P3HR-1 Epstein-Barr Virus with Heterogeneous DNA Is an Independent Replicon Maintained by Cell-to-Cell Spread

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We present results of biological experiments which indicate that the subpopulation of Epstein-Barr virus strain P3HR-1 with heterogeneous (het) DNA consists of self-contained replicons which multiply alongside, but independently of, Epstein-Barr virus strain HR-1 containing standard DNA. When a population of HR-1 virions containing het DNA was introduced into X50-7 cells, the input heterogeneous DNA increased in abundance, as did the DNA of the endogenous virus of X50-7 cells. The input standard HR-1 viral DNA, however, was not amplified. When parental HR-1 cells or a cellular subclone containing het DNA were grown for several weeks in the presence of human serum with neutralizing antibody, the het DNA was lost from the culture; standard HR-1 DNA, however, was not affected by antiserum. Furthermore, virions containing het DNA could be serially propagated through cellular subclones of HR-1 cells which lack het DNA. After each serial passage, cells which acquired het DNA released virions with the ability to induce early antigens in Raji cells. These experiments define a novel in vitro life cycle of an Epstein-Barr virus variant which is maintained, not vertically by partitioning to daughter cells in cell division, but horizontally by cell-to-cell spread.

The nonimmortalizing P3HR-1 strain of Epstein-Barr virus (EBV) contains a subpopulation of virions with heterogeneous (het) DNA. Het DNA has the structural features of defective viral DNA: it contains DNA sequences which are rearranged by comparison to standard DNA, certain regions of the genome are represented and others are excluded from the het DNA, and some portions of het DNA are hypermolar (2, 8, 13).

Het DNA is biologically interesting. When virions containing defective DNA are added to cells with a latent genome, they activate expression of the latent genome. This phenomenon has been best illustrated by addition of virions containing het DNA [het(+)] to a line, designated X50-7, which contains a tightly latent genome of the transforming phenotype. X50-7 cells release transforming virus after addition of virions with het DNA, but not after addition of virus lacking het DNA [het(-)] (11). Furthermore, het(+) but not het(-) virus can induce early antigens in Raji cells (10, 13, 18). Certain het(-) clones of HR-1 cells release virus with ability to induce early antigen only after superinfection with het(+) HR-1 virus (11). Taken together, these experiments show that het sequences are responsible for disruption of latency and activation of expression of the endogenous genome.

Unlike the standard EBV episome, het DNA does not appear to be stably associated with HR-1 cells. From a population of HR-1 cells which contains het sequences, cellular subclones can be readily isolated which do not contain the het DNA, even though the clones continue to maintain standard EBV DNA (10). Even the single rare cellular subclone of HR-1 cells isolated so far, which contains large amounts of het DNA, can be subcloned to yield cell subclones which lack het DNA (11). These findings indicate that at least one requirement for latency in the EBV system, namely, the partitioning of genomes to daughter cells with cell division, is not met by het DNA. Unlike the standard genome, het DNA may not be present within all

cells of a het(+) culture. Furthermore, the presence of het sequences may invariably cause cell lysis due to activation of viral replication.

The cell cloning experiments suggest, but do not directly prove, that het DNA sequences and standard HR-1 viral DNA sequences are not directly linked to each other. However, het DNA sequences are invariably found in the presence of standard EBV sequences, another feature reminiscent of helper-dependent defective viruses. The present experiments were done to provide biologic evidence on the question of the linkage between the het DNA and standard DNA. Specifically, we wished to learn whether het(+) DNA and het(+) virus replicate independently or in concert with standard HR-1 virus. In the first set of experiments, HR-1 virus containing a mixture of standard and het DNA was used to superinfect X50-7 cells, and the fate of both input standard and het sequences was followed by Southern hybridization by using probes capable of distinguishing the two types of sequences. In the second set of experiments, parental HR-1 cells or a subclone containing het sequences were grown in the presence of antiserum to EBV. After growth in antibody, the presence of het and standard EBV DNA sequences was again determined by Southern blot hybridization. In the third group of experiments, het(+) virions were serially propagated.

MATERIALS AND METHODS

Cells and cell clones. X50-7 cells are human umbilical cord lymphocytes transformed in vitro by EBV (17). They have a tightly latent EBV genome which, however, can be induced to replicate by superinfection with het(+) HR-1 virus (11). HH514-16 cells (clone 16) are a clone of the P3HR-1 line, in which there is a low level of spontaneous virus synthesis (less than 1% of cells make viral capsid antigen). More virions are made when clone 16 cells are treated with phorbol ester (13, 20). Clone 16 cells lack het DNA, and the virus released from them is unable to induce early antigen in Raji cells. HH543-5 cells (clone 5) are a clone of the P3HR-1 line,

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which contains viral het DNA and spontaneously releases virions which are able to induce early antigen (13).

Abundance of viral DNA after superinfection of X50-7 cells. X50-7 cells were inoculated with virus stocks prepared from clone 5 or clone 16 cells or, as a control, with medium. Virus stocks were unconcentrated supernatant fluids from clone 5 or clone 16 cells harvested and filtered 7 days after subculture of the cells. Phorbol ester was not used to prepare virus stocks. Cellular DNA was harvested from equal numbers of superinfected X50-7 cells on days 0, 3, and 5 or 7 after superinfection by the method of Wahl (15). DNA concentrations were measured by optical density. About 10 µg of cellular DNA was digested with *Bam*HI, electrophoresed in 0.5% agarose, and transferred to nitrocellulose. Blots were hybridized with cloned standard fragments of EBV (strain FF41) DNA made radioactive by nick-translation (6). In some experiments, the probes were heterogeneous HR-1 DNA cloned as *Bam*HI fragments in pBR322 (J. Countryman and G. Miller, manuscript in preparation).

Neutralization experiments. Two neutralization experiments were performed. In the first, P3H3 cells or clone 5 cells were grown either in standard medium or in medium containing 10% EBV antibody-positive human serum or 10% EBV antibody-negative human serum. With each weekly feeding, the appropriate serum was replaced. DNA was prepared from the cells after 8 weeks and probed for the presence of het DNA sequences.

In the second experiment, only clone 5 cells were used. They were grown in either 10% antibody-positive, 10% antibody-negative human serum or standard medium for 43 days. Thereafter, they were grown without human sera. Cells were collected and DNA was made weekly for 15 weeks and probed for the presence of the *Bam*HI het fragment WZd with a plasmid containing *Eco*RI fragment B of EBV DNA.

Serial passage experiment. Clone 16 cells were set up at 5×10^5 cells per ml in two flasks. To one flask was added a 1/10 volume of clone 5 supernatant. (This supernatant caused 10 to 15% of Raji cells to develop early antigen.) To the other flask was added an equivalent amount of fresh medium. Both flasks were split 1:2 weekly. After 3 weeks, both flasks were washed three times in RPMI 1640 medium base, and the last wash was saved. Four days later, the medium from both flasks was removed and tested, together with the last wash, for early-antigen induction in Raji cells. Cell splits at a 1:2 ratio were continued weekly for 4 weeks, when the washes were repeated, and 5 days later the supernatants from both groups were again harvested and tested for early-antigen induction in Raji cells. After 9 weeks cellular DNA was prepared from both cultures. Cell-free supernatants from both flasks were prepared at day 71 by filtration. These were added at a 1/10 volume to fresh flasks of clone 16 cells. Cells were once again split weekly and washed repeatedly after 3 weeks. Three days later, medium was harvested and tested for early-antigen induction. Filtered supernatants were prepared from both cultures after 7 weeks and used to initiate the third passage. This process of cell splitting and washing was repeated as described above. At each passage level, cellular DNA was prepared and examined for the presence of het sequences.

RESULTS

Replication of het DNA after superinfection of X50-7 cells with P3HR-1 clone 5 virus. We have previously shown that, at the multiplicities of infection which are achieved when undiluted clone 5 virus is added to Raji cells, there is no

stimulation of Raji viral DNA synthesis, even though early antigens are induced (11). When the same amount of clone 5 virus is added to X50-7 cells, however, there is stimulation of viral DNA synthesis, at least some of which, on the basis of restriction endonuclease polymorphisms, can be shown to be due to the endogenous virus (11). Since Raji cells seem to be relatively nonpermissive for viral DNA synthesis, we carried out analysis of the replication of superinfecting standard and het HR-1 DNA in X50-7 cells (see Fig. 2-5).

In our first experiments, X50-7 cells were superinfected with clone 5 virus or as controls with clone 16 virus or were mock infected. Cellular DNA was harvested periodically and examined for viral DNA sequences which are known to be represented in het DNA. The *Bam*HI het DNA fragments of clone 5 virus are listed in Table 1, and their location on HR-1 DNA is shown in Fig. 1. (This data is reproduced from reference 11.) For example, when blots were probed with *Bam*HI-S, there was an obvious increase in the amount of *Bam*HI fragment S in X50-7 cells which had been superinfected 7 days previously with clone 5 virus. An increased hybridization signal with the 1.8-kilobase-pair (kbp) *Bam*HI het fragment S (Fig. 2A) was also evident. Similarly, when blots were probed with *Bam*HI-M, there was an increase in the amount of *Bam*HI-M DNA in the X50-7 cells superinfected with clone 5 virus. An increase in the amount of 7.0-kbp *Bam*HI het fragment MB' was also evident. It was first detected on day 3, and the hybridization signal was more intense on day 7. This het fragment must have internal heterogeneity, as it invariably appears as a fuzzy band on the gel. The results thus indicate that, upon superinfection of X50-7 cells, there is amplification of het sequences.

Similar findings were obtained when the probe used was one of the het DNA fragments rather than a standard EBV DNA fragment (Fig. 3). When a blot was probed with *Bam*HI het fragment WZd, it was evident that, with time, there were marked increases in the quantities of het WZd only in X50-7 cells to which clone 5 virus had been added. In the same cultures it can be seen (Fig. 3) that there were increases in other viral DNA fragments (*Bam*HI-W and *Bam*HI-Z) to which the het DNA is homologous. However, there was no comparable increase in *Bam*HI het fragment C. Neither endogenous X50-7 viral DNA nor het DNA increased in amount in X50-7 cells which were mock infected or infected with virus from clone 16 cells.

Endogenous but not superinfecting standard DNA replicates. It is apparent (Fig. 2 and 3) that, after superinfection of X50-7 cells with clone 5 virus, there was an increase in the abundance of certain standard EBV DNA fragments, such as *Bam*HI-S, *Bam*HI-M, *Bam*HI-W, and *Bam*HI-Z. The

TABLE 1. Clones of novel *Bam*HI restriction endonuclease fragments present in the heterogeneous DNA of P3HR-1 clone 5 EBV

Size (kbp) ^a	Homologous <i>Bam</i> HI fragment(s) ^b
7.4	C
7.0	M, B', H
2.8	W Z d
1.8	S

^a These are the insert sizes of clones of het DNA in pSV₂ neo, determined by migration on 0.5% agarose in comparison with markers of a *Hind*III digest of phage λ DNA.

^b These homologies were determined by hybridizing plasmids containing *Bam*HI fragments of EBV (strain FF41) DNA to Southern blots containing HR-1 viral DNA.

FIG. 1. Map of EBV DNA HR-1 and FF41 strains showing the positions of heterogeneous DNA. Boxes with similar shading are linked, e.g., *Bam*HI-M to *Bam*HI-B' and *Bam*HI-W to *Bam*HI-Z. Asterisks indicate DNA fragment length polymorphisms, which distinguish HR-1 from FF41 DNA.

results of these experiments do not tell us whether this increase represents the endogenous X50-7 viral genome or the superinfecting HR-1 clone 5 viral genome, since these particular DNA fragments are the same size in both strains (12). There are, however, certain obvious restriction endonuclease polymorphisms which can distinguish the two viral strains (12). For example, in HR-1 there is no *Bam*HI site between fragments B and G. In HR-1 there is a ~16.2-kbp BG fragment rather than two individual fragments, B (~9.6 kbp) and G (~6.6 kbp). Therefore, we used *Bam*HI fragments B and G to probe Southern blots of superinfected X50-7 cells (Fig. 4). When *Bam*HI-B was used as the probe, there was a clear increase in the amount of *Bam*HI fragment B, but no significant change was observed in the amount of the BG fragment from the standard HR-1 virus. To make this point clear, we placed the three interval samples from X50-7 cells infected with clone 5 virus in adjacent slots in the gel and probed the blot with *Bam*HI-G. The hybridization signal on *Bam*HI-G, representing the endogenous X50-7 genome, increased on days 3 and 7 after superinfection, but there was no alteration in the intensity of the signal from HR-1 *Bam*HI-BG, which represents input standard HR-1 viral DNA.

Use of *Bam*HI-H as a probe for replication. The results of the experiment in which *Bam*HI-H was used as a probe are particularly revealing (Fig. 5). *Bam*HI-H can distinguish between the endogenous X50-7 genome, which has a full-sized *Bam*HI-H, and the superinfecting HR-1 genome, which has a deleted *Bam*HI-H fused to *Bam*HI-W (fragment WH) (1). Furthermore, *Bam*HI-H weakly detects a heterogeneous region, the *Bam*HI het fragment MB', presumably owing to homology of the *Not*I repeats (4, 7). This experiment again shows that the endogenous X50-7 *Bam*HI-H increases after superinfection with clone 5 virus, but the standard superinfecting *Bam*HI-WH does not change in amount. In the superinfected X50-7 cells, there was a slight increase in the fuzzy *Bam*HI het fragment MB' which was detected by *Bam*HI-H. There also appeared to be an increase in a fragment about the size of *Bam*HI-B'. This is probably the result of two events, replication of the endogenous *Bam*HI fragment B' in X50-7 cells and replication of the superinfecting HR-1 *Bam*HI fragment B', which is part of het DNA. In het HR-1 DNA there is a ~20-kbp *Eco*RI fragment which contains *Bam*HI-M attached to a stretch of the right side of the genome including *Bam*HI fragments B', W'I', and A

(Fig. 1) (H. Jenson, M. Rabson, J. Countryman, and G. Miller, manuscript in preparation).

These experiments demonstrate that, under conditions in which the superinfecting het (HR-1) DNA increases in amount, the superinfecting standard (HR-1) DNA does not, unless it happens to be linked to het sequences.

Neutralization of het sequences. The results of the foregoing experiments show that het DNA replicated preferentially and independently of standard DNA when the two were added together to X50-7 cells as part of superinfecting virus stock. But what of the replication of the two types of EBV DNA within the HR-1 line itself?

The results of our previous cell cloning experiments, outlined above, suggest to us that het HR-1 DNA replicates independently of standard HR-1 DNA. Furthermore, we have previously found that some clones of HR-1 cells which lack het DNA can be superinfected with virus containing het DNA, a finding which suggests that in the HR-1 line itself the het sequences might be maintained as a carrier culture by cell-to-cell spread (11). If this is the case, then we should be able to eliminate het DNA selectively by culturing HR-1 cells or the het(+) clone 5 cells in the presence of EBV antibody.

In the first such experiment, parental HR-1 cells or clone 5 cells were cultured in human serum with or without EBV neutralizing antibody or in medium without human serum for 8 weeks. The cell DNA was analyzed for the presence of het sequences by probing Southern blots with plasmids containing appropriate EBV DNA fragments. For example (Fig. 6), the *Bam*HI het fragment WZd, detected by a plasmid containing *Eco*RI-B, was present in the cultures of HR-1, in clone 5 cells grown in medium with fetal bovine serum, and in cells grown with antibody-negative human serum, but was absent from cultures containing the human serum with EBV antibody. The standard HR-1 fragments recognized by *Eco*RI-B were not altered in the antiserum-treated cultures. Identical results were obtained with *Eco*RI-F as a probe. Standard *Bam*HI fragments recognized by this probe (*Bam*HI-L, -M, and -S) were present in all the cultures. However, only in cultures which received antibody-negative human serum or media was the *Bam*HI het fragment S seen. Thus, we concluded that het DNA had been neutralized by the EBV antibody.

Time course of disappearance and reappearance of het DNA after addition of EBV antibody. In the previous experiment,

FIG. 2. Replication of heterogeneous EBV DNA after superinfection of X50-7 cells with P3J-HR-1 clone 5 virus. Cells were infected with clone 5 (c5) or clone 16 (c16) HR-1 virus or mock infected. DNA harvested on days 0, 3, and 7 was digested with *Bam*HI and transferred to nitrocellulose. The blots were probed with *Bam*HI-S or *Bam*HI-M. Note the increase in abundance, with time, of EBV DNA only after superinfection with clone 5 virus. (A) Increase in *Bam*HI-S and *Bam*HI het fragment S; (B) increase in *Bam*HI-M and *Bam*HI het fragment MB'.

cells were maintained in the continuous presence of anti-serum for 8 weeks and then analyzed for het DNA. We next wished to know how quickly het DNA disappeared after addition of antiserum and whether, after the removal of antiserum, het DNA reappeared (Fig. 7). The results of this experiment showed that, on day 7 after the addition of neutralizing antiserum to clone 5 cells, the het DNA was still present; by 2 weeks after the addition of antiserum, however, the het DNA disappeared from the culture with the neutralizing antibody but was still present in the culture with antibody-negative human serum. Het DNA remained absent as long as antibody-positive human serum was kept in the cultures (about 6 weeks); the cultures were then washed free of human serum and maintained in regular medium with fetal bovine serum. Samples of cells were harvested periodically and checked for the presence of het DNA. Het DNA remained absent in the culture which had been treated with neutralizing antibody for another 7 weeks. By 9 weeks after removal of the antiserum, however, het DNA reappeared in this culture and remained thereafter.

Serial propagation of het(+) virus. Although the results of the foregoing experiments suggest that het(+) virions are maintained by cell-to-cell spread, other interpretations of the effects of antibody are possible. For example, antibody could inhibit the intracellular generation of het DNA. For a more direct proof of the idea of cell-to-cell spread, we tried to learn whether het(+) virus could be serially propagated from one het(-) culture to the next. Het(-) clone 16 cells were infected with het(+) clone 5 virus, and a parallel culture was held uninfected. After a week, the inoculum was removed by washing, and the cultures were maintained for about 2 months. At this time, cellular DNA was prepared and supernatants from both cultures were passed to new clone 16 cells. This process was repeated for three serial passages (Table 2). During these passages, het DNA never appeared spontaneously in clone 16 cells (Fig. 8). The het DNA (as represented by *Bam*HI het fragment WZd) however, appeared after each passage in the series initiated with clone 5 virus. Total cellular DNA analyzed 11 days after the fourth passage had acquired het DNA. Furthermore, after each serial passage, supernatants harvested from clone 5 superinfected clone 16 cells, but not those from mock-infected clone 16 cells, were capable of inducing early antigen in Raji cells (Table 2). This result confirms our previous data that het(+) virus is responsible for early-antigen induction in Raji cells (10, 11, 13).

The results of these experiments show that virions containing het DNA sequences could be passed sequentially from one culture to the next and with time appeared to stimulate replication of standard HR-1 sequences.

DISCUSSION

Our findings indicate that the het(+) component of the clone 5 HR-1 virus population is a novel form of EBV. It does not seem to set up latency; in fact, it disrupts latency. It is maintained by cell-to-cell spread, presumably in the presence of helper virus, whereas standard HR-1 genomes are passed onto daughter cells. Het(+) clone 5 virus repre-

FIG. 3. Replication of *Bam*HI het fragment WZd DNA after superinfection of X50-7 cells with P3HR-1 clone 5 virus. Experimental conditions were as described in the legend to Fig. 2. Note that in cells superinfected with clone 5 virus there were increases in abundance of the *Bam*HI fragments Z and W and increases in the het fragment WZd. We are uncertain of the identity of the prominent band seen on day 3, which is slightly larger than the HR-1 *Bam*HI fragment WH.

sents a valuable variant which will aid in deciphering mechanisms of EBV DNA replication, packaging, and latency.

Preferential replication of het DNA. To study viral DNA replication, we took advantage of known DNA polymorphisms which distinguish the EBV endogenous to X50-7 cells from the standard HR-1 genome and the het HR-1 genome. The viral DNA which replicates appeared to be from two sources. The endogenous X50-7 DNA replicated, presumably because it was activated to do so by elements encoded by the het(+) virus. The superinfecting het(+) virus replicated, probably because it contained multiple origins of replication or in some way was at an advantage for the utilization of its origins.

We do not yet know where on the EBV genome the origins for viral DNA synthesis are located. Henry and co-workers have identified several regions of the genome which seem to function as autonomously replicating sequences in *Saccharomyces cerevisiae* (9). Of these sequences, *Bam*HI-Y, *Bam*HI-R, and *Eco*RI-DIJ, only the latter seems to be represented in heterogeneous clone 5 DNA. Yates et al. recently found that sequences in *Bam*HI-C are sufficient to permit other selectable sequences (e.g., the Tn5 transposon) to be maintained as plasmids in cell lines carrying EBV (19). Therefore, *Bam*HI-C must contain a certain type of origin of

FIG. 4. Endogenous X50-7 viral DNA but not standard P3HR-1 DNA replicated after superinfection of X50-7 cells with P3HR-1 clone 5 virus. X50-7 cells were infected with clone 5 or clone 16 virus or were mock infected. DNA was prepared on days 0, 3, and 7, digested with *Bam*HI, and analyzed by Southern blotting. Blots were probed with *Bam*HI-B (A) or *Bam*HI-G (B), which are capable of distinguishing the superinfecting HR-1 virus, which in turn has a fused *Bam*HI fragment BG from the genome in X50-7 cells in which *Bam*HI fragments B and G are present as separate fragments. Note a progressive increase in the amounts of fragments B and G in X50-7 cells superinfected with clone 5 virus. No comparable increase in the abundance of the input BG fragment is seen. The DNA samples used were the same as those shown in the blots of Fig. 2 and 3.

FIG. 5. Replication of endogenous X50-7 and superinfecting heterogeneous but not standard P3HR-1 DNA. Experimental design and samples were identical to those shown in Fig. 2-4. The probe, *Bam*HI-H, detected the input standard HR-1 viral genome as *Bam*HI-WH and the endogenous X50-7 genome as *Bam*HI-H. There was progressive increase in *Bam*HI-H and no change in *Bam*HI-WH.

replication. Het DNA from HR-1 virus contains sequences homologous to *Bam*HI-C (2, 11). Whether the same sequences which are capable of functioning as an autonomously replicating sequence or those which are required for maintaining a plasmid are also utilized for viral DNA replication is not known. In any event, it is apparent that het DNA from clone 5 virus contains one or more origins of DNA replication. It should be possible to learn which sequences present in het DNA function as origins by introducing plasmids which contain het sequences into X50-7 or clone 16 cells, both of which, according to data we present here, are permissive for het DNA replication.

There have been previous clues in the literature that defective HR-1 DNA may replicate preferentially. These

FIG. 6. Neutralization of EBV with heterogeneous DNA in P3H3 and clone 5 cells by human serum with EBV antibody. Parental P3H3 cells or clone 5 cells were grown in the presence of human serum with EBV antibody (+), without EBV antibody (-), or only in fetal bovine serum (0). At the end of 8 weeks, cell DNA was prepared, digested with *Bam*HI, and probed for the presence of het DNA with *Eco*RI-B or *Eco*RI-F. Het DNA is absent from cultures grown in the presence of antibody-positive human serum.

FIG. 7. Disappearance and reappearance of EBV with heterogeneous DNA after addition and removal of EBV antibody. Cultures were grown in the presence (+) or absence (–) of human serum with EBV antibody or in regular medium (0) for 6 weeks. Thereafter, cultures were washed and maintained in medium without human serum. Samples were periodically harvested and analyzed for the presence of het DNA. Note that the het fragment WZd was found after 7 days, disappeared, and reappeared on day 105. The lanes containing DNA from antiserum-treated cultures on days 84 and 91 were underloaded. On overexposed autoradiograms, however, het fragment WZd was absent on day 84 and present on day 91.

clues have come from analysis of viral DNA made upon superinfection of Raji cells at high multiplicity. Cho et al. found preferential accumulation of EBV DNA fragments which were similar to those that contribute to heterogeneity of HR-1 virus (3). These experiments differ from ours in several ways: (i) Raji or NC37 cells were used as the targets; (ii) the very high multiplicities of HR-1 virus used were presumably needed to overcome a block to viral DNA replication in Raji cells; and (iii) accumulation of viral DNA fragments after superinfection was studied with whole viral DNA as a probe. (The experiments were done before

recombinant EBV DNA clones were available.) Siegel and co-workers looked at the size of replicating viral DNA in superinfected Raji cells (14); there appeared to be several size classes, one of which could possibly be defective DNA.

Why does standard HR-1 DNA not replicate upon superinfection of X50-7 cells? The conclusion that het(+) virus replicates autonomously is in part supported by the finding (Fig. 2-5) that standard viral DNA does not replicate under conditions of exposure of X50-7 cells to stocks of clone 5 virus containing virions with both standard and defective DNA. Several possible explanations may be discussed, although at present we do not have data which enable us to choose between the several alternatives.

(i) We do not yet know the relative proportion of virions with standard DNA and virions with het DNA in the clone 5 virus stock, and we have not yet developed methods for physical separation of the two types of virions. If virions with het DNA are present in great excess, then the preferential replication of het DNA might be due to a higher input multiplicity.

(ii) We do not yet know the complete structure of het DNA from clone 5 virus, although we have worked out a structure of about 36 kilobase pairs which contains all the known het fragments except that homologous to *Bam*HI-C (Jenson et al., manuscript in preparation). Het DNA is packaged in virions (13; Fig. 6–8; Table 2). Assuming that the het monomer is ~50 kbp, and assuming a “head-full” requirement for packaging, then het DNA might be packaged as a trimer (5). When this trimer enters a cell, it might have a significant advantage in DNA replication over standard DNA simply because it contains more copies of the sequences which function as origins.

(iii) There might exist a mechanism by which the endogenous EBV genome or cellular products in X50-7 cells prevent replication of a superinfecting virus. Such a mechanism

TABLE 2. Serial passage of clone 5 HR-1 virus in clone 16 HR-1 cells as assayed by early-antigen induction in Raji cells

Passage no.	Days after inoculation of passage	Dilution of original inoculum ^a	% of Raji cells EA ^b -positive after exposure to supernatant from clone 16 cells			
			Uninfected		Infected	
			Wash	Media	Wash	Media
1	21	10 ⁻⁴	0	0	0	4
	54	10 ⁻⁸	NT ^c	0	NT	10
2	24	10 ⁻¹³	0	0	0	2
3	36	10 ⁻¹⁶	NT	0	NT	8
	46	10 ⁻¹⁹	0	0	0	50
4	11	10 ⁻²²	0	0	0	2
	21	10 ⁻²³	0	0	0	10
	25	10 ⁻²⁶	0	0	0	30

^a Dilution calculation: each inoculation, 1:5; each cell split, 1:2; each wash, 1:10.

^b EA, Early antigen.

^c NT, Not tested.

might be related to mechanisms which exist to keep the X50-7 genome latent. Standard superinfecting HR-1 virus may be susceptible to this regulation, whereas het(+) virus may be able to escape such a suppression mechanism. If this hypothesis is correct, then het(+) virus might lack certain signals present in standard virus which allow standard virus to be inhibited.

(iv) A similar mechanism of interference might exist which is mediated, not by the endogenous X50-7 virus, but by the het(+) virus itself. Particularly if het(+) virus is in excess, it might prevent establishment of infection by het(-) virus.

(v) Standard HR-1 virus may contain a mutation which prevents it from initiating infection in other cells. This mutation would presumably lie somewhere in the early phases of infection before viral DNA synthesis. Het(+) virus may no longer be affected by this mutation, possibly as a result of rearrangements which have occurred in the het(+) genome.

Neutralization of het(+) virus. The results of experiments in which het(+) DNA was eliminated by human serum with neutralizing antibody are the strongest evidence that het(+) DNA is maintained in the HR-1 line and in its het(+) cell clone 5 by cell-to-cell passage of an encapsidated virus. An explanation that is much less likely, but that should not be excluded, is that antiserum acts not by preventing cell-to-cell spread but by selectively blocking the replication of intracellular het DNA but not that of standard DNA. Antiserum does not affect the presence of the standard EBV

sequences which are maintained by partitioning to daughter cells.

Antiserum did not eliminate the het DNA immediately (Fig. 7), indicating that, at times, het DNA is intracellular and inaccessible to antiserum. Presumably, however, cells containing het DNA die owing to viral replication.

Since the neutralization experiment suggests that het(+) DNA is not partitioned to daughter cells in association with standard DNA, how is it possible that we previously isolated a cell clone (clone 5) which contains het sequences (13)? The most feasible explanation is that this cell clone, which was derived by limiting dilution in liquid medium, was isolated in the presence of het(+) virus. When the original cell divided, one of the progeny became infected with het(+) virus, and thereafter the clone was a carrier culture. This would also explain why it has been possible to segregate het(-) subclones from clone 5 cells (11).

In the experiment illustrated in Fig. 7, het(+) DNA reappeared in the culture about 9 weeks after removal of the antiserum. There are three possible explanations for the reappearance of het sequences. The het(+) clone 5 virus might have been incompletely neutralized and the small amount remaining took 9 weeks to reestablish itself in the culture by spreading. This seems most likely. Another explanation is that clone 5 cells spontaneously regenerated het sequences. This latter explanation seems less likely since, in subclones of clone 5 cells which no longer contain het(+) DNA, it does not reappear (11; unpublished observations). A third possible mechanism is that het(+) DNA might be maintained intracellularly in a few cells. Subsequently, it might be induced to make virions which spread in the culture.

Serial passage of clone 5 virus. This experiment provides an example of a purely replicative *in vitro* life cycle of an EBV variant, albeit a defective one. In the four passages we have carried out so far, a number of phenomena merit discussion. Late into a viral passage, clone 16 cells which had been superinfected with clone 5 virus had a higher content of standard viral DNA than did comparable uninfected cells (Fig. 8). This finding is compatible with the idea that the presence of het(+) DNA stimulates viral replication in HR-1 cells. It takes several weeks for het(+) virus to become established in clone 16 cells (11). Supernatants harvested early in the passage always had less ability to activate early antigen than those harvested late in the passage (Table 2). Thus, establishment of infection of clone 16 cells by superinfecting virus must be relatively inefficient. Late in passages 3 and 4, however, virus with high EAI ability was recovered. This result raises the possibility that passage enriches for variants with increased ability to superinfect and to induce early antigen in Raji cells. So far, however, the het sequences which have been seen during the four serial passages have been identical in size to those found in the original clone 5 virus stock (Fig. 8; unpublished observations).

Clone 16 cells are an appropriate host for clone 5 virus. Nonetheless, clone 16 cells are not fully permissive, since even late in a passage the majority of cells are viable. Either clone 16 cells have partial resistance to superinfection or they continually differentiate into subpopulations which vary in susceptibility to infection by clone 5 het(+) virus. Otherwise, we would expect clone 16 cells to die of the cytopathic effects of het(+) virus. In fact, the same process must occur in clone 5 cells, which also remain viable.

So far, the serial propagation of het(+) clone 5 virus has been tested only in clone 16 cells. In future experiments, it

FIG. 8. Serial passage of clone 5 het(+) HR-1 virus through het(-) clone 16 HR-1 cells. Clone 16 cells were infected with clone 5 virus or medium, and serial passages from each group to uninfected clone 16 cells were made. Intracellular DNA, prepared 54 days after passage 1, 48 days after passage 2, and 11 days after passage 4, was analyzed for het DNA by Southern blotting. Het DNA appeared in each passage. After passage 1 and 2 cellular DNA obtained from cultures with het DNA had a greater abundance of standard HR-1 DNA fragments.

will be interesting to study the requirements for serial propagation. Is the entire EBV genome needed? Do different EBV genome variants all permit replication of the defective sequences? Can the requirements for het(+) virus replication be sufficiently clearly defined so that an EBV variant can be engineered which does not establish latency, does not immortalize, and replicates independently of any helper function?

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