Organization of the Epstein-Barr Virus DNA Molecule

III. Location of the P3HR-1 Deletion Junction and Characterization of the *NotI* Repeat Units That Form Part of the Template for an Abundant 12-*O*-Tetradecanoylphorbol-13-Acetate-Induced mRNA Transcript

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A 1,400-base-pair (bp) region within the *Bam*HI H fragment of Epstein-Barr virus (EBV) (B95-8) DNA consists of a cluster of tandemly duplicated direct repetitions characterized by single sites for the *NotI* restriction endonuclease. Nucleotide sequencing revealed a 125-bp repeat unit of 84% guanine-plus-cytosine content which is theoretically capable of extensive secondary structure formation. The flanking sequences adjacent to the *NotI* repeat cluster contained an additional 38-bp portion of repeat unit DNA, thus establishing that EBV(B95-8) contains a nonintegral number of *NotI* repeats totalling 11.3 copies. Restriction site mapping of the homologous cloned *Bam*HI "H" fragment from the nontransforming EBV(P3HR-1) isolate revealed that a contiguous 6,650-bp region including the entire *NotI* repeat cluster has been deleted from the *Bam*HI-H, -Y, and -W regions of the P3HR-1 genome. By nucleotide sequencing across the novel junction, we have precisely identified the P3HR-1 deletion boundaries in *Bam*HI-H and the internal repeat and suggest that a complex pattern of direct and inverted partial DNA homologies may have been involved in the original recombination event. The cloned *Bam*HI H fragment and isolated *NotI* repeat unit have also been used as probes to detect homologous mRNA transcripts in the B95-8 and Raji cell lines of EBV-transformed lymphoblasts. These experiments showed that the *NotI* repeats form part of the template for a polyadenylated 2.5-kilobase mRNA transcript which becomes much more abundant after 12-*O*-tetradecanoylphorbol-13-acetate treatment of the cultures. The direction of transcription of this mRNA and the nucleotide sequence of most of its template and 5' flanking regions have been determined. The probable promoter for the 2.5-kilobase mRNA initiates transcription efficiently in an in vitro assay and contains several TAATGA-like elements that may be indicative of herpesvirus immediate-early class promoters. The need to repress expression of this gene during latency suggests a possible novel regulatory role for tandem repeat structures inside a eucaryotic virus transcription unit. The deletion in EBV(P3HR-1), which has been associated with the loss of lymphocyte "immortalizing" capacity in this isolate, eliminates part of the coding region as well as the *NotI* repeats from the 2.5-kilobase mRNA transcript, but the promoter and proximal 340-bp portions of the template remain.

A characteristic feature of Epstein-Barr virus (EBV) is its ability to transform human B-lymphocytes into continuously growing lymphoblastoid cell lines. Efforts to determine which regions of the genome are involved in transformation-related functions have, in general, taken one or the other of the following approaches: (i) identification of those regions which are transcribed in latently infected lymphoblastoid cell lines or (ii) characterization of

the DNA segment which is deleted in the unusual nontransforming EBV(P3HR-1) isolate.

At least three separate regions of the EBV genome encode polyadenylated RNA in latently infected cell lines: a section encompassing the 3.1-kilobase (kb) internal repeat cluster and including the *Bam*HI C, W, Y, and H fragments, a small region which is defined by the *Bam*HI K fragment and located toward the middle of the U_L segment of the genome, and finally the

regions immediately adjacent to the DNA termini. A number of the individual RNAs coded by these regions have been characterized in terms of size and direction of transcription (18, 19, 33). In addition, EBV-transformed cell lines contain two small, EBV-specified, polymerase III transcripts which are very similar in structure to the adenovirus-associated VA RNAs and are present in great abundance (1, 21, 28).

The deletion in EBV(P3HR-1) maps in the U_L segment of the genome adjacent to the 3.1-kb internal repeat (IR) cluster (3, 10, 16, 27). This deletion appears to correlate with the loss of "immortalizing" ability since transforming virus can be regenerated by superinfection of nonproducer Raji cells with P3HR-1 virus, and in all cases the transforming virus regained those sequences deleted from P3HR-1 DNA, presumably by recombination with the resident Raji genomes (12). More detailed structural analyses have shown that the P3HR-1 deletion encompasses part of a 3.1-kb IR unit, all of the *Bam*HI Y fragment, and a portion of the *Bam*HI H fragment (4, 14). We have previously shown that the EBV(B95-8) *Bam*HI H fragment also contains a set of 11 tandem duplications of the *Not*I repeats (14). These repeats share partial homology with the *Pst*II (DS_R) repeats described by Hudewenz et al. (17). In the present study we defined the boundary of the EBV(P3HR-1) deletion by comparing nucleotide sequence analyses of cloned EBV(B95-8) *Bam*HI-H and the homologous fragment from EBV(P3HR-1) and demonstrated that the deletion eliminates the *Not*I repeat cluster from the EBV(P3HR-1) genome. In an attempt to understand the function of the *Not*I repeats and their relevance to EBV transformation, we also analyzed the structure of the repeat cluster by nucleotide sequencing and found that it encodes a major viral mRNA transcript.

MATERIALS AND METHODS

Cells and virus. Raji, P3HR-1, and B95-8 lymphoblast cultures were grown at 35°C in RPMI-1640 medium containing 10% fetal calf serum (Sterile Systems, Logan, Utah). Induction of lytic viral functions was achieved by the addition of 40 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml (Consolidated Midland Corp., Brewster, N.Y.) 2 to 3 days before harvesting (34). Virion DNA used for the construction of plasmid DNA clones was isolated as described previously (14, 15).

RNA isolation. Pelleted cells were rapidly denatured with a solution of 4 M guanidinium thiocyanate plus 0.1 M 2-mercaptoethanol (7). The total cell RNA was then separated away from the bulk protein and DNA by sedimentation through a CsCl cushion (13). The RNA pellet was suspended in 0.5 M NaCl-0.5% sodium dodecyl sulfate-100 mM Tris (pH 6.4), and contaminating proteins were extracted with a phenol-chloroform-isoamyl alcohol mixture (25:24:1). The

RNA was then ethanol precipitated, and the polyadenylate-containing fraction was selected using an oligodeoxythymidylate column (2). ³²P-labeled RNA was prepared from cells which had been concentrated 10-fold and incubated for 6 h in low-phosphate Eagle minimal essential medium containing ³²P_i at 100 μCi/ml.

Gel electrophoresis, probes, and hybridization. Preparation of plasmid DNAs, restriction enzyme digestion and electrophoresis of cleaved DNA, isolation of DNA fragments, and preparation of in vitro-labeled DNA probes were performed as previously described (14, 15). RNA samples, which had been denatured with purified glyoxal (25), were electrophoresed through 1.4% agarose gels in a circulating buffer of 10 mM sodium phosphate (pH 7.0) and subsequently transferred to nitrocellulose with 20× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described by Thomas (30). Hybridization of DNA probes to RNA blots was performed in 50% formamide-5× SSC-50 mM sodium phosphate (pH 6.5)-250 μg of salmon sperm DNA per ml-1× Denhardt solution-10% dextran sulfate. The same buffer without dextran sulfate was used for the preincubation step. After 24 h of incubation at 45°C, blots were washed in 2× SSC-0.1% sodium dodecyl sulfate at room temperature four times and 0.1× SSC-0.1% sodium dodecyl sulfate for 1 h at 50°C (32). Hybridization of [³²P]RNA against nitrocellulose filter transfers (8, 30) of cleaved plasmid DNA samples was carried out in a hybridization buffer of 6× SSC-40% formamide-2× Denhardt solution-250 μg of yeast RNA per ml-500 μg of salmon sperm DNA per ml. The same mixture was used for the preincubation step. After hybridization for 48 h at 45°C, the filters were washed for 2 h in 3× SSC at 60°C, 2× SSC plus 25 μg of RNase A per ml at room temperature, and 2× SSC plus 0.5% sodium dodecyl sulfate at 60°C. Finally, the filters were rinsed in 0.1× SSC at room temperature, dried, and exposed for autoradiography.

DNA sequencing. DNA nucleotide sequencing was conducted by the procedure of Maxam and Gilbert (24). For 5' protruded restriction sites, terminal phosphate was removed using bacterial alkaline phosphatase and subsequently replaced using T4 polynucleotide kinase and [^γ-³²P]ATP in a forward reaction (5). For 3' protruded restriction sites, terminal end labeling was performed using deoxyterminal transferase and [³²P]cordycepin (32). In each case a uniquely labeled terminus was derived by asymmetrically cleaving the labeled fragment with a second restriction endonuclease.

In vitro transcription. Analysis of promoter sites in vitro was conducted using a HeLa cell lysate system (23) purchased from Bethesda Research Laboratories, Bethesda, Md. For each reaction 2.5 μg of linear DNA template was added to 30 μl of HeLa cell lysate in the presence of radioactive ribonucleotide triphosphates. Runoff transcripts were analyzed using glyoxal-agarose gel electrophoresis and autoradiography.

RESULTS

***Not*I repeat cluster is deleted in EBV(P3HR-1).** The P3HR-1 genome contains a large deletion which removes part of the 3.1-kb *Bam*HI-W IR cluster and extends into the U_L segment of the

genome (4, 10, 16). In an earlier study (14) it was shown that this deletion removes all of the *Bam*HI Y fragment and that portion of the *Bam*HI H fragment located between the *Bam*HI-Y/H junction and the *NotI* repeat cluster. In those experiments the P3HR-1 deletion was mapped by an indirect approach using cloned B95-8 DNA fragments and a P3HR-1 probe comprising *in vitro*-labeled total viral DNA. Interpretation of the results was complicated by the inability of the *NotI* fragment to form hybrids on filters when incubated with homologous probes and by the known cross-homology between the *Bam*HI H fragment and a distant region of the U_L segment of P3HR-1 DNA (11, 17, 26). (The cross-hybridizing region is deleted from the B95-8 genome.) For these reasons it could not be determined whether the entire *NotI* repeat cluster was included in the P3HR-1 deletion. To examine this question more directly, we used a cloned P3HR-1 *Bam*HI "H" fragment (pDH61) encompassing the deletion. Figure 1b shows some representative fragment profiles generated by restriction enzyme cleavage of pDH61, and a summary of all of the cleavage mapping data is shown in Fig. 1c, which compares the structure of the viral insert in pDH61 with that of the B95-8 *Bam*HI H and W fragments. The major points are as follows. (i) The *Bgl*II, *Hinc*II, *Pvu*II, *Kpn*I, and *Sac*II restriction sites which are present in the left-hand 2,300 base pairs (bp) of the B95-8 *Bam*HI H fragment are conserved in P3HR-1 *Bam*HI-H (pDH61 also contains an additional *Pst*I site not present in the B95-8 clone). (ii) Those restriction sites which are located to the right of the *Sac*II sites in B95-8 *Bam*HI-H either are absent in pDH61 (e.g., *Not*I, *Sph*I, and *Bst*EII) or their relative positions differ (e.g., *Stu*I, *Nco*I, and *Sac*I). (iii) In pDH61 those restriction sites which map beyond the *Sac*II sites, namely, the *Pst*I, *Stu*I, *Sac*I, *Bgl*II, and *Xho*I sites, form a pattern which is characteristic of a section of the 3.1-kb *Bam*HI-W IR unit. The pivotal *Sac*II sites have been designated as those from *Bam*HI-H. An alternative possibility, that a *Sac*II site derived instead from the 3.1-kb repeat DNA, was eliminated initially on the basis of the size of the double digest fragments. For example, the *Sac*II-*Bam*HI and *Sac*II-*Pst*I fragments of pDH61 were both 250 bp smaller than would be expected if the *Sac*II site had come from the fused 3.1-kb repeat DNA. The above assignment of the *Sac*II sites was subsequently confirmed by nucleotide sequence data (see below).

We concluded that the P3HR-1 deletion boundary is located in the 200-bp region between the *Sac*II and *Sac*I sites of the *Bam*HI H fragment, that the deletion creates a novel *Bam*HI H fragment in which DNA sequences

from a 3.1-kb IR unit are fused to the distal 2,300 bp of *Bam*HI-H, and that the entire *NotI* repeat cluster is eliminated from the P3HR-1 genome. The general features of this interpretation agree with those obtained by Bornkamm et al. (4), who compared a P3HR-1 *Sal*I-A cosmid clone with a *Sal*I-A clone from the EBV(M-ABA) strain.

Locating the EBV(P3HR-1) deletion boundary by nucleotide sequencing. Restriction site mapping of the *Bam*HI H fragment of P3HR-1 localized the position of the deletion junction to within 200 bp of the *Sac*II site. To further define the boundary of the deletion, we sequenced this segment of the B95-8 and P3HR-1 *Bam*HI H fragments by using plasmid DNA end labeled at the *Sac*I, *Sac*II, and *Nco*I sites (Fig. 2a). The results are presented in Fig. 2b. Compared to B95-8, the P3HR-1 DNA contained only three nucleotide changes between the *Sac*II site and a cluster of cytosines 130 bp to the right of *Sac*II, at which point the two sequences diverge. One of these base changes occurs within the *Stu*I site; thus, the lack of this cleavage site in P3HR-1 is not related to the deletion. After the cytosine cluster, the diverged P3HR-1 sequence is almost identical for at least 85 nucleotides to that of a portion of the 3.1-kb IR unit from B95-8 DNA (6, 18a). Because of partial homology between the *Bam*HI-H and 3.1-kb repeat sequences at the novel junction, the exact site of the deletion can only be defined to within the five nucleotides (-CCCAG-) located 1,363 to 1,368 bp from the *Bam*HI-Y/W junction. These experiments yield a minimum size estimation for the P3HR-1 deletion of 6,650 bp in sequence complexity; however, because the total number of 3.1-kb IR units is considerably reduced in P3HR-1, the actual size of the deletion may be greater by increments of 3.1 kb. Further analysis of the sequences at and around the deletion site revealed a complex arrangement of additional direct and inverted homologies. Within a 26-bp flanking region from *Bam*HI-H and a 23-bp flanking region for IR, we can detect (i) a perfect 7-bp inverted homology, (ii) an 11- of 12-bp direct homology, and (iii) a 10- of 12-bp inverted homology. Two alternative views depicting the arrangement of these homologies and possible secondary structure formation between the two boundary regions are presented in Fig. 2c.

Sequencing of the *NotI* repeat unit and flanking unique sequence DNA. To sequence the *NotI* repeat unit, we generated four classes of DNA fragments from a cloned *Bam*HI H fragment of B95-8 (pSL77) (see Fig. 2a). First, pSL77 DNA was subjected to partial digestion with *NotI*. The cleaved DNA was end labeled with [γ -³²P]ATP and then digested to completion with *Bgl*II, and two populations of radiolabeled molecules were isolated from agarose gels, one migrating in the

FIG. 1. (a) Comparison of cloned plasmids from the *Bam*HI-H regions of EBV(B95-8) and EBV(P3HR-1). Shown are ethidium bromide stains of fragments generated by restriction enzyme cleavage of the 5.8-kb *Bam*HI H fragment of B95-8 (pSL77) and the 4.1-kb

*Bam*HI "H" deleted fragment from the equivalent region of P3HR-1 (pDH61). The DNA was electrophoresed through a 1.4% agarose gel at 750 V/h. Selected fragment sizes are given in bp. Individual gel tracks contain the following: phage λ DNA cleaved with *Eco*RI (track 1) and *Hind*III + *Eco*RI (track 2); pSL77 DNA cleaved with *Bam*HI (track 3) and *Hind*III + *Not*I (track 4); pDH61 DNA cleaved with *Bam*HI (track 5), *Hind*III + *Not*I (track 6), *Bam*HI + *Sac*I (track 7), *Bam*HI + *Stu*I (track 8), *Bam*HI + incomplete *Sac*II (track 9), and *Pst*I (track 10). (b) Structure of the B95-8 and P3HR-1 genomes showing relative locations of the deleted regions (bounded by broken lines), the 3.1-kb IRs ($\square$), the *Not*I and *Pst*I repeats, and the *Bam*HI fragments used in these studies. That portion of the P3HR-1 deletion denoted by an asterisk represents the minimum size of the deletion, whereas the remainder may either have been eliminated during the deletion event or may reflect a preexisting strain difference in the number of IRs. (c) Derivation of the deleted *Bam*HI H fragment of P3HR-1. The diagram shows a comparison of the physical maps of the P3HR-1 viral DNA insert in pDH61 with those of the B95-8 viral DNA fragments in pSL77 (*Bam*HI-H) and pSL8 (*Bam*HI-Y and -W). Note the similarity of the pDH61 cleavage pattern to the left of the broken line with the left-hand portion of pSL77 and the similarity of that to the right of the broken line with the right-hand portion of the 3.1-kb IR in pSL8. The IR sequences are shown as solid bars. *Bam*HI-H, -Y, and -W map at adjacent positions between coordinates 0.65 and 0.71 in the B95-8 genome.

size range of 8.3 to 10.1 kb and one migrating at 2.8 to 4.1 kb. In the second variation, pSL77 DNA was cleaved to completion with *NorI* before end labeling and asymmetric cleavage with *BglII*. These four sets of fragments allowed us to sequence both rightward and leftward through the *NorI* repeat units themselves and rightward and leftward into the flanking unique sequence DNA. Possible minor variations among individual repeat units would not have been detected by this approach. The nucleotide sequence of the consensus 125-bp *NorI* repeat unit is given in Fig. 3a, where it is compared with the known sequence for the 102-bp *PstI* (DS_R) repeat (U. K. Freese, G. Laux, J. Hudewentz, E. Schwarz, and G. W. Bornkamm, submitted for publication). Noteworthy features of the *NorI* repeat sequence include its high guanine plus cytosine (G+C) content (84% as compared with 57% for the overall EBV genome) and its extensive homology with the *PstI* repeats. This homology includes a nearly perfectly conserved 9-bp tandem repeat. Approximately the final 50 bp of both repeat unit sequences (Fig. 3a) can be drawn as potentially self-complementary stem-loop structures of reasonable stability, containing 70% of the nucleotides in guanine · cytosine pairs (Fig. 3b).

The almost complete nucleotide sequence for a total of 2,300 bp in the region encompassing and flanking the *NorI* repeats in *BamHI*-H is presented in Fig. 4a and was derived by the sequencing strategy shown in Fig. 2a. Eleven complete copies of the *NorI* repeat sequences are located between positions 829 and 2,241 in Fig. 4a, based on direct counts from a partial cleavage ladder (14), but as indicated from the sequence data, the final nucleotide of repetitive sequence occurs 38 bases into a twelfth repeat unit. Thus, the *NorI* repeat cluster contains a nonintegral number of repeat units.

Transcription of the *NorI* repeat cluster after TPA induction. The 3.1-kb *BamHI*-W IRs represent major templates for transcription in latently infected lymphoblasts (20, 33). At 125 bp the *NorI* repeat is considerably smaller than the large IR sequence, but the question arose whether it too might form part of a transcription unit. RNA was extracted from B95-8 cells which had been induced with TPA. Polyadenylated RNA, selected by oligodeoxythymidylate chromatography, was denatured with glyoxal, electrophoresed on agarose gels, and transferred to nitrocellulose. The filters were hybridized with an in vitro-labeled probe of pSL77 DNA, which iden-

tified a major RNA species of 2.5 kb (Fig. 5, tracks 2 and 5). This same RNA also hybridized with a probe consisting of the isolated 125-bp *NorI* repeat unit DNA, indicating that the *NorI* repeats themselves form part of the template for the 2.5-kb RNA coded by *BamHI*-H.

RNA extracted from Raji and P3HR-1 cells was also examined for the presence of the 2.5-kb transcript, and the levels were compared with those in B95-8 cells. Before TPA induction, a 2.5-kb mRNA species homologous to the pSL77 DNA probe was not detectable in P3HR-1 cells and was present only in very low levels in B95-8 and Raji cells (Fig. 5, tracks 4 and 7). After TPA treatment, the 2.5-kb transcript could readily be identified in Raji cells and was much more abundant in B95-8 cells (tracks 5 and 6). We estimate that in these experiments the induced B95-8 cells contained 20- to 50-fold higher levels of this transcript than the uninduced B95-8 cell lines, whereas TPA treatment of Raji cells resulted in an approximately fivefold increase. P3HR-1 cells failed to synthesize the 2.5-kb transcript even after TPA induction, which is consistent with the observation that the *NorI* repeats, part of the template for this RNA, are deleted in P3HR-1. However, a 2.7-kb transcript was observed in the induced P3HR-1 cultures (track 3). This transcript presumably represents mRNA from the cross-hybridizing DS_R region (Freese et al., submitted for publication). The Raji genome also contains the cross-hybridizing DS_R region, but because only a single RNA band of 2.5 kb was detected in the TPA-induced Raji cells (track 6), we suspect that the Raji DS_R region may be reduced in size relative to the P3HR-1 DS_R region, with the result that the cross-hybridizing *PstI* and *NorI* repeat mRNAs comigrate in our gels.

Mapping of the 2.5-kb transcript and promoter. Two approaches were used to map the location of the template more precisely. First, Southern transfers of cleaved pSL77 DNA were hybridized with radiolabeled RNA extracted from B95-8 cells which had been incubated in medium containing inorganic ³²Pi. The 3,400-bp *SacI*-*BamHI*, 3,600-bp *SacII*-*BamHI*, and 3,200-bp *HincII* fragments hybridized strongly in these experiments, whereas the 2,500-bp *BamHI*-*SacI*, 2,300-bp *BamHI*-*SacII*, and 1,850 *HincII* fragments were only weakly positive (Fig. 6). The [³²P]RNA also hybridized weakly to the 1,060-bp *AvaI* fragment of pSL77. These hybridization results suggest that the RNA transcript extends from a position no more than 100 to 200

sequences bounding the region deleted in P3HR-1. On the left is an insertion element-like format with direct and inverted homologies indicated, and on the right is a stem-loop arrangement ($\Delta G = -28$ kcal) (29). The short arrows indicate one interpretation of the site of recombination drawn in this case as the A in the ambiguous -CCCAG- sequence.

a

b

FIG. 3. (a) Comparison of the 125-bp *NotI* and the 102-bp *PstI* repeat units aligned for maximal homology. The nucleotide sequence of the *PstI* repeat is from Freese et al. (submitted for publication). Altered bases within regions of high homology are denoted by asterisks. Arrows indicate the conserved 9-bp direct repeat. (b) Comparison of possible hairpin structures within the final 50 bp of the *NotI* (ΔG , = -31 kcal) and *PstI* (ΔG = -31 kcal) repeat unit sequences.

bp to the left of the *SacII* site, through the *NotI* repeat cluster, to a position no more than 200 to 300 bp beyond the adjacent *AvaI* and *HincII* sites. Since this map distance covers 2,500 to 2,600 bp, the bulk of the transcription unit would appear to occupy a contiguous unit without major splicing or intervening sequences. Note that the 1,650-bp *AvaI* fragment, which consists almost entirely of *NotI* repeat sequences, and the adjacent 480-bp *AvaI* fragment are both known from our previous DNA-DNA hybridization experiments to behave aberrantly in Southern filter transfers (14), and presumably this accounts for their unexpectedly low hybridization efficiency in this assay also. It may be relevant that these two fragments have extremely high overall G+C contents of 82 and 78%, respectively.

The location of the promoter and direction of transcription for the 2.5-kb mRNA were determined by in vitro transcription of pSL77 DNA by the procedure of Manley et al. (23). In this assay RNA transcription initiates faithfully, but termination signals are not recognized and transcription continues to the end of the linear DNA fragment. Figure 7 compares the in vitro transcripts obtained with pSL77 DNA cleaved with *BamHI*, *BstEII*, *HincII*, and *SacI*. The transcript from *BstEII*-cleaved DNA is 150 bp smaller than that generated from *BamHI*-cleaved DNA, and since the *BstEII* site maps 150 bp from the right-hand *BamHI* site of pSL77, the direction of transcription must be from left to right. This was confirmed by the results obtained with *HincII*- and *SacI*-cleaved DNA. On the basis of the in vitro transcription results, a

GGTACCACCN	CCTTGTGACA	CACCTTAAATG	AAACCCAACG	GGCTAAAATG	50
<u>KpnI</u> ACACACCTGA	ATTAACCAAT	AAGAAAGCCC	CACTCCTGAG	CAAACCTTAA	100
AGTATTACAC	AGAGACCCCA	AAAAGAGGAT	AAAAGACGGG	TGCCGGCCCG	150
GCTCGCCAGC	GTCGTCCAGA	CGCTCGGGGG	GTGCACACCT	CCCAGCCGGC	200
CCGGCGTCCT	TGCGGCGGCC	<u>AvaI</u> TCCCCCCCCA	TGAAGCGGGT	GGCGCGGGGT	250
CCCTGCCTGG	CACCAAGGTC	CGGCCTGGGA	GCGCACCCCC	CACCCCGGCC	300
GGGCCCTTGG	TGGCTCAGGC	CGCGGTCTCT	CCCCGTGAAC	GGGGCGCGCT	350
<u>ApaI</u> GGGCCGCGGC	TGCCCCCTGG	<u>SacII</u> ACCCCTAGGC	CTGTGCCATG	<u>BssHII</u> GGGACCCCCT	400
<u>SacII</u> GCCAGTCGGC	GCGGGGTCCC	AGGACCACGC	<u>StuI</u> CCCTCCCTCA	<u>NcoI</u> CTGCCCCCCC	450
CCCTGCCTTC	AGGTGCACCG	GACCAGCAGA	CCCGGCGGCT	ACCCCCAGGG	500
TGGGGCCAGC	GGACCGCACC	CACCTAGGTC	GGACYAGCGG	AYGCAGCATC	550
CCCGGATGAG	CTCCRRRRCC	ATGCAAGCGG	GGCACGCCCY	RGGGGAGGGT	600
<u>SacI</u> ACCGGTGGG	AGCAGGGTGG	GGGCGTCCCG	GGACCCAGC	CCCCAGCCGG	650
CAATCCATGC	GTACCGGGCC	AGCAGAGCAA	GCGGACCACG	CCCATTGAA	700
CCCTACCGGC	GGATGCAGCG	ACCCCCAGAG	GAGCCCCAGA	ACCAGGCAAG	750
CCGGGTACGC	CCTGGGGGAG	GGATCGGCGG	GGCTGGGGTC	YAGGGGACCA	800
CGCCCCCACC	CYGCATTCCA	AGTGCAGTGG	AGCGCCAGGA	ACCCCGGCTG	850
CCCCAGGACC	TGGCGGCGGC	GCAGCGGTGC	<u>HaeII</u> CCAGCGGGGC	CACCCCCCAC	900
CCGGAGCGGG	GCAGCGGCCC	AGCGGACCCA	CCGGCGGGCG	CCCGGTGCC	950
<u>HaeII</u> CCCGGAGCGC	CAGGAACCCC	GGCTGCCCCA	<u>NotI</u> GGACCTGGCG	GCGGCGCAGC	1000
GGTGCCACGC	GGGGCCACCC	CCCACCCGGA	GCGGGGCAGC	GGCCAGCGG	1050
←-----8 x 125 bp NotI repeats-----→					2050
ACCCACCGGC	GGCCGCCCGG	CTGCCCCCGG	AGCGCCAGGA	ACCCCGGCTG	2100
<u>NotI</u> CCCCAGGACC	TGGCGGCGGC	GCAGCGGTGC	<u>HaeII</u> CCAGCGGGGC	CACCCCCCAC	2150
CCGGAGCGGG	GCAGCGGCCC	AGCGGACCCA	CCGGCGGGCG	CCCGGTGCC	2200
<u>HaeII</u> CCCGGAGCGC	CAGGAACCCC	GGCTGCCCCA	<u>NotI</u> GGACCTGGCG	GTAGTGCAGT	2250
GTCCCCGCTG	CCCATGGAAT	GCTCAGACCC	CGGGTTGGTG	GCACTGTTGC	2300
GCCCGGCCCT	GTACACTACA	CTCTAAAAGT	<u>AvaI</u> AACCTGTCTA	CTTCGCCATG	2350
CTTCTTACAC	TACTCACCTA	CATGTCAACC	GCCTTACCC	TCCCATGGG	2400
ATGGCGGCGG	TTATGTTTTT	<u>HincII</u> CCATGTTGCG	GGTGCCGG...	<u>NcoI</u>	2450

FIG. 4. Nucleotide sequence of the noncoding strand of EBV DNA in the region flanking the *NotI* repeats. The sequence given begins at the presumed promoter sequence within the 670-bp *AvaI* subfragment of pSL77 and extends through the *NotI* repeat sequences and into the adjacent unique sequence DNA. Boxes surround two CAAT-like regulatory elements and one TATAAA-like regulatory element at -91, -77, and -29 nucleotides, respectively, upstream from a potential mRNA start site indicated by the vertical arrow at position 133. Three possible TAATGAGA-like sequences commonly found upstream of herpesvirus immediate-early promoters are indicated by overhead broken lines at -132, -112, and -95 relative to the potential mRNA start site. The 11.3 copies of the 125-bp *NotI* repeat sequences lie between positions 829 and 2,241, with the first and final nucleotides within each complete or partial repeat unit marked by square brackets. Positions marked with Y, R, or N indicate pyrimidine, purine, or unknown nucleotides, respectively.

FIG. 5. Identification of a 2.5-kb TPA-induced mRNA transcript from the *NotI* repeat region of B95-8 *Bam*HI-H. The autoradiograph shows the hybridization of in vitro-labeled pSL77 (*Bam*HI-H) DNA to nitrocellulose transfers of polyadenylated RNA after fractionation by electrophoresis through 1.4% agarose gels containing glyoxal. Individual tracks contain RNA isolated from the following B95-8 + TPA (Track 2); P3HR-1 + TPA (track 3); uninduced B95-8 (track 4); B95-8 + TPA (track 5); Raji cells + TPA (track 6); and uninduced Raji cells (track 7). Track 1 contains reference denatured end-labeled restriction fragments of pBR322 DNA with sizes given in nucleotides.

strong start site (presumably the promoter for the 2.5-kb RNA) is located to the left of the *Sac*II site, within the 670-bp *Ava*I subfragment of pSL77. The P3HR-1 deletion removes approximately 3,500 bp of *Bam*HI-H DNA, including the bulk of the template for this RNA transcript. However, because the 670-bp *Ava*I subfragment is retained in P3HR-1 DNA, we also tested the P3HR-1 *Bam*HI H fragment (pDH61) in the in vitro transcription assay. From the results obtained with the *Bam*HI-*Pvu*II, *Bgl*II, and *Sac*I digests (Fig. 7, tracks 6 through 8), it is evident that the promoter for the 2.5-kb RNA transcript is also retained in P3HR-1 and remains functional in vitro. A summary of the map location of the P3HR-1 deletion relative to the *NotI* repeat cluster and the 2.5-kb *NotI* transcript is presented in Fig. 8.

DISCUSSION

The *NotI* repeat cluster in EBV DNA is of interest because of its map location within that portion of the *Bam*HI H fragment which is deleted in a nontransforming isolate, its unusual multiple tandem repeat structure, and the fact that it is expressed abundantly as polyadenylated mRNA after TPA induction. The *NotI* repeat units themselves have an extremely high G+C content and are theoretically capable of assum-

ing a stable stem-loop secondary structure of the type often associated with DNA-protein interactions. This particular repetitive sequence region covers 1,400 bp and contains 11.3 copies of the 125-bp *NotI* repeat unit. The significance of a nonintegral number of repeats remains unclear, but since this is a feature shared also with the large 3.1-kb *Bam*HI-W IR cluster (14), the arrangement of extra partial copies appears to be an inherent feature of tandem repeat structures in EBV DNA. In most EBV isolates, the *Bam*HI H fragment exhibits homology with a distant portion of the *U_L* segment (11, 16, 26). Hudewentz et al. (17), using the nomenclature *DS_R* and *DS_L* for these homologous regions, found that the *DS_R* region contained up to 30 copies of a tandem repeat unit which had a single site for the *Pst*I restriction endonuclease. In heteroduplex experiments the tandem repeats of *DS_R* showed both hairpin formation and partial homology with the 1,500-bp region of *Bam*HI-H now known to contain the *NotI* repeats. Our comparison of the nucleotide sequences of the 125-bp *NotI* and 102-bp *Pst*I repeats confirms that they share common stretches totalling 87 bp and have an obviously close evolutionary relationship.

Hybridization experiments with unlabeled RNA in gels revealed that the *NotI* repeats also comprise part of the template for a 2.5-kb polyadenylated RNA transcript whose level in EBV(B95-8) cells is dramatically increased within 48 h after TPA induction. This RNA species almost certainly corresponds to the 2.5-kb transcript described by Hummel and Kieff (18) as being the most abundant early transcript in TPA-treated EBV(B95-8) cells. The *Bam*HI H fragment is also known to be a major template for transcription in nonproducer EBV-infected lymphocytes, but the only relevant transcript identified in the most stringently latent IB4 cells was a 3.1-kb RNA which is partially coded by the IRs (33).

Our experiments with ³²P-labeled RNA probes isolated from B95-8 lymphocytes localized the template for the 2.5-kb transcript to a contiguous region encompassing the entire *NotI* region of *Bam*HI-H. In addition, the in vitro runoff transcription results placed a strong rightward RNA start site (presumably the promoter for this 2.5-kb mRNA) 700 to 800 bp to the left of the first *NotI* repeat unit. In the nucleotide sequence of this region shown in Fig. 4, a modified Goldberg-Hogness TATAAA-like sequence can be identified at position 128, which would suggest a potential mRNA start site at the cytosine 28 nucleotides downstream from the GATAAA sequence. Additional analysis (not shown) comparing the size of an in vitro transcript ending at the *Bss*HII site with that of an

FIG. 6. Localization of the template for the 2.5-kb transcript within *Bam*HI-H. The autoradiographs illustrate hybridization of a ³²P-labeled, polyadenylated RNA (³²P polyA mRNA) probe extracted from TPA-induced B95-8 cells to pSL77 DNA cleaved with the following enzymes: (tracks 1 and 3) *Bam*HI + *Sac*II; (tracks 2 and 4) *Bam*HI + *Sac*I; (tracks 5 and 7) *Ava*I; and (tracks 6 and 8) *Hinc*II. Fragment sizes are given in base pairs. Solid bars in the mapping diagram represent the fragments that hybridized most intensely; the two high-G+C content *Ava*I fragments which behave aberrantly are indicated by asterisks.

S1-protected hybrid formed between the *in vivo* RNA (B95-8 cells, TPA induced) and an isolated *Bgl*III-*Bss*HII fragment from pSL77 revealed that in both cases the 5' end of the transcript was

located at 195 ± 10 nucleotides to the left of the *Bss*HII site (i.e., between 17 and 37 nucleotides downstream from GATAAA). At least two CAAT box-like sequences may be present at

FIG. 7. Determination of the location and orientation of a predominant RNA start site near the *NotI* repeats by in vitro runoff transcription. ^{32}P -labeled RNA was synthesized by incubating HeLa cell extracts with fragments of pSL77 (B95-8) or pDH61 (P3HR-1) plasmid DNAs in the presence of $[\text{}^{32}\text{P}]\text{GTP}$ and was then extracted and fractionated by electrophoresis through glyoxal-agarose gels. (a) The autoradiographs show RNA transcribed from pSL77 DNA cleaved with *Bam*HI (track 2), *Sac*I (track 3), *Bst*EII (track 4), and *Hinc*II (track 5) and from pDH61 DNA cleaved with *Bam*HI + *Pvu*II (track 6), *Bgl*II (track 7), and *Sac*I (track 8). The size markers shown in track 1 are ^{32}P -end labeled restriction fragments of pBR322 and pTJ148. (b) Relative locations of the RNA start sites (solid circles) and the direction of transcription (arrow). The dashed line marks the position of the P3HR-1 deletion junction, and the sizes of RNA products are given in nucleotides. Restriction sites are indicated as follows: *Bam*HI (Ba), *Bgl*II (Bg), *Bst*EII (Bs), *Hinc*II (H2), *Not*I (N), *Pvu*II (P2), *Sac*I (S1), and *Sac*II (S2).

FIG. 8. Summary diagram showing the location of the *NotI* repeat cluster and the 2.5-kb *NotI* transcript in relation to the P3HR-1 deletion and flanking sequences as determined in this work. Note that in addition to the 6,650-bp deletion, the P3HR-1 genome also lacks five or six complete copies of the 3.1-kb IR relative to B95-8. The identification and location of the region of homology with the *PstI* (DS_R) repeats and of the inverted repeat loop in the IR sequence (short arrows) come from the data of Hudewentz et al. (17). The probable promoter for the 3.1-kb spliced transcript expressed during latency in IB4 and Raji cells was identified in the IR sequence by Jones and Griffin (18a) and Cheung and Kieff (6). The polyadenylation signal for the 2.5-kb TPA-induced mRNA has not yet been identified, but an AATAAA sequence on the other strand, presumably associated with the 3.1-kb mRNA, occurs approximately 510 bp to the right of the final *NotI* site (unpublished data).

-75 (CCACT) and -89 (CCAAT) nucleotides upstream from the start site. In addition, the TAAATGACA at -132 and the TTAATGAAA at -112 (and possibly also AATAAGAAG at -89) closely resembled the characteristic multiple TAATGAGA-like elements found in several immediate-early promoters of herpes simplex virus type 1 (22) and in the single immediate-early (IE94 gene) promoter from cytomegalovirus (Colburn) (K.-T. Jeang and G. S. Hayward, manuscript in preparation). This EBV promoter also falls within the 900-bp segment described by Hudewentz et al. (17) as having the highest degree of homology with the DS_R region. No polyadenylation signal for the 2.5-kb mRNA has yet been detected, but since our sequence extends only 2,300 bp from the presumed RNA start site, this would not be unexpected. The 2.5-kb RNA is polyadenylated, and it should therefore code for a polypeptide product. A potential translation signal (ATG) is found at +74 from the mRNA start site (Fig. 4, position 229). Although the downstream *NotI* repeats are completely free of termination signals, all three reading frames appear to possess terminators before the repeat cluster, and therefore we suspect that, unless splicing occurs, the tandem repeat units themselves might not serve as protein-coding sequences. The entire region between the promoter and the *NotI* repeats also has an unusually high G+C content (75%).

The P3HR-1 deletion fuses sequences from the *Bam*HI H fragment to a site within the 3.1-kb IR unit. At the novel junction both the equivalent EBV(B95-8) sequence and the IR sequence have a matching pentanucleotide, -CCCAG-. In addition, the two flanking EBV(B95-8) sequences encompassing the deletion boundaries display a surprisingly complex pattern of direct and inverted homologies. In one view the structure closely resembles that of a typical insertion element (Fig. 2b), although the deletion event itself did not follow the expected pattern for excision of an insertion element. Alternatively, the deletion can be envisaged as occurring in the middle of the stem in a large stem-loop structure. [It should be noted here that EBV(B95-8) is not the parent of the EBV(P3HR-1) strain, but there is no reason to suspect that the actual parent, EBV(Jijoye), differs significantly from B95-8 at the deletion boundaries.] It is interesting that the promoter for the 2.5-kb transcript is retained in EBV(P3HR-1) even though most of the template sequences, including the *NotI* repeats, are lost in the deletion. This creates the possibility of the formation of a novel fusion polypeptide(s) that would be unique to the EBV(P3HR-1) strain. Although both the *NotI*(DS_L) repeats and the *PstI*(DS_R) repeats encode major transcripts, neither of these genes appears individually essential for virus replication or for the maintenance of viral latency. For

example, in contrast to EBV(P3HR-1), the EBV(B95-8) isolate, which replicates normally and transforms lymphocytes efficiently, lacks the entire *PstI* repeat region.

The unusual structural features of the genes encompassing the *NotI* (or *PstI*) repeat clusters suggests the potential to serve a regulatory function during latent infections, either through interactions at the DNA level with specific viral or cellular repressor protein(s) or perhaps through protein-RNA interactions. The idea of a regulatory role is especially attractive if the 2.5-kb mRNA does indeed prove to code for a pivotal immediate-early product that initiates the productive lytic infection pathway. One could also argue that the *NotI* repeats may represent novel forms of enhancer elements which lie inside a transcription unit rather than at the expected 5' position, although our results do show that the absence of these downstream elements in EBV(P3HR-1) DNA has no effect on the efficiency of transcription initiation from the *NotI* promoter *in vitro*. Whether the absence of the *NotI* repeat cluster from the EBV(P3HR-1) genome is directly related to the loss of transforming ability in this isolate remains to be determined. Since the P3HR-1 deletion removes all but the proximal 339 bp of the template for the 2.5-kb mRNA, the protein product of this transcript must also be considered a candidate for a role in lymphocyte immortalization. However, the gene for the 3.1-kb latency transcript, which is also partially deleted in P3HR-1, seems more likely to play a direct role in that process.

After our work was completed, Dambaugh and Kieff (9) and Jones and Griffin (18a) also presented sequence data for the *NotI* repeats of B95-8 DNA. The Jones and Griffin sequence agrees with our data, but that of Dambaugh and Kieff differs in that it lacks one C residue (Fig. 3a, position 63). The latter authors and Freese et al. (submitted for publication) have also detected a 2.5-kb mRNA homologous to the *NotI* repeats.

ACKNOWLEDGMENTS

We are grateful to Beverly Griffin and Karl Freese for providing sequence data on the 3.1-kb *BamHI* repeat and the 102-bp *PstI* repeat, respectively, before publication. We thank Gary Hayward for helpful discussions, Beverly Plunkett and Tom Lietman for competent technical assistance, and Nancy Standish for preparation of the manuscript.

K.-T.J. is a predoctoral student supported by the Medical Scientist Training Program at the Johns Hopkins University School of Medicine (GM 07309). This work was funded by Public Health Service grant ROI CA 30356 to S.D.H. from the National Institutes of Health.

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