Activation of expression of latent Epstein–Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA

(immunoblotting/defective virus)

JILL COUNTRYMAN* and GEORGE MILLER†*

*Department of Molecular Biophysics and Biochemistry, and †Departments of Pediatrics, Epidemiology and Public Health, Yale University, New Haven, CT 06510

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ABSTRACT We previously found that a form of Epstein–Barr virus with rearranged DNA induces replication of latent Epstein–Barr virus. We now have found that one of three fragments of this rearranged DNA, when cloned in recombinant plasmids and used to transfect cells, can activate expression of several polypeptides from a latent viral genome. The 33-kDa protein that is the product of the active fragment is likely to be responsible for disruption of latency.

Latency is a biological property common to the herpesviruses, which persist in their host after initial infection. Viral genomes, which are quiescent during latency, can be activated to replicate mature virus by a variety of stimuli. In the familiar case of herpes simplex virus, such stimuli include sunlight, elevated temperature, and section of the trigeminal nerve (1).

The biochemical mechanisms that maintain latency and that are modulated upon reactivation are not understood. Excellent experimental models exist for establishment and maintenance of herpes simplex latency in ganglionic neurons of the mouse, but it is difficult to carry out biochemical analysis in vivo (2). Progress has been made in development of in vitro models of herpes simplex latency in human fibroblasts and, recently, in neuronal cultures (3). However, viral replication must be suppressed by addition of interferon and inhibitors of viral DNA synthesis; furthermore, only some of the cultured cells contain viral genomes (4).

Latency of the Epstein–Barr herpesvirus (EBV) in human B lymphocytes can be established, manipulated, and analyzed in cell culture. A few viral functions are expressed during latency, but mature transcripts of genes representing products made when virus is synthesized are not present. EBV latency can be activated by a number of diverse stimuli, including phorbol ester tumor promoters, butyrate, anti-immunoglobulins, and a factor in serum (5–8). Induction of viral replication is accompanied by synthesis of many new mRNAs and polypeptides (9).

Findings in a series of recent experiments indicated that a form of rearranged EBV DNA, which we call heterogeneous or het DNA, might provide clues about viral genes and gene products that play a role in latency of EBV. het DNA was found in a cell line designated P3J-HR-1 (HR-1) which spontaneously synthesizes considerable amounts of EBV. It was initially found that het DNA was not associated with all HR-1 cells; cellular subclones of HR-1 cells lacking het DNA could readily be isolated. Those cell clones which lacked het DNA spontaneously synthesized small amounts of virus, although much more virus could be recovered after induction with phorbol ester (10). Virus released from HR-1 cells did not immortalize lymphocytes, a defect which correlated with a sizeable genomic deletion (11). Instead, HR-1 virus is measured by its capacity to induce early antigens (EA) in Raji cells, which already contain an EBV genome. Subcloned HR-1 virus without het DNA did not cause EA expression in Raji cells. A rare HR-1 subclone with large amounts of het DNA spontaneously released virus that caused EA expression in Raji cells (12). Thus spontaneous virus production, the ability to induce EA in Raji cells, and the presence of het DNA are linked.

In further experiments, viral populations containing only standard HR-1 DNA were compared with those harboring both standard and het DNA for their ability to induce expression of a latent EBV with an immortalizing phenotype. Only virus stocks containing het DNA activated a latent genome in trans (13).

het DNA has the structural properties of defective viral DNA, although its biotype is activating rather than interfering. The genome is extensively rearranged and portions of it are repeated (14). Included in the het DNA we have been studying is a 16-kilobase-pair (kbp) palindrome (unpublished observation). We have now asked whether we could identify, within het DNA, specific regions that are responsible for the disruption of latency.

MATERIALS AND METHODS

Viral het DNA Fragments. The virions of P3J-HR-1 HH543-5 (clone 5) contain, in addition to standard HR-1 DNA, four novel BamHI heterogeneous (het) restriction fragments. The sizes of these four het fragments are 7.5, 7.0, 2.8, and 1.8 kbp. The 7.5-kbp het fragment is homologous to BamHI fragment C (Chet), and the 1.8-kbp fragment is homologous to BamHI fragment S (Shet). Both of these appear to represent standard fragments from which deletions have occurred. The other two het fragments are homologous to BamHI fragments that are not contiguous on the genome. The 7.0-kbp het fragment is homologous to standard BamHI fragments M and B' (MB'het); the 2.8-kbp het DNA fragment is homologous to BamHI fragments W and Z (WZhet) (13).

DNA was isolated from clone 5 virions and the four novel BamHI fragments were cloned in pBR322 and pSV2neo (15). The cloned fragments were identified on the basis of their size, their hybridization to cloned standard EBV DNA fragments, and their hybridization to viral DNA in cells with or without heterogeneous DNA. The 7.5-kbp BamHI frag-

Abbreviations: EBV, Epstein–Barr virus; EA, early antigen(s); EA-D, diffuse EA; EA-R, restricted EA; VCA, viral capsid antigen; MA, membrane antigen; PMA, phorbol 12-myristate 13-acetate; kbp, kilobase pair(s); het DNA, a form of rearranged EBV DNA ('heterogeneous DNA'). Chet, Shet, MB'het, and WZhet are het DNA fragments corresponding to the standard EBV BamHI fragments C, S, M and B', and W and Z, respectively.

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ment Chet was not stable in large-scale preparations. The Shet, MB'het, and WZhet fragments were used in gene-transfer experiments. WZhet and its two BamHI/HindIII subfragments were also cloned in pSVOD (16).

**Cells and Transfection.** D98/HR-1 cells, a gift from R. Glaser (Ohio State University), are hybrids between adenine phosphoribosyltransferase-minus D98 cells and the HR-1 Burkitt lymphoma line (17). They express the EBV nuclear antigen (EBNA), a product of the latent cycle, but only a rare cell makes antigens of the viral replicative cycle. D98/HR-1 cells are readily transfectable by the calcium phosphate method (18). Mouse LTK- (thymidine kinase minus) cells, obtained from W. Summers (Yale University), were maintained in Eagle's minimal essential medium containing antibiotics and 5-10% fetal bovine serum. To induce the replicative cycle in D98/HR-1 cells, phorbol 12-myristate 13-acetate (PMA) was used at 20 ng/ml for 72 hr. Cloned het viral DNA (4-6 μg) was introduced into confluent cultures on coverslips in 60-mm dishes by the calcium phosphate precipitation method (19). To obtain stable transformants, D98/HR-1 cells were transfected with either pSV2neo or pSV2neo-WZhet DNA. The neomycin analog G418 (geneticin) was introduced, at a concentration of 400 μg/ml in medium, 72 hr after transfection. Cells were maintained in selective media at all times. Seven weeks after initial selection, cells were analyzed for antigen production.

**Antigen Expression.** At intervals after transfection, the coverslips were removed, washed twice in phosphate-buffered saline, and fixed for 10 min in acetone/methanol (2:1, vol/vol) or acetone alone. Coverslips were stored at -20°C until reacted with either polyvalent sera or mouse monoclonal antibodies. Monoclonal antibodies R3.3 and K8.1, against the diffuse (EA-D) and restricted (EA-R) components of EA, and L.2, against viral capsid antigen (VCA), were obtained from G. Pearson (20). A fourth monoclonal antibody, 72A1, specific for membrane antigen (MA), was from G. Hoffman (21). The appropriate fluorescein-conjugated anti-human or anti-mouse antibodies were purchased.

**Immunoblots.** Cells were transfected in 100-mm dishes with 12 μg of cloned het DNA. Transfected cells were scraped into 0.5 ml of NaDodSO4 sample buffer (0.1% 2-mercaptoethanol/10% (vol/vol) glycerol/2% NaDodSO4/0.1% bromophenol blue/625 mM Tris Cl, pH 6.8) either 72 hr (LTK- cells) or 24-36 hr (D98/HR-1 cells) after transfection and boiled for 10 min. Approximately one-third of each total sample was subjected to NaDodSO4/10% PAGE. Protein transfer and detection of the antigen–antibody complex on the filter were as described (22).

**RESULTS**

**Antigen Expression After Gene Transfer with Cloned het Fragments in Cells Without an EBV Genome.** To determine whether het DNA encoded any antigenic proteins, the three het fragments cloned in pSV2neo were introduced into LTK- mouse cells and HeLa cells. After 72 hr, antigens were sought by indirect immunofluorescence with a polyvalent human serum, W.C., which reacts with a large number of replicative proteins (23). In the mouse and HeLa cells, MB'het and WZhet expressed antigens that were restricted to the nucleus (Fig. 1 B and D and data not shown). The Shet fragment did not induce any antigen in the LTK- or HeLa cells.

**Antigen Expression After Gene Transfer with Cloned het Fragments in Cells Containing an EBV Genome.** We next asked whether any differences in antigen expression could be seen when the het fragments were transfected into a background containing a latent genome. The same three het fragments cloned in pSV2neo were therefore introduced into D98/HR-1, a hybrid cell which contains latent HR-1 genomes. Again, WZhet and MB'het caused appearance of antigens detected by the polyvalent human serum, and Shet was without effect. However, the antigen seen after introduction of pSV2neo-WZhet into D98/HR-1 cells was not exclusively nuclear but was observed in cytoplasm in most of the cells (Fig. 1C). pSV2neo-MB'het, which induced a prominent nuclear antigen in many mouse and HeLa cells, now induced a rare, fainter nuclear antigen in D98/HR-1 cells (Fig. 1 A and B).

As shown in Table 1, many more antigen-positive D98/HR-1 cells were induced after transfection with pSV2neo-WZhet than with pSV2neo-MB'het. In LTK- and HeLa cells, the two fragments induced similar numbers of nuclear-antigen-positive cells.

**FIG. 1.** Indirect immunofluorescence of antigens induced by het fragments. (A and C) D98/HR-1 cells transfected with pSV2neo-MB'het and pSV2neo-WZhet, respectively. (B and D) LTK- cells transfected with pSV2neo-MB'het and pSV2neo-WZhet, respectively. All antigens were detected by using polyvalent human antibody W.C. and indirect anti-Ig immunofluorescence. (Magnifications: x250 in A, x100 in B-D).
positive cells (data not shown). het fragments carried on the pSV2neo vector were more effective at inducing antigen expression than when carried on pBR322.

Reactivity of Antigens Induced by het DNA to Monoclonal and Polyclonal Antibodies. Differences in the morphology and number of antigen-positive cells found after introduction of pSV2neo-WZhet into a background with or without EBV DNA could be explained by two models. In the first, WZhet expresses more, and different, antigens in D98/HR-1 cells than in LTK- cells due to "helper" functions supplied in trans by the latent EBV genome in D98/HR-1 cells. In the second, WZhet induces expression of antigens from the latent HR-1 genome. To help decide whether the antigens seen in D98/HR-1 cells were encoded or induced by WZhet, we used a panel of monoclonal antibodies.

These monoclonal antibodies, directed against EA-D, EA-R, VCA, and MA, were used to characterize the antigens found in LTK- and D98/HR-1 cells after introduction of pSV2neo-WZhet and pSV2neo-MB'het (Table 2). None of these four monoclonal antibodies detected the nuclear antigens induced in LTK- cells by pSV2neo-WZhet or by pSV2neo-MB'het. However, all four monoclonal antibodies, directed at four distinct replicative proteins, reacted with the antigens seen in D98/HR-1 cells transfected with pSV2neo-WZhet (Fig. 2). Since the 2.8-kbp WZhet fragment is probably too small to encode all these antigens, it is likely to induce their expression from the endogenous genome.

Only human polyclonal sera with reactivity to EA were able to detect the antigens expressed by pSV2neo-MB'het and pSV2neo-WZhet in LTK- and HeLa cells. Serum with antibody to VCA and not EA were unreactive. However, human sera with antibody only to VCA did react with a large number of D98/HR-1 cells transfected with pSV2neo-WZhet. Thus the products directly encoded by these two fragments are evidently EA, but the products induced by pSV2neo-WZhet in D98/HR-1 cells include late functions.

These experiments suggested that the antigens induced by pSV2neo-WZhet were derived from the endogenous genome. Furthermore, a specific function must be required to induce the viral genome, because pSV2neo-MB'het, which also encodes a nuclear EA, does not bring about induction.

Kinetics of Antigen Induction by pSV2neo-WZhet. To determine the optimal time for analysis of the viral polypeptides, a preliminary experiment assessed the kinetics of antigen induction by pSV2neo-WZhet in D98/HR-1 cells (Table 3). Based on the total numbers of antigen-positive cells detected by the polyclonal human serum, antigen expression was maximum by 24 hr after transfection. At this time, about 45% of antigen-positive cells reacted with R3.3, the monoclonal antibody to EA-D, and about 14% reacted with 72A1, the monoclonal antibody to MA. At 48 hr, the total number of antigen-positive cells was the same; but the fraction containing EA-D decreased slightly, to about 40%, and the proportion with MA reactivity increased, to about 30%. By 5 days after transfection, the total number of antigen-positive cells decreased. Replicative proteins were synthesized very rapidly; therefore, we analyzed polypeptides 24 hr after transfection.

Viral Polypeptides Made in LTK- and D98/HR-1 Cells After Transformation with pSV2neo-WZhet (Fig. 3). Immunoblotting was used to identify protein(s) made following gene transfer with pSV2neo-WZhet. A single 33-kDa protein was made after introduction of pSV2neo-WZhet into LTK- cells. A similar sized protein is synthesized in COS-1 cells (unpublished data). No such protein was seen in cells transfected with pSV2neo alone. However, after gene transfer of pSV2neo-WZhet into D98/HR-1 cells, at least six new viral polypeptides, ranging in size between 35 and 60 kDa, were identified. These polypeptides were absent from D98/HR-1 cells that had been transfected only with pSV2neo. The pattern of polypeptides made in D98/HR-1 cells after addition of pSV2neo-WZhet was similar to that seen after treatment of the cells with the phorbol ester PMA, which also induces viral replication (5). Against the background of induced viral polypeptides, it was difficult to tell whether the 33-kDa polypeptide that was found in LTK- cells was also made in D98/HR-1 cells.

Since the pSV2neo plasmid carries a drug-resistance marker, we obtained a population of D98/HR-1 cells resistant to G418 after transfection with either pSV2neo or pSV2neo-WZhet. Those D98/HR-1 cells containing the latter plasmid again expressed a group of viral polypeptides, the pattern of which was similar to that seen transiently after transfection. A polypeptide of about 4 kDa, which was observed in the transient transfection of D98/HR-1 cells with pSV2neo-WZhet, was missing in the corresponding drug-resistant transformants. About 6 weeks after preparation of the samples represented in Fig. 3B, the drug-resistant D98/HR-1 cells spontaneously stopped making viral antigens.

Antigen Expression After Introduction of WZhet DNA and Two Subfragments in Vector pSVOd. Indirect evidence suggested that expression of a protein encoded by WZhet was responsible for activation of latency. The WZhet fragment in the pSV2neo vector always caused more cells to express antigen than did the same het fragment when inserted into pBR322 (Table 1) or pSVOd (Table 4). Neither of the latter plasmids markedly enhance eukaryotic gene transcription (23). To determine whether the entire 2.8-kbp WZhet fragment was required for the interruption of latency, the WZhet fragment was digested at its single HindIII site and the two subfragments, one of 1.8-kbp and the other of 1.0-kbp, were subcloned, as BamHI--HindIII subfragments, in pSVOd. These constructs and the entire WZhet fragment cloned in pSVOd were used to transfet LTK- and D98/HR-1 cells.

Table 1. Antigen expression in D98/HR-1 cells after gene transfer with fragments of heterogeneous EBV (HR-1) DNA

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>No. of antigen-positive cells per coverslip</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSV2neo</td>
<td>1</td>
</tr>
<tr>
<td>pSV2neo-Shet</td>
<td>2</td>
</tr>
<tr>
<td>pSV2neo-MB'het</td>
<td>10</td>
</tr>
<tr>
<td>pSV2neo-WZhet</td>
<td>239</td>
</tr>
<tr>
<td>pBR322</td>
<td>4</td>
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<tr>
<td>pBR322-Shet</td>
<td>0</td>
</tr>
<tr>
<td>pBR322-MB'het</td>
<td>1</td>
</tr>
<tr>
<td>pBR322-WZhet</td>
<td>13</td>
</tr>
</tbody>
</table>

Human antiserum W.C. was used.
Neither subfragment induced antigens in LTK− or D98/HR-1 cells, whereas the full-sized WZhet fragment inserted into pSV0d induced antigen in both systems (Table 4 and data not shown). Once again the observed antigen was nuclear in the LTK− cells and predominantly cytoplasmic in the D98/HR-1 cells. These data are consistent with the idea that interruption of the WZhet fragment eliminates the nuclear antigen and that this encoded protein is required for the activation of latency.

**DISCUSSION**

We have analyzed the mechanism of herpesvirus latency with the powerful techniques of molecular genetics. Our results show that expression of latent EBV can be activated by gene transfer of a 2.8-kbp fragment of rearranged het DNA. Virion populations that contain a mixture of standard and het DNA activate viral DNA replication, antigen expression, and virion synthesis in cells containing latent EBV (12, 13, 24). Several possible mechanisms of activation of latency by virion populations with het DNA can now be excluded on the basis of our data obtained by use of DNA-mediated transfection. Activation is not due to proteins contained on the virions themselves and does not require interaction between superinfecting standard and het genomes. Disruption of latency can apparently be effected by a single component, WZhet, and does not require a group of products or the two other novel het fragments Shet and MB’het. A major conclusion of these experiments is that most of the products that appear after introduction of WZhet arise from the endogenous latent genome and not from the het fragment. The antigens that are made—EA, VCA, and MA—are detected by monoclonal antibodies that do not react with the nuclear product encoded by WZhet itself (Figs. 1 and 2 and Table 2). Furthermore, two antigens, EA-D and MA, have been mapped, by hybrid-arrested in vitro translation, to DNA fragments not contained on WZhet (20, 25). Only a single polypeptide of 33 kDa is encoded by WZhet itself, but in cells

**Table 3. Kinetics of appearance of antigen after transfection of D98/HR-1 cells with pSV2neo-WZhet**

<table>
<thead>
<tr>
<th>Time after transfection, hr</th>
<th>No. of antigen-positive cells per coverslip</th>
<th>Polyvalent human serum W.C.</th>
<th>Mouse monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytoplasmic</td>
<td>Nuclear*</td>
</tr>
<tr>
<td>pSV2neo-WZhet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>24</td>
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<td>pSV2neo</td>
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<tr>
<td>72</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ND, not done.

*Exclusively nuclear.*

**Fig. 2.** Antigens in D98/HR-1 cells induced by pSV2neo-WZhet and detected by murine monoclonal antibodies. (A and B) R3.3, anti-EA-D. (C and D) 72A1, anti-MA. (Magnifications: ×250 in A, C, and D; ×100 in B.)
containing latent EBV, many new polypeptides are induced (Fig. 3). We have not yet determined whether introduction of WZhet leads to synthesis of mature virions from D98/HR-1 cells. Virion production takes place at low frequency and would best be detected by bioassay. However, there is no bioassay for virions in the D98/HR-1 cells. The genomes are nonimmortalizing and, since they lack het DNA, they do not induce EA in Raji cells.

**Mechanism of Induction.** Our data do not indicate the level at which latency is regulated. The principal difference between lymphoid cells that have a latent genome and those whose genome is replicating is in the number of different mRNAs they contain (9). However, there is also evidence that more nuclear viral RNAs are found than appear as mature cytoplasmic mRNAs (26). Thus WZhet probably activates transcription, but it might also affect posttranscriptional processing and/or transport.

The results pose a number of questions about how WZhet might activate expression. What is the importance of the rearrangement in het DNA? It is likely that this rearrangement is required, since standard HR-1 virions with unarranged DNA do not disrupt latency (13). Are transcription and a product from WZhet required to activate latency, or is the DNA alone sufficient? Induction occurred at a low level when WZhet cloned in pBR322 or in pSV0d was used, but the signal was considerably weaker than when the fragment was introduced into the pSV2neo vector (Tables 1 and 4). Only the latter plasmid contains a complete set of "enhancer" sequences, which appear to be needed to produce the phenomenon. In het virions, these enhancers may be provided by sequences outside of WZhet. pSV2neo-WZhet interrupted by the restriction endonuclease EcoRI, which does not cleave the WZhet DNA but cuts the vector, was unable to activate expression in a transient assay (data not shown). Thus, the DNA itself is probably not active (e.g., by binding an inhibitory molecule).

The results suggest that the 33-kDa protein encoded by WZhet is responsible for activation of the latent genome. This protein shares antigenic epitopes with a "standard" EBV polypeptide, since human sera with antibody to EBV react with it. However, we do not know whether an identical polypeptide is made during the course of the normal replicative cycle. The 33-kDa protein may also be a novel viral fusion protein that results from the juxtaposition of two nonadjacent coding regions. Whether the WZhet product directly activates transcription of one or more viral genes or alters an inhibitor encoded by the virus or by the cell is a problem for the future.

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**Table 4.** Antigen expression in D98/HR-1 cells after gene transfer with pSV0d-WZhet and its two subfragments

<table>
<thead>
<tr>
<th>Plasmid</th>
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<td>pSV2neo-WZhet</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>pSV0d-1.0WZhet*</td>
<td>2</td>
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</table>

*Contains 1.8-kbp subfragment.

†Contains 1.0-kbp subfragment.

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