EBV DNA carried by the Raji cell line has a deletion in the DBP ORF (Quinn & McGeoch, 1985) hence reactivity may be early.
‡ ND, Not determined.

EBV- and HSV-coded proteins

In order to characterize the polypeptides found in EBV-infected cell lines that showed cross-reactivity with the HSV alkaline DNase and ICSP 34/35, extracts of the EBV-negative cell line...
Fig. 1. Indirect immunofluorescence analysis of EBV-infected and non-infected cell lines. Cell lines B95-8 (a, c, f and g), Ball-1 (b), Raji induced with TPA and sodium butyrate (d and h) or Raji (e) were fixed as described and reacted with the following rabbit sera to the HSV proteins: alkaline DNase (a, b and c); ICSP 34/35 (d and e); major DBP (f); gB (g); TK (h). (c) Cells reacted with human EBV VCA-positive serum. Cells that reacted with serum were detected using either anti-rabbit fluorescein isothiocyanate-conjugated serum (a, b, d, e, f and h) or with an anti-human rhodamine conjugate (c). All sera were at a dilution of 1:50, with the exception of the TK antiserum which was at a dilution of 1:20. Dilutions of goat serum and conjugates are described in Methods. Bar markers represent 100 μm (a, b, c, d, g, h) and 500 μm (e, f).
EBV- and HSV-coded proteins

Fig. 2. Analysis of lymphoid cells for the presence of proteins with antigenic cross-reactivity to HSV gB. The EBV-negative cell line Ball-1 (a) and the EBV-infected cell lines Raji induced with TPA (b), P3HR-1 (d) and BL18 (e) were fixed with acetone. Cells were incubated with normal swine serum to prevent non-specific reaction of serum with the cell lines, and then with HSV-1 gB antiserum. Reactivity of the gB antiserum with the cell lines was detected using peroxidase-linked goat anti-rabbit IgG which was reacted with Hanks-Yates substrate (Towbin et al., 1979). The results of the same experiment using gD serum reacted with the P3HR-1 cell line (f), and of indirect immunoperoxidase staining using a human EBV VCA-positive serum on the P3HR-1 cell line (c) are also shown.

Ball-1 and EBV-positive cell lines Raji and P3HR-1 were examined by Western blot analysis. An antiserum to the HSV major DBP was also used for comparison. The results are shown in Fig. 3. No cross-reacting polypeptide was detected in the Ball-1 cell line with any antiserum used (for example, Fig. 3 lanes 7 and 8). Similarly, no cross-reacting polypeptides were detected in Raji cells that had not been induced with TPA (Fig. 3 lane 5). In contrast, however, antiserum to the HSV DNase reacted with a doublet of proteins of approximately 64K found in EBV producer cell lines (for example P3HR-1; Fig. 3 lane 2), sera to the HSV ICSP 34/35 reacted with a protein of 50K in TPA-induced Raji cells (Fig. 3 lane 6) and sera to the HSV major DBP reacted with a protein of 140K in the P3HR-1 cell line (Fig. 3 lane 1). The type of EBV-infected cell responding with sera to either the HSV DNase or ICSP 34/35 is the same as is seen when
Fig. 3. Western blot analysis of EBV-infected and non-infected cell lines. Extracts were prepared from: Ball-1, induced (lane 4); Ball-1 (lane 3); Raji, induced lane 6; Raji (lane 5); P3HR-1, induced (lanes 1, 2 and 8) and P3HR-1 (lane 7). These were separated by SDS-PAGE and transferred to nitrocellulose as described in Methods. Blots were then incubated with rabbit antiserum to the HSV proteins, ICSP 34/35 (lanes 3 to 8), alkaline DNase (lane 2) and major DBP (lane 1), and reactive proteins (†) were detected using anti-rabbit immunoperoxidase-linked antibody and Hanke-Yates substrate (Towbin et al., 1979). Dilutions of the primary antisera were 1:50, 1:100 and 1:200 respectively; dilution of the anti-rabbit immunoperoxidase was 1:200 in each case. Lane 9 shows an Mr marker track with values ($\times 10^{-3}$) indicated alongside. Lanes shown were cut from a single nitrocellulose blot prior to reacting with primary antisera. The amount of protein loaded in each track was 50 μg.

examining these cell lines by immunofluorescence (see Table 1). This may suggest that the EBV protein cross-reacting with HSV DNase is equivalent to an HSV γ pattern of synthesis, whereas that reacting with HSV ICSP 34/35 has a β pattern of expression. In the case of the major DBP, no specific reactivity was observed in EBV-infected cells when using immunofluorescence, but a 140K protein was found which specifically reacted with antiserum to the HSV major DBP in EBV producer cell lines in Western blots.

Further characterization of the gB-related EBV polypeptide was performed using the anti-gB serum in a Western blot analysis of uninfected and EBV-infected cell extracts (data not shown). Two EBV-infected cell extracts were shown to contain a 75K polypeptide that cross-reacted weakly with the HSV gB serum. A similar set of experiments using non-denatured protein extracts to detect any heat-sensitive oligomeric form of the EBV gB analogous to those found with HSV-1, HSV-2, bovine mammillitis virus or equine herpesvirus type 1 (Snowden et al., 1985) indicated that no such oligomer exists.

DISCUSSION

There are now several reports of homologous genes between HSV and EBV. In particular, amino acid sequence comparisons between well characterized HSV gene products have shown that EBV may encode proteins homologous to the HSV ribonucleotide reductase, major DBP, DNA polymerase, major capsid protein, alkaline DNase, gB and gH (Gibson et al., 1984; Pellett et al., 1985; Quinn & McGeoch, 1985; McGeoch & Davison, 1986; Davison & Scott, 1986a, b). In addition, Costa et al. (1985) have detected a 2-7 kb mRNA species transcribed from HSV
DNA which has homology to the EBV DNA sequence. The results presented here provide evidence for five distinct HSV gene products having homologues that are present in EBV-infected cells, namely the alkaline DNase, ICSP 34/35, major DBP, TK and gB. Fig. 4 summarizes the relationships between homologous genes of EBV and HSV, and Table 1 indicates which techniques have been used to demonstrate homologies. An examination of the respective physical and genetic maps of HSV and EBV (Fig. 4) shows that the relative positions of genes in each virus have some similarities. However, detailed DNA sequence analysis will be necessary in order to speculate on genome rearrangement in HSV and EBV, in a similar manner to that between EBV and varicella-zoster virus (VZV) (Davison & Taylor, 1987; Davison & Scott, 1986a).

There are previous examples of viruses that were considered divergent which, after more appropriate analysis, have subsequently been shown to be significantly conserved. For example, the three papovaviruses simian virus 40 (SV40), BK virus and polyoma virus show little DNA cross-hybridization under conditions of high stringency. However under conditions of low stringency (T_m = 50 °C), similar to those used here, the DNA of the three viruses exhibits extensive homology (Howley et al., 1979). Furthermore, initial antigen analysis of the proteins encoded by SV40, BK and polyoma viruses showed little cross-homology but a subsequent study clearly showed that antigenic cross-reactivity does occur between the proteins of these three
viruses (Jenson et al., 1980). In the case of these papovaviruses, the genetic and antigenic conservation was confirmed by DNA sequence analysis (and subsequent protein prediction). Since the HSV genes used in this study are relatively well characterized, it is interesting to contrast the properties of these gene products with what is known about the corresponding EBV enzymes or genes.

The finding of a 140K protein in EBV-infected cells which cross-reacts with the HSV major DBP agrees with our previous report of the extensive conservation of the major DBP in other members of the herpesvirus group (Littler et al., 1981; Yeo et al., 1981). In addition, recent DNA sequence analysis of HSV and EBV demonstrates that they both contain extensively conserved major DBP genes (Quinn & McGeoch, 1985). Thus, this protein would appear to be a herpesvirus group-specific antigen with an essential role in virus replication.

We have demonstrated that EBV producer cell lines contain a protein doublet with a mobility of about 64K in SDS–PAGE which shows antigenic cross-reactivity with the HSV alkaline DNase. Further, the EBV ORF BGLF5 predicts a protein with significant amino acid sequence homology with the HSV enzyme (McGeoch et al., 1986; Littler et al., Abstracts, 10th International Herpesvirus Workshop, 1985). There is considerable evidence for an EBV-associated alkaline DNase activity which is biochemically distinct from any host cell enzyme, but which resembles that of the HSV enzyme (Clough, 1980; Tan et al., 1982a, b). In HSV-infected cells the role of the virus-coded DNase is not completely clear; however, it does appear to be important in virus DNA replication (Banks et al., 1983; Francke et al., 1978; Francke & Garrett, 1982; Moss et al., 1979; Moss, 1986). It is likely therefore that the EBV enzyme is linked to active virus production and may have a role similar to that of the corresponding HSV enzyme, possibly in resolution of replicative intermediates of viral DNA.

The use of the HSV gB serum indicates the presence of a protein in EBV-transformed cells that shares antigenic cross-reactivity with HSV-1 gB. These practical results are therefore in agreement with the theoretical prediction of Pellett et al. (1985) who, from DNA sequence analysis, have proposed that the EBV BALF4 ORF possesses regions of sequence homology with the HSV-1 gB gene and also apparent amino acid sequence conservation. However, the Mr of the EBV gB that we detected (75K) differs from that reported by Gong et al. (1987), who reported a cross-reacting gB protein of 110K. It is worth noting that in vaccinia virus recombinants expressing the cytomegalovirus gB homologue, the protein that was expressed had an Mr of 145K and 55K. Pulse-chase experiments showed that the 145K protein was post-translationally processed to the 55K form via a 66K intermediate (Cranage et al., 1986). A similar phenomenon may be operative in the EBV system leading to a lower apparent Mr.

We can conclude, however, that the conservation of the gB-related polypeptide extends not only to members of the alphaherpesvirinae, (Snowden et al., 1985) and betaherpesvirinae (Kouzarides et al., 1987), but also apparently to those of the gammaherpesvirinae. The conservation of the gB proteins throughout the herpesvirus group not only suggests a fundamental and essential role of this protein in virus replication but also has implications for the role of gB in host immunity to herpesviruses and its use as a vaccine against herpesvirus infection.

There is little indication of the function of the HSV ICSP 34/35, whose main identifiable property is its high affinity for the HSV DNA polymerase (Powell & Purifoy, 1977; Vaughan et al., 1985). It is interesting that type-specific monoclonal antibodies for the HSV ICSP 34/35 suggest that the ICSP 34/35 molecule found in different strains varies considerably in its mobility on polyacrylamide gels (P. Vaughan, personal communication; Vaughan et al., 1984). It is likely therefore that the reactivity between the serum raised to the HSV ICSP 34/35 and the EBV 50K antigen will be limited to some highly conserved sites on the protein. Indeed, our use of low stringency DNA hybridization locates the homologous region of the EBV genome to either the BamH1 O and/or P fragments. Examination of this region of EBV shows three unassigned ORFs (BOLF1, BORF1 and BPLF1). As no published sequence information is available for this region of the HSV genome a suitable alternative is a comparison with the equivalent region of the VZV genome. Comparison of the amino acid sequence of these three ORFs with the sequence of VZV (Davison & Taylor, 1987) shows that BORF1 and VZV ORF 20
have weak homology. In the case of the EBV ORFs BORF1 and BPLF1, no homology was detected with any VZV ORFs. However, the alignment of the EBV and VZV genomes indicates that these two EBV ORFs co-align with the VZV ORFs 21 and 22 in location, direction and size. This suggests that these ORFs may have a similar function in viral replication and may have conserved secondary or tertiary structures which could result in immunological homology.

From the results presented here, and in other reports, it appears that several of the enzymes and proteins involved in HSV and EBV DNA replication, nucleotide metabolism and particle structure are conserved (Baer et al., 1984; Gibson et al., 1984; Littler et al., 1981, 1986; Quinn & McGeoch, 1985). It also appears likely that these proteins are maintained throughout the herpesvirus group. In addition, we believe that the approach taken in this study is a valuable method for determining the genetic relationship between EBV and HSV and could be applied for further studies between these, or other, herpesviruses.

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REFERENCES


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