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Epstein-Barr Virus with Heterogeneous DNA Disrupts Latency

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By cloning the HR-1 Burkitt lymphoma line, we previously uncovered two distinct biological variants of nontransforming Epstein-Barr virus (EBV). The most commonly cloned variant has a low rate of spontaneous viral synthesis and is unable to induce early antigen in Raji cells (EAI^-). A rare variant spontaneously releases virus which is capable of inducing early antigen in Raji cells (EAI^+). Since EAI^- virus lacks heterogeneous DNA (het^-) and EAI^+ virus contains heterogeneous DNA (het^+), we suggested that spontaneous viral synthesis and induction of early antigen are biological properties which correlate with the presence of het sequences. The present experiments provide three new lines of experimental evidence in favor of this hypothesis. (i) Revertant subclones of the EAI^+ het^+ variant which have lost the het DNA concomitantly lost EAI ability. Thus, het DNA is not stably associated with the cells as are the episomes. (ii) het DNA was acquired by two het^- subclones of the HR-1 line after superinfection with EAI^+ virus. After superinfection, these clones synthesized EAI^+ het^+ virus. Thus, het DNA may be maintained in the HR-1 line by cell-to-cell spread. (iii) Virus with het DNA activated full expression of endogenous latent EBV of the transforming phenotype in a line of immortalized neonatal lymphocytes designated X50-7. By use of restriction endonuclease polymorphisms unique to both the superinfecting and endogenous genomes, we show that the genome of the activated virus resembles that of the virus which was endogenous to X50-7 cells. This result suggests that het sequences result in transactivation of the latent EBV. het DNA had homology with EBV sequences which are not normally contiguous on the physical map of the genome. het DNA was always accompanied by the presence of DNA of nonheterogenous HR-1. Thus, het DNA is a form of "defective" EBV DNA. However, the biological effect of this defective DNA is to enhance rather than to interfere with EBV replication. This is a novel property of defective virus.

The unusual P3J-HR-1 strain of Epstein-Barr virus (EBV) is released from a cell clone of the Jijoye Burkitt lymphoma line (10). Presently available stocks of P3J-HR-1 do not immortalize lymphocytes (15, 16). This deficiency is correlated with a deletion of about 6,800 base pairs by comparison with its transformation-competent parent Jijoye (2, 13, 19) and other immortalizing EBVs (1, 6, 7, 19).

P3J-HR-1 also differs from other EBVs in its ability to activate early antigen (EA) expression in cell lines, such as Raji, which harbor EBV in a latent state (8). It is not yet clear whether EA induction (EAI) in Raji is the result of expression of the superinfecting or the endogenous genome.

Partial denaturation mapping (3) and restriction endonuclease analysis (1, 7, 9, 23, 25) show that HR-1 viral DNA is heterogeneous (het). Restriction enzyme analysis of parental HR-1 DNA reveals fragments which are present in both hypermolar and submolar quantities, by comparison with standard DNA fragments. We initiated our studies of het DNA by asking whether it was found in virus released from all cells of the HR-1 line or only from a subpopulation of cells. Therefore, we cloned HR-1 cells and examined viruses obtained from cellular subclones (9). These clonal HR-1 viruses differed from parental HR-1 virus both in genome structure and in biological properties. The first set of clonal HR-1 viruses that we isolated lacked het DNA. They were also low spontaneous synthesizers of virus. Even when large quantities of virus were recovered from some of these clones by induction of the viral replicative cycle with phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (29), this biotype of HR-1 virus was still unable to induce EA in Raji cells (9). An EAI^- clone of HR-1 cells has also been isolated by Yano et al. (28).

We finally isolated, by limiting dilution, a clone of HR-1 cells which spontaneously released large amounts of virus which induced EA in Raji cells (20). This clone seemed to be present at a frequency of 0.5%. The genome of this variant contained, in molar or greater amounts, het restriction endonuclease fragments of EBV DNA which were present as submolar fragments in the parental line and were absent from clones which were EAI^-. Furthermore, het DNA is present in DNA prepared from virions released by this clone (unpublished data). This result indicated that we had cloned out a rare variant which had properties generally associated with the parental HR-1 virus. The results provide support for the hypothesis that the presence of het DNA, spontaneous viral synthesis, and the ability to induce EA in Raji cells are linked (Table 1).

Several questions prompted the present set of experiments which were aimed at defining the biological properties of het^+ virus. Are the het DNA sequences associated with the cells in a stable fashion, as are the standard EBV episomes? How are the het DNA sequences maintained in the HR-1 line? What clues could we obtain about the way in which het DNA might activate viral expression? Does het DNA have the same consequences on all latent EBV genomes?

MATERIALS AND METHODS

Cells and cell clones. As a source of parental HR-1 virus, we used the P3H3 line which we obtained in 1981 from W. Henle. The prototype of a cell clone of P3H3 which releases EAI^- virus is HH514-16 (20). This clone is hyperresponsive to TPA; upon induction with TPA, it releases ~7 ng of viral DNA per ml in the supernatant fluid. The HR-1 clone which

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TABLE 1. Summary of biological properties of parental HR-1 line and its variant subclones

HR-1 variant	No. of similar clones	het DNA	Biological properties		
			Transformation	EA induction	Spontaneous viral synthesis
Parental line	NA[a]	+	−	+	+
Clone HH514-16	199	−	−	−	+
Clone HH543-5	1	+	−	+	+

[a] NA, Not applicable.

sheds EAI^+ virus (ca. 2 ng of viral DNA per ml) is HH543-5 (20). Six subclones of HH543-5 were derived in a limiting dilution experiment on feeder layers of human placental cells. The six subclones arose in a 96-well microtiter plate seeded with one HH543-5 cell per 10 wells.

As target cells for superinfection, we used two lymphocyte lines in which the EBV genome is latent and the Burkitt lymphoma line Raji and X50-7, which are human umbilical cord lymphocytes transformed in vitro by EBV (27). We also used EBV genome-negative BJAB and fresh lymphocytes as targets. A line of umbilical cord lymphocytes derived by transformation with culture fluids from X50-7 cells which had been superinfected with HH543-5 virus was designated HH830.

Viral stocks. Virus was prepared from unconcentrated supernatant fluids of the parental P3H3 line or the HH543-5 clone by filtration. Virus was similarly prepared from clone HH514-16 7 days after subculture in the presence of 200 ng of TPA per ml.

Viral antigen expression. Antigens representing the viral replicative cycle were detected by indirect antiimmunoglobulin immunofluorescence. The antibodies were either anti-viral capsid antigen positive (VCA^+)/EA^-, anti-VCA^+/EA^+ human sera or murine monoclonal antibodies to EA diffuse (R3.3), EA restricted (K8.1) (17), membrane antigen (MA) (72A1) (11), or VCA (L2), which were generously provided by G. Pearson. The second reagent was a fluorescein isothiocyanate-conjugated rabbit anti-human immunoglobulin for human sera or rabbit anti-mouse immunoglobulin for mouse monoclonals.

Expression of biologically active virus. Virus was tested for its ability to induce EA by mixing 1 ml of supernatant fluid with 1 ml of Raji cells and assaying the Raji cells for antigen expression with a VCA^+/EA^+ human serum after 3 days of incubation at 37°C. Transforming virus was detected by exposing fresh human umbilical cord lymphocytes in microtiter cultures to filtered supernatant fluids.

TABLE 2. Spontaneous viral synthesis and ability to induce EA in six subclones of HH543-5

Clone	% Cells VCA^+	% Raji cells EA^+ after clone supernatant
HH543-5	25	8
HH795-1	2	0
HH795-2	1	0
HH795-3	1	0
HH795-4	<1	0
HH795-5	<1	0
HH795-6	<1	0

Analysis of viral DNA in cell lines. Viral DNA sequences were detected by Southern blotting. Total cellular DNA was extracted by the procedure of Wahl et al. (26). About 10 μg was digested with *Bam*HI or *Eco*RI, electrophoresed in a 0.5% agarose gel, and transferred to nitrocellulose. Viral sequences were detected by hybridization with recombinant plasmids containing EBV DNA fragments radiolabeled with ^{32}P by nick translation.

Determination of the homologous regions of EBV DNA represented in het DNA. Filters were prepared containing either *Bam*HI or *Eco*RI digests of intracellular DNA from the EAI^+ het^+ clone HH543-5. Individual cloned *Bam*HI or *Eco*RI EBV DNA fragments were used as probes on these filters. The sizes of the *Bam*HI and *Eco*RI het DNA fragments were determined by comparison with markers of standard EBV viral DNA fragments and lambda phage DNA.

RESULTS

Loss of het DNA sequences from subclones of clone HH543-5. We initially asked whether the het DNA sequences, spontaneous viral production, and the EAI^+ phenotype were stable upon subcloning. Six subclones of clone HH543-5 (sometimes called clone 5) were obtained by limiting dilution. These clones were tested for VCA with two different VCA^+/EA^- human sera. Whereas HH543-5 had 25% VCA^+ cells, the six subclones all had low levels of VCA from <1 to 2% of the cells (Table 2). Supernatant fluids from these six subclones were unable to induce EA in Raji cells (Table 2). We then examined intracellular viral DNA in the subclones

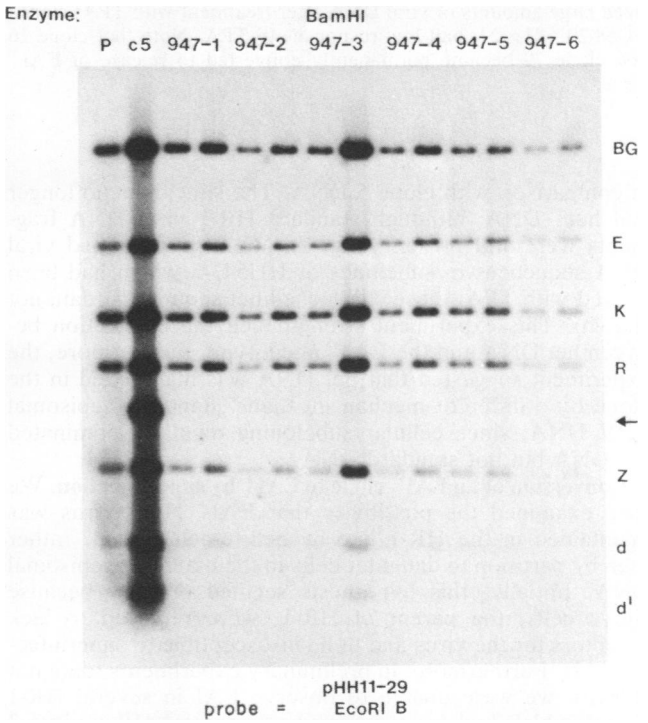

FIG. 1. Absence of EBV het DNA in cellular subclones of clone HH543-5. A Southern blot containing *Bam*HI-digested DNA from the HR-1 parental line, clone 5, and six subclones of clone 5 (947-1 through 947-6) was probed with a plasmid containing the *Eco*RI B fragment. Note that the prominent het band (arrow) present in clone 5 is absent in the subclones. All of the other *Bam*HI fragments expected to be recognized by *Eco*RI-B are present.

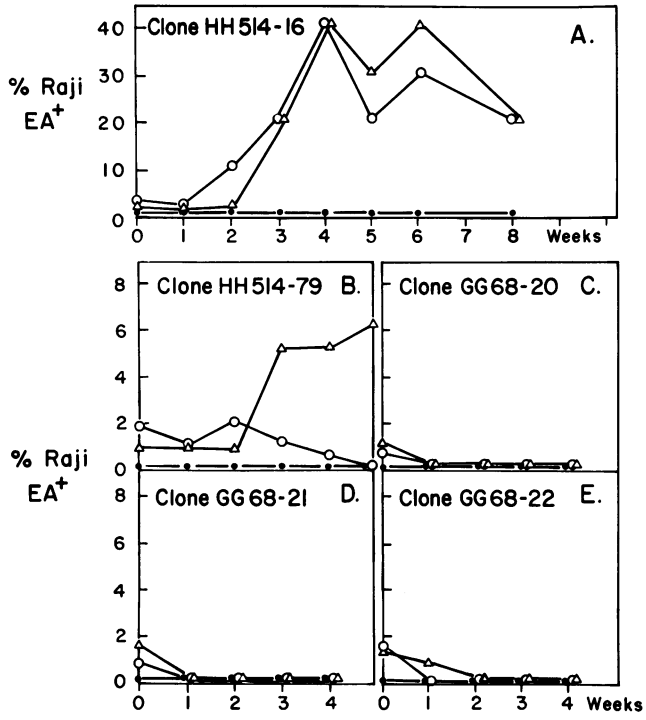

FIG. 2. Release of EA-inducing virus by cellular clones of the HR-1 line after superinfection with clone 5 (△), parental HR-1 virus (○), or medium (●). Fluids were harvested weekly and tested for EAI in Raji cells. None of the 5 clones tested was a spontaneous producer of EAI$^+$ virus. Clones HH514-16 and HH514-79 synthesized large amounts of viral DNA after treatment with TPA; clones GG68-20, -21, -22, had low responses to TPA. Note that clone 16 and clone 79 became permanently converted to release of EAI$^+$ virus.

in comparison with clone 5 DNA. The subclones no longer had het$^+$ DNA, although standard HR-1 viral DNA fragments were still present (Fig. 1). We also examined viral DNA sequences in subclones of HH543-5 which had been treated with TPA; these still lacked het sequences (data not shown). This experiment strengthened the correlation between het DNA and the EAI$^+$ phenotype. Furthermore, the experiment suggested that het DNA was maintained in the clone by a different mechanism than "standard" episomal EBV DNA, since cellular subcloning regularly eliminated het DNA but not standard DNA.

Conversion of an EAI$^-$ clone to EAI$^+$ by superinfection. We then examined the possibility that EAI$^+$ HR-1 virus was maintained in the HR-1 line by cell-to-cell spread, rather than by partition to daughter cells in the manner of episomal DNA. Initially, this hypothesis seemed unlikely because Jijoye cells, the parent of HR-1, were reported to lack receptors for the virus and to be insusceptible to superinfection (12). Furthermore, in preliminary experiments (data not shown), we were unable to observe EAI in several HR-1 clones which had been exposed to parental HR-1 virus 3 days previously. Nonetheless, we carried out the experiments whose results are illustrated in Fig. 2, first with clone HH514-16 (clone 16) as the recipient cells and later with other clones. Cells were exposed either to parental HR-1 virus, to HH543-5 virus, or to medium. Every week for 8 weeks supernatant fluids were collected, saved, and tested at the same time for their capacity to induce EA in Raji cells (Fig. 2A). Two weeks after addition of parental virus and 3 weeks after infection with clone 5 virus, clone 16 cells acquired the ability to release EAI$^+$ virus. Thereafter, the cultures of clone 16 cells were stably converted to the EAI$^+$ phenotype. Examination of the viral DNA sequences in the cells showed that the mock-infected clone 16 cells remained het$^-$, whereas clone 16 cells which had received clone 5 or parental HR-1 virus became EAI$^+$ het$^+$ (Fig. 3).

Clone HH514-16 was a "superinducible" clone in response to TPA. We wondered whether this biological property correlated with its susceptibility to superinfection with clone 5 virus. Therefore, we tested four additional HR-1 subclones for their response to superinfection with clone 5 and parental HR-1 virus. One other superinducible clone, HH514-79, was permanently converted to EAI$^+$ by superinfection with clone 5 virus (Fig. 2B); in this experiment, clone 79 remained EAI$^-$ after addition of parental HR-1 virus and after mock infection. The EAI$^+$ superinfected culture of clone 79 also became het$^+$ (data not shown). Three HR-1 clones (GG68-20, -21, -22) which were moderately inducible (9) could not be converted either with clone 5 or parental HR-1 virus (Fig. 2B); these clones did not acquire het DNA after superinfection. Thus, it seems that some, but not all, HR-1 cellular subclones can be superinfected with EAI$^+$ virus and thereby acquire het DNA. With the limited results so far, ability to be stably superinfected with het$^+$ DNA correlates with response of the clones to TPA.

Antigen expression in different lymphoid lines after superinfection with clone 5 virus. The next set of experiments

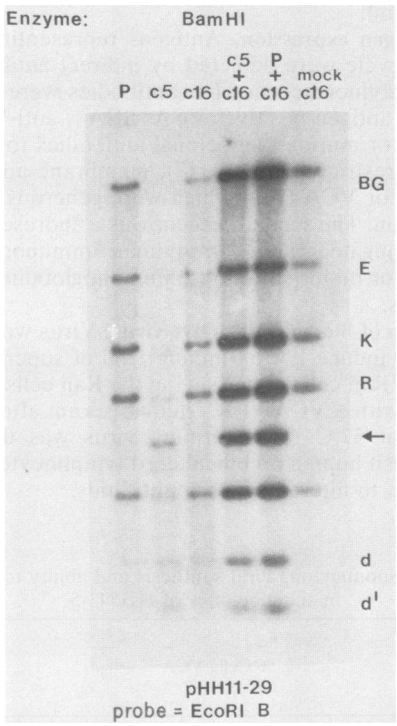

FIG. 3. Acquisition of het DNA in clone 16 cells after superinfection with clone 5 or parental HR-1 virus. Shown is a Southern blot containing intracellular DNA prepared from parental HR-1 cells (P), HH543-5 (c5), HH514-16 (c16), and from clone 16 cells superinfected with virus from clone 5 or parental HR-1 virus (P) or mock infected. Note that the superinfected clone 16 cells have acquired the 1.7-megadalton (Md) het fragment (arrow).

TABLE 3. Antigen expression 3 days after infection with HR-1 clone HH543-5 virus

Target cell line	Origin	EBV DNA	Human serum		Murine monoclonal antibody to:			
			EA^+/VCA^+	EA^-/VCA^+	$EA\text{-}D^{+a}$	$EA\text{-}R^{+a}$	MA^+	VCA^+
X50-7	HUCL[b]	+	+	+	+	+	+	+
HH889	HUCL	+	+	−	+	+	−	−
Raji	BL[c]	+	+	−	+	+	−	−
FF500-28	HUCL	+	−[d]	−[d]				
FF465	HUCL	+	−[d]	−[d]				
FF467	HUCL	+	−[d]	−[d]				
HR-1 clone HH514-16	BL	+	−					
BJAB	BL	−	−	−				
Primary HUCL		−	−	−				

[a] D, Diffuse; R, restricted.
[b] HUCL, Human umbilical cord lymphocytes.
[c] BL, Burkitt lymphoma.
[d] Level of spontaneous expression of EA which was not affected by addition of clone 5 virus was ca. 1%.

focused on the question of whether lymphocyte lines which contain EBV lineages other than HR-1 respond to superinfection with clone 5 HR-1 virus in a way similar to that observed in clones of the HR-1 lineage. Do the cells make virions of the EAI^+ phenotype? Can the cells be permanently converted to carry the het^+ DNA sequences?

Initially, we examined antigen expression with polyclonal human and monoclonal mouse antibodies in nine different types of B lymphocytes which had been infected with clone 5 virus 3 days before (Table 3). Cells without an EBV genome (BJAB and fresh lymphocytes) did not display antigens of the productive cycle after infection with the virus at the multiplicity we used. Three lines of umbilical cord lymphocytes (FF465, FF467, and FF500) which had been transformed in vitro by the FF41 strain and which were low-level spontaneous synthesizers of EAs did not increase their level of antigen expression after superinfection with clone 5 virus. However, three EBV genome-containing lines responded: Raji and two lines of umbilical cord lymphocytes, X50-7 and HH889. Raji and HH889 produced only EA after superinfection; immunofluorescence with the murine monoclonals showed that this was EA of both the diffuse and the restricted types. The X50-7 line, by contrast, responded to superinfection with synthesis of all the antigens of the replicative cycle, EA, VCA, and MA, as detected both by human antibodies and mouse monoclonals. None of these lines produced viral antigens after exposure to clone 16 virus (data not shown).

We then attempted to convert five of the lines to permanent carriage of het DNA and EAI^+ virus by chronic exposure to clone 5 virus (Fig. 4), as we had done previously with HR-1 subclones (Fig. 2). The three low-level spontaneous producer lines, FF500, FF467, and FF465, did not show any significant change in antigen expression after superinfection. Both Raji and X50-7 stayed antigen positive for 2 weeks, and then spontaneous expression of antigen disap-

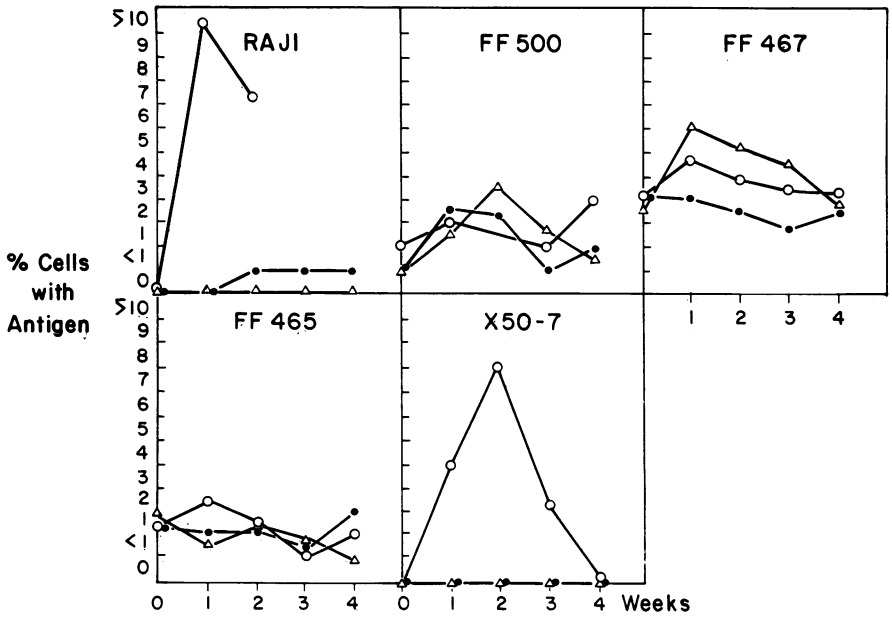

FIG. 4. EA expression after superinfection of several different lymphoid lines with HR-1 virus. Cells were superinfected either with EAI^+ virus (clone 5) (○) or EAI^- virus (clone 16) (●) or were mock infected (△). Cell smears were made weekly and examined for EA expression. Raji cells infected with clone 5 virus were lost after week 2 due to contamination. Note that Raji and X50-7 cells responded to superinfection with clone 5 virus with antigen synthesis. Other lines did not. Antigen synthesis in X50-7 cells was not stable.

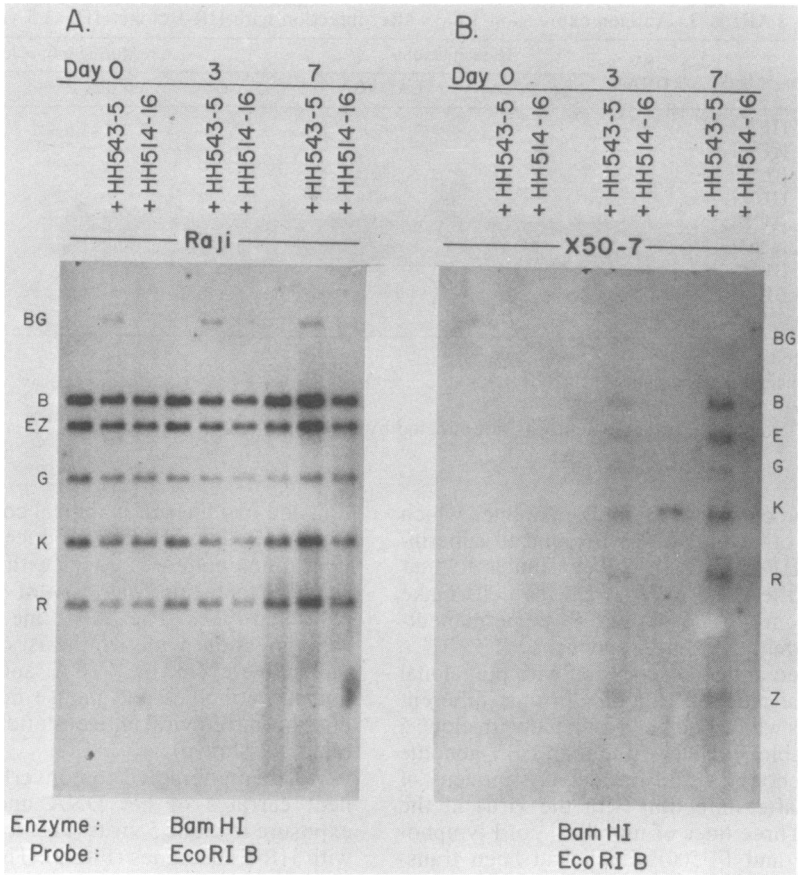

FIG. 5. Content of EBV DNA in Raji (A) and X50-7 cells (B) after superinfection with HR-1 clone 5 virus. The same amount of intracellular DNA (10 μg) was loaded in each lane. DNA was digested with *Bam*HI and probed with *Eco*RI-B. Each cell line was uninfected, infected with EAI^+ virus (clone 5), or infected with EAI^- virus (clone 16), and DNA samples were prepared on days 0, 3, and 7. Note that superinfection of Raji cells did not alter the content of viral DNA. Superinfection of X50-7 cells with clone 5 virus resulted in amplification, on days 3 and 7, of EBV DNA sequences present in X50-7 cells.

peared. Intracellular DNA which was prepared from X50-7 cells exposed for 4 weeks to clone 5 virus, clone 16 virus, or medium did not show either polymorphisms of the superinfecting clone 5 virus (see below) or the presence of het DNA (data not shown). Thus, whereas virus with het DNA activated expression of EA in Raji and the complete replicative cycle in X50-7 cells, clone 5 virus did not stably convert either cell line to the EAI^+ het^+ phenotype.

Viral DNA content in Raji and X50-7 cells after superinfection with clone 5 virus. The two cell lines were infected with either clone 16 (het^-) or clone 5 (het^+) virus or were uninfected. Total cellular DNA was harvested on days 0, 3, and 7 after superinfection and was examined on a Southern blot (Fig. 5). In the Raji line, there was no change in the content of viral DNA after superinfection with clone 5 virus; some of the input viral DNA could be seen (the BG fragment in Fig. 5A), but this did not increase in amount. The result in X50-7 cells was different. This line has a low level of episomes. On days 3 and 7 after superinfection with clone 5 (het^+) but not with clone 16 (het^-) virus, there was amplification of the endogenous viral DNA sequences (Fig. 5B). This was confirmed by probing with several probes besides *Eco*RI-B (data not shown). There was at least a 10-fold increase in the content of viral DNA.

These results corroborated the data about antigen induction in the two lines (Table 3). Thus, in Raji cells, addition of clone 5 virus leads only to EA synthesis and not to viral DNA or late antigen expression. By contrast, in X50-7 cells, superinfection results in synthesis of viral DNA as well as late antigens (Table 4).

Characteristics of a virus recovered from superinfected X50-7 cells. We wished to learn whether activation of viral expression upon superinfection of X50-7 cells with het^+ EAI^+ clone 5 virus was due to the input clone 5 virus or to the endogenous virus. X50-7 cells permitted us to study this question since they underwent the full cycle of viral replication after superinfection. Three days after superinfection, when X50-7 cells were producing EA, VCA, and MA, fluids from these cultures were harvested, filtered, and transferred to fresh umbilical cord lymphocytes. A transformed cell line (designated HH830) was established after 4 weeks. The presence of a number of polymorphisms in the *Bam*HI and *Eco*RI restriction endonuclease cleavage sites of the episomal EBV DNA in X50-7 cells and the HR-1 viral DNA permitted comparison of the genome of the recovered virus with that of the endogenous X50-7 virus and the HR-1 virus (Table 4, Fig. 6). The recovered virus was compared with HR-1 clone 5, HR-1 clone 16, and X50-7 cell virus by Southern blot analysis of their intracellular DNAs. Several different probes were used which identify known polymorphisms of the HR-1 lineage, such as fusion of *Bam*HI fragments W′ and I′, fusion and deletion of *Bam*HI frag-

ments W and H, and fusion of *Bam*HI fragments B and G (7, 19). All of the characteristics of the viral DNA in HH830 cells were identical to those in X50-7 cells; this finding indicated that superinfection with clone 5 HR-1 virus resulted in rescue of the endogenous virus. Furthermore, no het DNA was identified in the virus which was rescued.

EBV homologous sequences which are represented in het DNA. Although a more complete description of the composition of het EBV HR-1 DNA will be presented in a subsequent paper, a preliminary analysis is needed for a discussion of the biological properties of het DNA and the mechanism by which het DNA might disrupt latency. *Bam*HI digests of clone 5 DNA contained four novel *Bam*HI fragments which were not seen in similar digests of clone 16 DNA (Table 5). In addition, the *Bam*HI digest of clone 5 DNA showed that the *Bam*HI-W′ I′ fragment was present in hypermolar amounts (Fig. 6C). There were four novel *Eco*RI fragments of clone 5 HR-1 DNA which were not identified in clone 16. *Bam*HI or *Eco*RI digests of clone 5 DNA were probed with plasmids containing individual cloned restriction fragments of EBV DNA. Thus, a summary was prepared (Table 5, Fig. 6) which demonstrates that the novel het DNA fragments contain sequences which represent only a portion (less than 25%) of the genome. On the basis of hybridization, it appears that several of the het fragments contain sequences from EBV regions which are not adjacent on the physical map (Fig. 7). For example, sequences represented in *Bam*HI-M are linked to *Bam*HI-B′, and *Bam*HI-W is linked to *Bam*HI-Z. The exact arrangement and composition of the het DNA sequences is still under investigation.

TABLE 4. Characterization of the DNA of the virus (HH830) recovered from X50-7 cells after superinfection with clone 5 HR-1 virus

Intracellular DNA on blot	*Bam*HI fragment detected with the following probe:																							
	*Eco*RI-C										*Eco*RI-B									*Eco*RI-F				
	A	B′	W1′	het (4.6 Md)	H	I′	WH	V	W′	BG	B	E	G	K	R	het (1.7 Md)	Z	c	d	het (4.6 Md)	M	L	S	het (1.2 Md)
HR-1 clone 16	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	−	+	?	−	+	+	+	−
HR-1 clone 5	+	+	−	+	+	−	−	+	−	+	−	−	−	−	+	+	+	?	+	+	+	+	+	+
X50-7	+	+	−	+	+	−	−	+	−	+	−	−	−	−	+	+	+	?	+	+	+	+	+	−
HH830	+	+	−	−	+	+	−	+	+	−	+	+	+	+	+	−	+	?	−	+	+	+	+	−

DISCUSSION

Evidence that het DNA activates latency. This set of experiments shows that het EB viral DNA in the HR-1 viral strain is responsible for induction of EA in Raji cells and for activation of the complete viral replicative cycle in X50-7 cells. In our earliest experiments on this problem, we found that subclones of the HR-1 line which did not contain het DNA sequences also failed to release virus which was competent to induce EA in Raji cells (9). We showed that this was not merely a matter of the quantity of virus released, because large amounts of viral DNA were found in the supernatant fluid of EAI^- clones which had been treated with TPA. Furthermore, we showed that this DNA was encapsidated, for it sedimented as virus on a sucrose gradient and was resistant to exposure to DNase (20). The correlation between het DNA and EAI was strengthened when we found that a rare clone of HR-1 cells which released virus competent to induce EA also contained het sequences (20). In the present report, we provide additional correlative data. Subcloning of the rare EAI^+ het^+ clone results in concomitant loss of het DNA, EAI, and spontaneous viral synthesis (Table 2, Fig. 1). Furthermore, addition of HR-1 virus which contains het DNA to cells which lack such sequences results in simultaneous conversion of some HR-1 clones to het^+ EAI^+ (Fig. 2 and 3). Still missing from this skein of evidence is the demonstration that direct transfer of specific het DNA fragments themselves, without additional parts of the EBV genome, can induce EA or the complete viral productive cycle in the appropriate cell line.

Nature of the association of het DNA with various lymphoid lines. The experiments suggest that, both in the HR-1 parental line and in subclone HH543-5, het DNA is maintained by a mechanism different from that which maintains

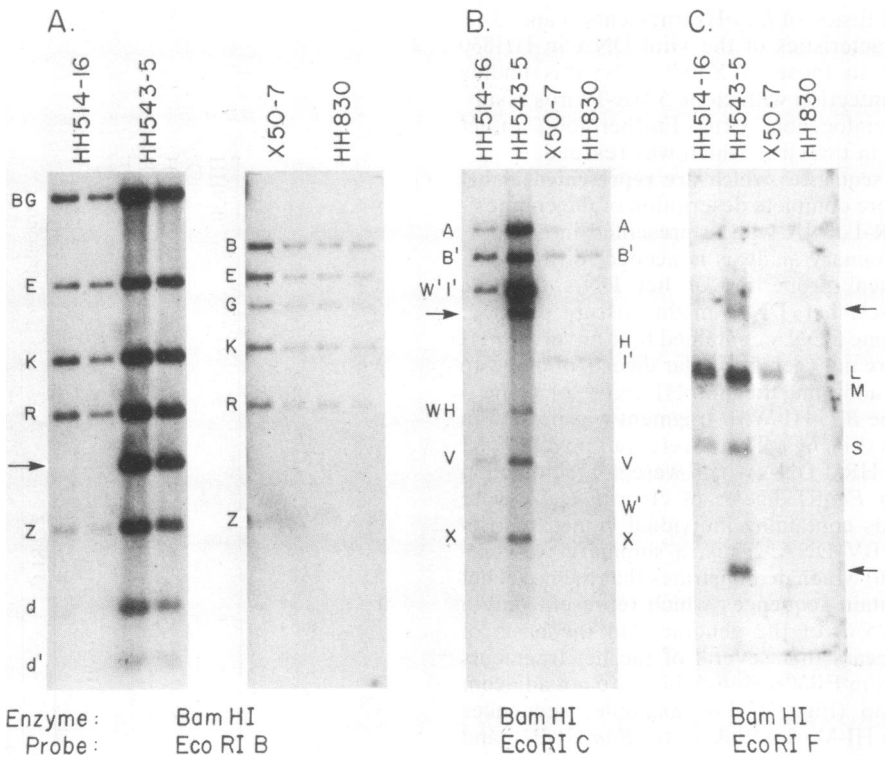

FIG. 6. Characteristics of the virus recovered from X50-7 cells after superinfection with clone 5 virus. This virus is maintained in a transformed line of umbilical cord lymphocytes designated HH830. Shown are Southern blots of *Bam*HI-digested intracellular DNA prepared from HH514-16, HH543-5, X50-7, and HH830. The probes are *Eco*RI-B (A), *Eco*RI-C (B), and *Eco*RI-F (C). Note that the recovered virus (HH830) did not contain het sequences (arrows). Note also that the recovered virus had the restriction endonuclease polymorphisms associated with the X50-7 cell line (Table 4).

the standard EBV DNA fragments which constitute the episome. In neither the parental line nor in the het$^+$ subclone is het DNA stable, for the majority of subclones of each is het$^-$.

Whereas standard episomal EBV DNA is passed along in cell division to daughter cells, het DNA is likely to be maintained, at least in part, by cell-to-cell spread. We have not yet proved this point directly by eliminating het DNA by culturing the HR-1 or clone 5 cells in the presence of antibody. However, the results illustrated in Fig. 2 and 3 show that it is possible to superinfect certain HR-1 clones with het$^+$ virus. This result contradicts previous findings which held that HR-1 cells do not have receptors for EBV. Not all HR-1 clones are able to be superinfected by clone 5 virus. Of five clones we studied, three were not converted into producers of het$^+$ EAI$^+$ virus upon exposure to clone 5 virus. These clones may lack receptors for virus, or there may be other mechanisms to account for their resistance to superinfection. If all HR-1 cells were uniformly susceptible to superinfection by het$^+$ EAI$^+$ virus, one would expect that all of the cells in the line would be virus producers and would be lysed, but they are not. Even conversion of the two susceptible clones was a process which occurred slowly and was only evident 2 to 3 weeks after exposure to clone 5 virus (Fig. 2). Thus, superinfection by het$^+$ EBV is a relatively inefficient process.

TABLE 5. Novel restriction endonuclease fragments found in viral DNA of the EA-inducing clone (HH543-5) of P3JHR-1 EBV

New *Bam*HI fragments		New *Eco*RI fragments	
Size (mol wt × 10^6)	Homologous *Bam*HI fragments	Size (mol wt × 10^6)	Homologous *Bam*HI fragments
~5.0	C		
~4.7[a]	B', M	15.2[a]	M, B', W', I', A
~1.7[a]	W, Z, d	13.9[a]	W, M, S, d, Z
1.2	S	5.6	Z
		2.8	C, W

[a] Fragment which contains homologous sequences from *Bam*HI fragments which are not adjacent on the genome.

The failure of het DNA to become stably associated with some lines which it can nonetheless superinfect raises the possibility that special features of the HR-1 lineage are responsible for the stable acquisition of het sequences. The HR-1 virus contains a sizable deletion adjacent to the internal repeat (1, 7, 19). het DNA is not found in the Jijoye line whose virus lacks this deletion (18). Therefore, the capacity to generate and maintain het sequences may be a consequence of the absence of functions encoded in the deleted DNA.

Different outcomes of het superinfection of EBV carrier lines. Apart from the differences between HR-1 and all other lines in their ability to carry the het sequences in stable fashion, there are striking differences in the response of X50-7 and Raji cells to transient introduction of the same amounts of virus with het DNA sequences. In Raji cells, superinfection with het$^+$ virus at the multiplicity we used results only in EA synthesis, not in viral DNA replication or late antigen

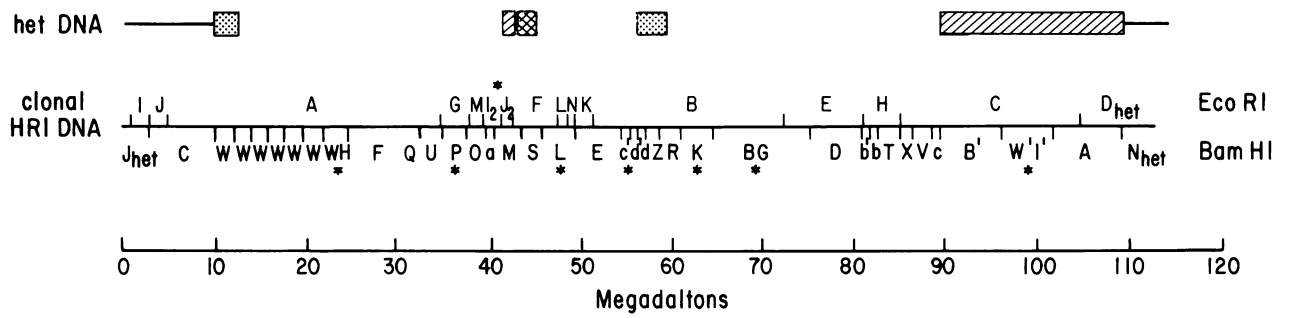

FIG. 7. Location of het DNA sequences in HR-1 virus. Shown is a physical map of clonal HR-1 DNA. The asterisks indicate polymorphisms in DNA restriction fragments which distinguish HR-1 DNA from that of FF41, a transforming virus (19). The boxes represent het DNA which is present in hypermolar quantity (Table 5). Boxes with similar shading symbolize het DNA sequences which appear to be adjacent to each other, e.g., *Bam*HI-W is contiguous with *Bam*HI-Z, and *Bam*HI-M is contiguous with *Bam*HI-B'. The lines represent het DNA which does not seem to be hypermolar.

expression (Table 3, Fig. 5). By contrast, in X50-7 cells, introduction of het sequences results in full expression of the genome, with synthesis of viral DNA, late proteins, and transforming virions (Table 3, Fig. 5 and 6).

The latter system is the one which sheds the most light on the consequences of introduction of het sequences. The transforming virus which is recovered from X50-7 cells after superinfection with het$^+$ virus is different in biological properties from the input virus, which is nontransforming. Furthermore, the recovered virus has the genome structure of the virus which is endogenous to X50-7 cells (Fig. 6). Thus, it is clear that the consequence of introduction of het$^+$ virus into X50-7 cells is *trans* activation of the endogenous genome. This result makes it likely that EA expression in Raji cells after superinfection is also due to *trans* activation of resident Raji EA genes. However, this point is not established experimentally by our data. In Raji cells, there appears to be a block which prevents the resident viral genome from entering viral DNA replication. The simplest model which would explain our findings, both in Raji and X50-7 cells, is that introduction of het sequences releases a single block of expression on an early gene. This then results in a cascade of expression of genes leading to viral maturation. However, in Raji cells, there is a second point of inhibition at the level of viral DNA replication.

How might het DNA work? Several models could be suggested as the basis for further study of how het DNA might activate latency. Amplification and repetition of certain EBV DNA sequences might bind molecules either of viral or cellular origin which serve to control expression of the latent genome. Alternatively, these amplified sequences might code for a product which positively activates the expression of the genome in a manner analogous to the herpes immediate early protein ICP4 (4, 18). Either of these general models could explain the apparent *trans* mode of action of het sequences in X50-7 cells (Table 4, Fig. 5). The rearrangement of het DNA may permit the expression of functions which are not ordinarily expressed by the genome. This might occur, for example, if het DNA brought together a strong constitutive early promoter with a gene whose product was ordinarily closely regulated. Either amplification or rearrangement of het DNA might result in altered patterns of methylation, which in turn could alter expression (24). Although het sequences are known to contain rearranged viral genes, they may also have acquired cellular DNA which behaves in a regulatory way. Whatever the mechanism, het DNA makes available for study selected regions of EBV DNA which appear to regulate expression of the genome.

het EBV DNA is defective DNA with unusual biological properties. The studies we have done so far on the structure of het DNA indicate that this DNA shares many properties previously seen in herpesvirus defective DNAs (5, 14, 21, 22). A limited number of regions of the genome are represented in het DNA (Table 5, Fig. 6) (7). The *Bam*HI and *Eco*RI het fragments bring together noncontiguous regions of the genome which appear to have been rearranged. Several of the het *Bam*HI fragments are present in increased molarity, indicating that they are repeated. het DNA fragments are always accompanied by standard HR-1 DNA fragments, a finding which suggests that het DNA requires standard DNA as a "helper." All of these are general properties of defective DNAs; however, the usual defective virus interferes with viral replication. EBV het$^+$ virus appears to activate viral replication, a novel property for defective virus.

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