JOURNAL OF VIROLOGY, May 1983, p. 424–433
0022-538X/83/050424-10$02.00/0

Vol. 46, No. 2

RNA Encoded by the IR1-U2 Region of Epstein-Barr Virus DNA in Latently Infected, Growth-Transformed Cells

VICKY VAN SANTEN, ANDREW CHEUNG, MARY HUMMEL, AND ELLIOTT KIEFF*
Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637

Received 22 October 1982/Accepted 28 January 1983

The IR1-U2 region of Epstein-Barr virus DNA consists of multiple copies of a 3.1-kilobase (kb) repeat sequence, IR1, which maps to the left of a 3.3-kb unique region, U2. Although hybridizations of cytoplasmic polyadenylated RNA from latently infected cells to viral DNA indicate that the IR1-U2 region encodes a substantial fraction of the viral RNA in these cells, only a single low-abundance 3-kb cytoplasmic polyadenylated RNA has been identified on Northern blots. Further analysis of the cytoplasmic polyadenylated RNA encoded by the IR1-U2 region indicates that (i) the RNA is transcribed from left to right; (ii) there are only three copies of the 3-kb RNA per cell; and (iii) the RNA is spliced. The RNA hybridizes to possibly contiguous 0.56- and 1.3-kb U2 domains. These domains and part of IR1 hybridize to the 3-kb cytoplasmic RNA. DNA between IR1 and the 0.56-kb U2 domain does not hybridize to the 3-kb RNA. The CCAAT-34 nucleotide-TATAA sequence in IR1 may be part of the promotor for the 3-kb cytoplasmic polyadenylated RNA since (i) it enables left-to-right transcription of IR1 by a HeLa cell extract, and (ii) latently infected cells contain giant polyadenylated nuclear RNAs which differ in size by 3 kb, as would be expected if transcription initiates in any copy of IR1 and continues through the rightward remaining copies into U2.

Infection of normal human B lymphocytes with Epstein-Barr virus (EBV) results in persistent latent infection and cellular growth transformation (14, 36). Latent growth-transforming infection is characterized by the presence of the complete viral genome (21, 39, 50) as covalently closed circular episomes (28, 34); by the expression of polyadenylated polyribosomal RNA from the IR1-U2, IR3, and U5 regions (19, 22, 24, 25, 37, 48); and by the expression of new intranuclear (42) and cell membrane (45) antigens. A similar state of virus infection characterizes latently infected B lymphocytes in normal seropositive humans (33) and malignant African Burkitt tumor cells (10).

Probes which include IR1 or U2 (*Bam*HI fragments V, X, and H; Fig. 1) identify a 3-kilobase (kb) RNA in cytoplasmic extracts of a latently infected cell line, IB4 (24, 48). The importance of the EBV IR1-U2 region in inducing growth transformation is also suggested by studies of the P3HR-1 EBV strain. The P3HR-1 genome is deleted for the entire U2-IR2 region (7, 18, 23, 38, 40). The virus produced by P3HR-1 cells can infect lymphocytes but cannot induce growth transformation (32, 41).

cDNA made from the 3′ end of IB4 cell RNA hybridizes more to the EBV DNA *Bam*HI fragment H, which contains the right part of U2 and IR2, than to *Bam*HI fragments V or X, which contain IR1 and the IR1-U2 juncture, respectively, suggesting that the 3′ end of the 3-kb RNA is in U2 or IR2 (48). Since the cDNA also hybridizes to *Bam*HI fragments V and X, the data are also compatible with the existence of several RNAs with different 3′ ends. The sequence of IR1 reveals a CCAAT-34 nucleotide-TATAAA sequence and a TATA sequence which could be part of promoters for rightward transcription from IR1 into U2 (8). Other important features of IR1 include multiple potential splice donor and acceptor sites, a 1.1-kb open reading frame, and an unusual 500-base pair dyad symmetry which would have a profound effect on the secondary structure of RNA encoded by IR1 (8).

The objective of this report is to define further (i) the sites for initiation of transcription from IR1 in vitro; (ii) the direction of transcription from IR1 and U2 in latently infected growth-transformed IB4 cells; (iii) the number and size of RNAs transcribed from IR1 and U2 in IB4

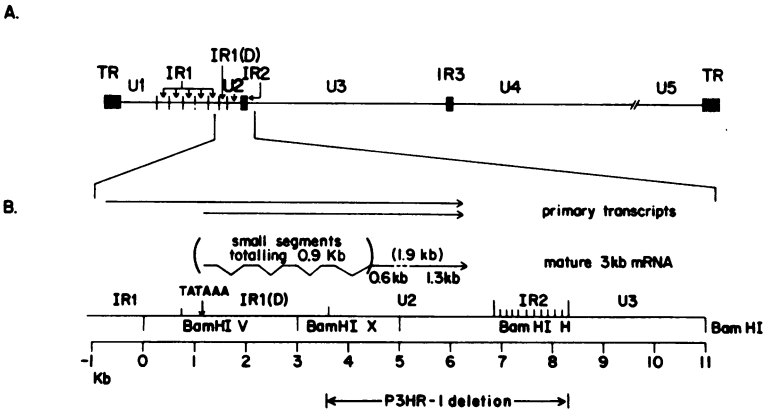

FIG. 1. (A) Schematic diagram of the EBV genome. The genome is divided into five regions of unique sequence complexity (U1 through U5) bounded by direct tandem copies of DNA sequences at the termini (TR) and at internal sites within the molecule (IR1 through IR4). The discontinuity in the map between U4 and U5 reflects the fact that the B95-8 isolate of EBV is deleted for a region of the DNA which contains a fourth internal repeat, IR4. The deletion has no known effect on the biological properties of the virus. IR1(D) indicates the final incomplete copy of IR1. (B) Model for the 3-kb mRNA transcribed from the EBV IR1-U2 region in IB-4 cells. A *Bam*HI restriction enzyme map of the IR1, U2, and IR2 regions is shown. TATAAA indicates the position of the promoter-like sequence at nucleotide 1137. RNAs transcribed from this region are shown above the map. Some of the primary transcripts begin in copies of *Bam*HI fragment V to the left of the one shown on the map. In this model, splicing of long primary transcripts generates a 3-kb mRNA. The 0.6- and 1.3-kb RNA segments could be a continuous 1.9-kb segment. The uncertain continuity of these segments is indicated by the dotted line in the 1.9-kb segment. Other RNA segments spliced onto the 1.9-kb segment have not been demonstrated and are indicated in parentheses. The polyadenylic acid 3' end (not shown) probably constitutes the remaining 0.2 kb of the 3-kb mature mRNA.

cells; and (iv) whether the 3-kb polyadenylated RNA previously identified in the cytoplasm of IB4 cells is spliced.

MATERIALS AND METHODS

Cell culture. The IB4 cell line was established by in vitro transformation of human cord blood lymphocytes by the B95-8 isolate of EBV and is negative for antigens expressed early and late in EBV replication (24). Cells were maintained by adding an equal volume of RPMI 1640 medium (GIBCO Laboratories) supplemented with 10% fetal calf serum (K. C. Biologicals) every 3 days. RNA was prepared from cells 3 days after feeding.

Recombinant DNA. Recombinant EBV (B95-8) DNAs were used for hybridization to RNA blots and in vitro transcription. Plasmids pDK14, pDF322, and pDK286 are clones of *Bam*HI fragments V, X, and H, respectively (9). For S1 nuclease analysis, restriction fragments from the library were subcloned into the vector plasmid pKH47 (6). The recombinant EBV-pBR322 plasmids were cut with *Bam*HI to free the EBV DNA. Before ligation into the *Bam*HI site of pKH47, either the EBV DNA was isolated by electrophoresis in agarose gels or the pBR322 vector was destroyed by cutting with *Eco*RI, an enzyme that does not cut within the EBV DNA. Ligated DNA (ligase was obtained from Bethesda Research Laboratories) was transfected into *Escherichia coli* HB-101 by the calcium phosphate technique (15). The subclones of *Bam*HI fragments V, X, and H were named pKH47-V, pKH47-X, and pKH47-H, respectively.

Plasmid DNA was prepared from bulk cultures (12). After ethanol precipitation, DNA to be used for S1 nuclease analysis was suspended in 10 mM Tris (pH 8.0)–1 mM EDTA and was stored at −70°C. Restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs and were used as recommended by the manufacturers.

Preparation of RNA. Cytoplasmic and nuclear fractions were prepared from IB-4 cells (48). DNA was sheared with a Tekmar Tissuemizer for the preparation of nuclear RNA (5). Vanadylribonucleoside complex (10 mM) was used as an RNase inhibitor (4). RNA was stored in 20 mM Tris (pH 7.4)–2 mM EDTA–0.2% sodium dodecyl sulfate at −70°C.

For some experiments, nuclear RNA was further freed of DNA by pelleting in cesium chloride. RNA in 1 ml of 10 mM Tris (pH 7.4)–2 mM vanadylribonucleoside complex with CsCl added to a refractive index of 1.4017 was overlaid with a CsCl solution with a refractive index of 1.4003 and was sedimented in an SW41 rotor at 34,000 rpm for 6 h. The RNA was not degraded during cesium chloride centrifugation since the 45S ribosomal RNA precursor was still visible in gels of CsCl-purified nuclear RNA.

In vitro transcription. *Bam*HI fragment V DNA (2 μg) was incubated at 30°C for 60 min with 30 μl of HeLa cell extract (Bethesda Research Laboratories) in 50 μl of a solution consisting of 5 mM ATP, 5 mM CTP, 5 mM GTP, 0.05 mM UTP, 10 μCi of [α-^{32}P]UTP, 10 mM creatine phosphate, and 0.14 mM EDTA (29, as specified by the manufacturer). After completion of the reaction, protein was removed by phenol and chloroform isoamyl alcohol extraction in the presence of 0.5% sodium dodecyl sulfate–7 M urea. Nucleic acid was precipitated, suspended, and

subjected to electrophoresis in 1.4% agarose methyl mercury gels (3).

Preparation and hybridization of RNA blots. Nuclear polyadenylated and non-polyadenylated RNAs were separated by one cycle of oligodeoxythymidylic acid [oligo(dT)]-cellulose column chromatography (2, 35). Cytoplasmic polyadenylated RNA was purified by one or two chromatography cycles. The yield of cytoplasmic polyadenylated RNA was between 1 and 2 mg per 8×10^9 cells. Denaturation of RNA, electrophoresis in formaldehyde-agarose gels (27), and blotting onto nitrocellulose (46) were as previously described (48). The size of nuclear RNAs was determined by plotting the square root of the size against the log of the electrophoretic mobility (27). Ribosomal precursor RNA sizes of 6.9 and 14 kb and ribosomal RNA sizes of 5.2 and 2 kb were used as standards (49). Electrophoresis of cytoplasmic and nuclear RNA was in 0.8 and 0.6% agarose gels, respectively. Specific DNA restriction fragments were recovered from agarose (HGTP, Seakem) gels by electrophoresis against a dialysis membrane. Fragments were then labeled with ^{32}P by nick translation (43) and hybridized to RNA blots as previously described (48) without dextran sulfate.

The number of IR1-U2 3-kb cytoplasmic polyadenylated RNA molecules per cell was determined from the relative levels of hybridization of the RNA and known amounts of DNA to recombinant viral DNA probes and from the RNA yield per cell.

S1 nuclease analysis. S1 nuclease analysis (6) of RNA was performed by using strand-separated DNA. Individual strands of EBV restriction fragments subcloned into the vector plasmid pKH47 were purified by binding to oligo(dT) or oligodeoxyadenylic acid [oligo(dA)]-cellulose (16). Nonradioactive strand-separated DNA (25 to 100 ng) was hybridized to 50 μg of IB-4 cytoplasmic polyadenylated RNA or yeast RNA in 10 μl of 50% (vol/vol) recrystallized formamide–0.4 M NaCl–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.5]–1 mM EDTA–0.1% sodium dodecyl sulfate at 50°C for 3 h. Nonhybridized DNA sequences were removed by digestion in 0.3 ml of 0.28 M NaCl–30 mM $NaC_2H_3O_2$ (pH 4.4)–4.5 mM $ZnSO_4$–20 μg each of native and denatured salmon sperm DNA per ml, with S1 nuclease (Boehringer-Mannheim Corp.) at 1,000 to 2,000 U/ml, for 30 min at 37°C. After ethanol precipitation, RNA was destroyed by incubation in 0.1 M NaOH at 70°C. The RNA-cDNA was electrophoresed on 1.5% alkaline agarose gels (31), transferred to nitrocellulose (44), and detected by hybridization to nick-translated probes made from EBV DNA restriction fragments purified from recombinant DNA plasmids. A *Hin*fI digest of plasmid pDK286 or pKH47-H DNA was included in each gel and blot to provide size markers. In some experiments, the RNA-cDNA was also electrophoresed in formaldehyde agarose gels as described for RNA (48). Both types of denaturing gels yielded identical results for the size of the protected DNA.

RESULTS

Nuclear RNAs encoded by the IR1-U2 *Bam*HI fragments V, H, and H. *Bam*HI fragment V, X, and H probes hybridized almost exclusively to the same nuclear RNAs (Fig. 2). A trace of the 3.0-kb cytoplasmic RNA was present in the polyadenylated nuclear RNA fraction (identified as 2.9 kb in the autoradiogram shown in Fig. 2). Two classes of RNAs larger than 3 kb could be distinguished. The largest nuclear RNAs were polyadenylated and range in size from approximately 22 to 14 kb in decrements of approximately 3 kb. Many RNA species between 5 and 13 kb were in both the polyadenylated and non-polyadenylated fractions. It was, therefore, not possible to discern whether the stepladder of polyadenylated RNAs continues through the range of 5 to 13 kb.

*Bam*HI fragment H includes not only the right part of U2 but also IR2 and the left 2.7 kb of U3 (Fig. 1). In addition to those nuclear RNAs in common with *Bam*HI fragments V and X, *Bam*HI fragment H identifies a 2-kb nuclear polyadenylated RNA and a more abundant 1.1-kb nuclear non-polyadenylated RNA. These last RNAs are encoded by the U3 region of *Bam*HI fragment H, whereas the RNAs in common with *Bam*HI fragments V and X do not extend into IR2 or U3. Thus, probes which include mostly the U2 part of *Bam*HI fragment H hybridize to the same RNAs as *Bam*HI fragments V and X, whereas probes from *Bam*HI fragment H U3 hybridize to the 1.1- and 2.0-kb RNAs but not to the other RNAs (Fig. 2; other data not shown). IR2 does not hybridize to any of the nuclear RNAs of latently infected cells (11). All of the nuclear RNAs were confirmed to be RNA and not DNA by their density in neutral cesium chloride.

Direction of transcription of the 3-kb cytoplasmic polyadenylated RNA. To determine the direction of transcription of the cytoplasmic polyadenylated RNA, *Bam*HI fragments V, X, and H were cloned into plasmid pKH47 (16) so that the strands could be separated. Plasmid pKH47 is a derivative of pBR322 with adenine and thymine homopolymers inserted into the opposite strands so that the strands can be separated by using oligo(dT) and oligo(dA) columns (16). To relate the separated EBV DNA strands which were cloned into pKH47 to the strands of the EBV genome, it was necessary to determine the relative orientation of the EBV and pKH47 restriction endonuclease sites and to identify which pKH47 strands contain polydeoxyadenylic acid or polydeoxythymidylic acid. The strategy used to identify the polydeoxyadenylic acid-containing strand of pKH47 is shown in Fig. 3. The orientation of EBV DNA in pKH47-V, pKH47-X, and pKH47-H was then determined by restriction endonuclease analysis.

Each of the separated strands of plasmids pKH47-V, pKH47-X, and pKH47-H was then hybridized to cytoplasmic polyadenylated RNA

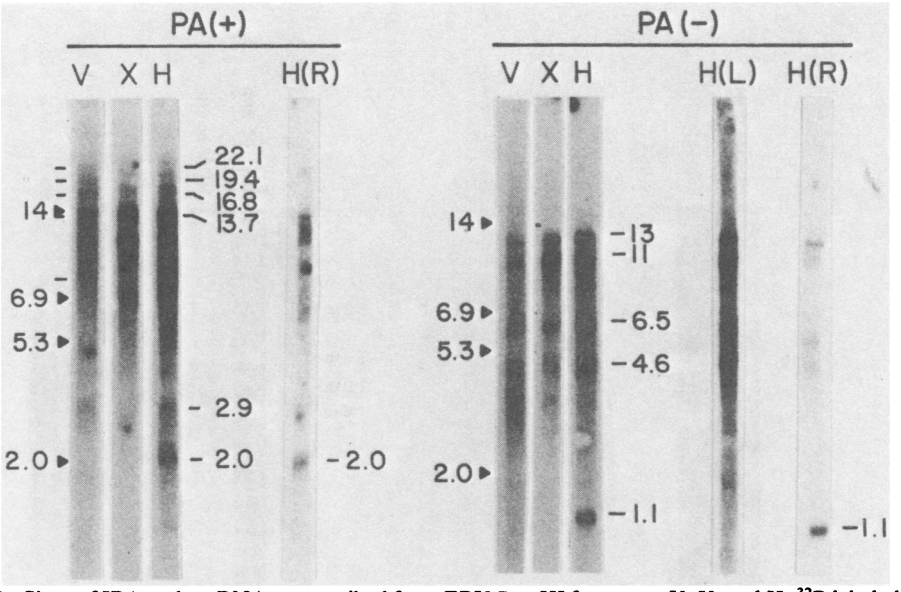

FIG. 2. Sizes of IB4 nuclear RNAs transcribed from EBV *Bam*HI fragments V, X, and H. ^{32}P-labeled probes of *Bam*HI fragments V, X, and H were hybridized to blots of polyadenylated [PA(+)] and non-polyadenylated [PA(−)] nuclear RNA. The *Bam*HI fragment V probe is *Pst*I fragment b (see Fig. 5). H(L) and H(R) indicate probes made from the *Sst*I-a and *Sst*I-b fragments of *Bam*HI fragment H (see Fig. 7). Ribosomal and precursor ribosomal RNAs from IB-4 cells (▲) were used as size standards (49). RNAs detected by hybridization to the DNA fragment probes are indicated by lines. RNAs identified in both polyadenylated and non-polyadenylated RNA are not indicated on the blots of polyadenylated RNA.

and treated with S1 nuclease. RNA protected parts of the EBV DNA R strand of all three clones, indicating that RNA is transcribed from left to right from *Bam*HI fragments V, X, and H (see Fig. 5 through 7). Cytoplasmic RNA did not protect any of the L strand from S1 digestion.

In vitro transcription from IR1. The observations that cytoplasmic polyadenylated RNA is transcribed from the R strand of *Bam*HI fragments V, X, and H and that the same fragments encode large nuclear RNAs differing in size by the length of IR1 suggest that transcription may be initiated in any copy of IR1. A CCAAT-34 nucleotide-TATAAA sequence similar to many RNA polymerase II promoter sequences and a less similar TATA sequence have been defined in IR1 and are in the correct orientation to promote transcription from left to right (8). Utilization of these promoter-like sequences was tested by using a HeLa cell extract (29) and *Bam*HI fragment V DNA. *Bam*HI fragment V is one complete copy of IR1. The relevant maps of *Bam*HI fragment V are shown in Fig. 4. The CCAAT-34 nucleotide-TATAAA sequence would promote transcription of a 1.9-kb runoff product, whereas the TATA sequence would promote transcription of a 1.4-kb RNA (or a 1.6-kb RNA if the promoter functions in the opposite direction). Transcripts of approximately 1.9 kb were detected (Fig. 4A). The synthesis of this RNA was inhibited by 0.8 μg of alpha-amanitin per ml, suggesting that the 1.9-kb runoff product is transcribed by RNA polymerase II. As ex-

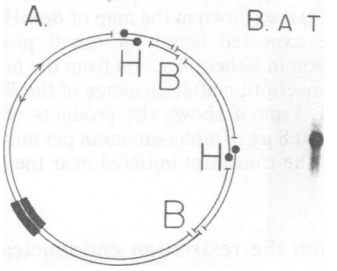

FIG. 3. Determination of the oligo(dA)- or oligo(dT)-binding strand of plasmid pKH47. (A) Schematic diagram of the pKH47-X DNA used in the experiment. The plasmid DNA was cleaved with *Hin*dIII (H), labeled with ^{32}P at the 5′ ends (●) (30), and then cleaved with *Bam*HI (B). The oligo(dA)- and oligo(dT)-binding sites are indicated by closed rectangles. Only the strand with a counterclockwise 5′ to 3′ direction contains a labeled end which would be covalently attached to oligo(dA) or oligo(dT). This plasmid DNA was denatured and strand separated by binding to oligo(dA)- and oligo(dT)-cellulose. (B) Autoradiogram of an agarose gel containing the material recovered from oligo(dA)-cellulose (A) and oligo(dT)-cellulose (T). The data indicate that the 5′ labeled end is on the poly(A)-containing strand.

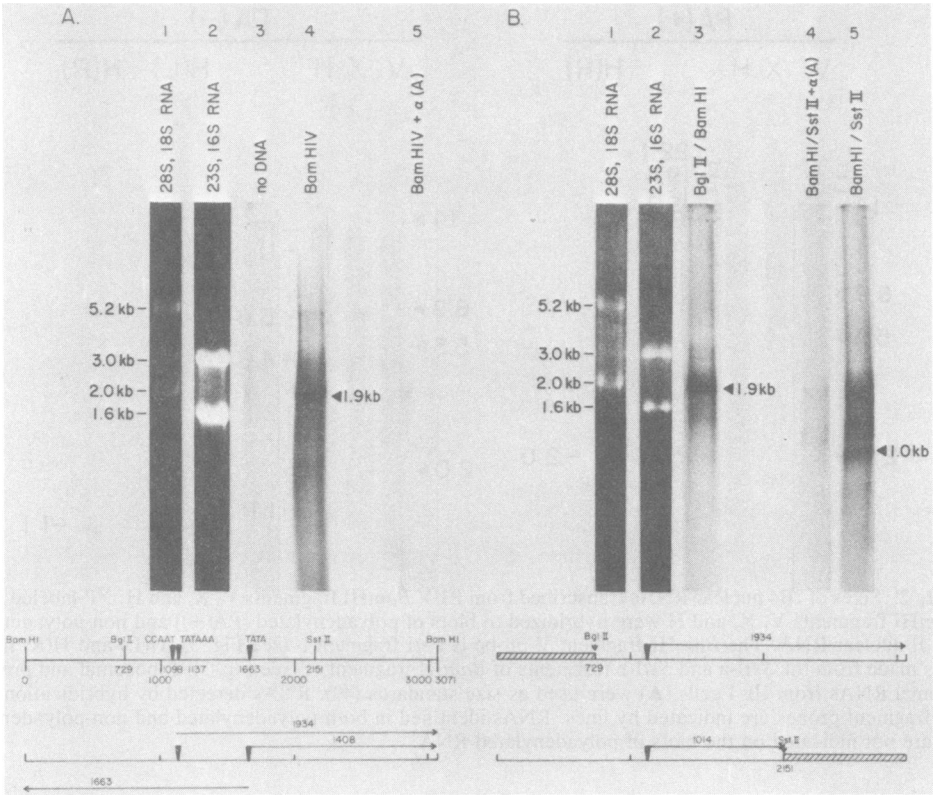

FIG. 4. Transcription from EBV *Bam*HI fragment V in vitro. *Bam*HI fragment V DNA purified from plasmid pDK14 (9) was transcribed by a HeLa cell extract. The labeled transcripts were electrophoresed in methyl mercury agarose gels and detected by autoradiography. Marker human and *E. coli* ribosomal RNAs are shown in lanes 1 and 2. In A, the products of transcription from *Bam*HI fragment V DNA are shown in lane 4. Negative controls in which no *Bam*HI fragment V DNA was added (lane 3) or in which RNA polymerase II was inhibited by 0.8 μg of alpha-amanitin per ml (lane 5) are shown. Promoter-like sequences identified in the nucleotide sequence (8) are shown in the map of *Bam*HI fragment V (A). The numbers indicate nucleotides. The map below shows the expected length of runoff products if transcription begins near each promoter-like sequence. Transcription in either direction from the promoter-like sequence at nucleotide 1663 is shown. In B, products of in vitro transcription after cleavage of the *Bam*HI fragment V template DNA with *Bgl*II (lane 3) or *Sst*II (lane 5) are shown. Lane 4 shows the products of transcription from *Sst*II-cleaved *Bam*HI fragment V DNA in the presence of 0.8 μg of alpha-amanitin per ml. A diagram of the expected effect of cleavage of the template DNA on the size of the transcript initiated near the promoter-like sequence at nucleotide 1137 is also shown (B).

pected from the restriction endonuclease sites, cleavage of *Bam*HI fragment V with *Bgl*II had no effect on the length of the runoff product, whereas cleavage with *Sst*II shortened the runoff product to 1 kb (Fig. 4B). This indicates that the CCAAT-34 nucleotide-TATAAA sequence is part of a functional promoter. A small amount of 1.9-kb RNA was made without *Bam*HI fragment V. This endogenous template activity was unaffected by 0.8 μg of alpha-amanitin per ml.

S1 nuclease analysis of cytoplasmic RNA encoded by *Bam*HI fragments V, X, and H. The DNA sequences of *Bam*HI fragments V, X, and H which encode cytoplasmic polyadenylated RNA were mapped by S1 nuclease analysis with the strand-separated DNAs of plasmids pKH47-V, pKH47-X, and pKH47-H. The R strand of each fragment was hybridized with IB4 cytoplasmic polyadenylated RNA, and unhybridized DNA was degraded with S1 nuclease. The RNA-protected DNA was electrophoresed in denaturing agarose gels, blotted onto nitrocellulose, and detected by hybridization to ^{32}P-labeled EBV DNA fragments. The RNA protected a 1.75-kb segment of *Bam*HI fragment V and the full-length 3.1-kb *Bam*HI fragment V (Fig. 5). Minor protected DNAs of 1.75 to 3.1 kb were also detected. When only DNA to the right of the *Sst*I site (see map in Fig. 5) was used in the hybridization, the full-length protected DNA and the slightly smaller minor species were reduced in size to 2.4 kb, and the RNA still

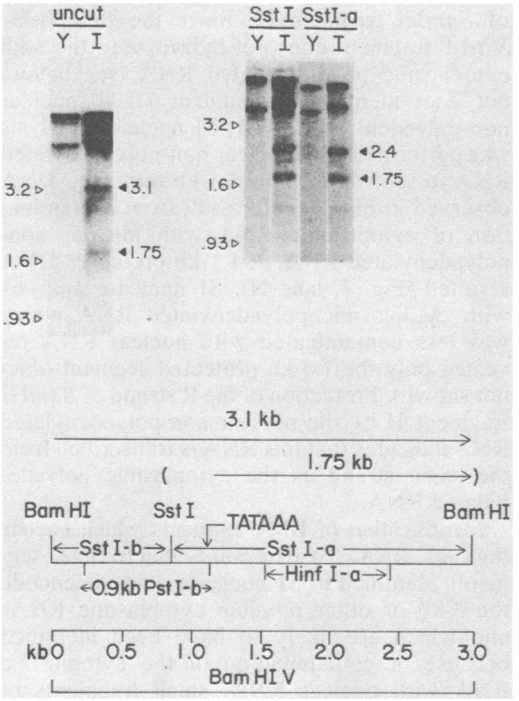

FIG. 5. S1 nuclease analysis of IB-4 cytoplasmic polyadenylated RNA transcribed from the R strand of EBV *Bam*HI fragment V. Hybridizations were done with yeast RNA as controls (Y) and with IB-4 cytoplasmic polyadenylated RNA (I). In *Sst*I hybridizations, plasmid pKH47-V was cleaved with *Sst*I before strand separation and hybridization to RNA. The *Sst*I site is shown in the map of *Bam*HI fragment V below. After *Sst*I restriction, EBV DNA sequences on both sides of the restriction site remained covalently attached to polydeoxyadenylic acid, were recovered by binding to oligo(dT)-cellulose, and were hybridized to RNA. In *Sst*I fragment a hybridizations, the DNA was also cleaved with a restriction endonuclease which cleaves within the vector so that only the *Sst*I fragment a part of *Bam*HI fragment V is recovered and hybridized to RNA. The blots shown were made from alkaline gels. The positions of marker DNAs (△) and DNAs protected by IB-4 RNA and not by yeast RNA (▲) are shown. Sizes are indicated in kb. A restriction enzyme map of *Bam*HI fragment V indicating the map position and size of protected DNAs is shown below. TATAAA indicates the promoter-like sequence at position 1137 in Fig. 3. The possible range of position of the 1.75-kb protected DNA segment is indicated by parentheses. The direction of transcription of the RNA is indicated by arrows. The 0.9-kb *Pst*I fragment b and *Hin*fI fragment a, used for hybridization to blots of cytoplasmic RNA in Fig. 7, are also shown.

protected a 1.75-kb segment. Thus, the RNA protecting the 1.75-kb segment is transcribed entirely from DNA to the right of the *Sst*I site, as would be expected if the RNA begins near the CCAAT-34 nucleotide-TATAAA sequence and extends to the rightward *Bam*HI site. Furthermore, the data indicate that the minor species and the full-length protected DNA differ only at their 5′ end, which is to be left of the *Sst*I site.

RNA protected a 0.56-kb segment of *Bam*HI fragment X and a family of DNA segments ranging in size from 1.1 kb to full-length *Bam*HI fragment X (Fig. 6). Cleavage of *Bam*HI fragment X DNA with restriction endonucleases *Xho*I and *Hpa*I, which cut progressively closer to its right end, progressively removed the higher-molecular-weight components of the family and had no effect on the size of the 0.56-kb protected segment. Identical results were obtained by using only the parts of *Bam*HI fragment X to the right of the *Xho*I and *Hpa*I sites. Cleavage of pKH47 with *Hin*dIII removes DNA to the left of the *Hin*dIII site in *Bam*HI fragment X (Fig. 6) while leaving the *Hin*dIII-b fragment

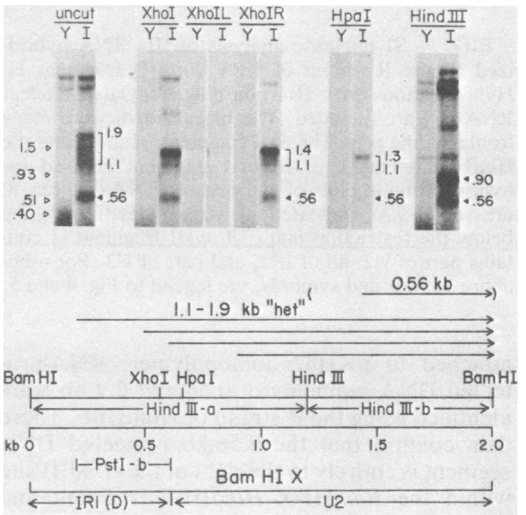

FIG. 6. S1 nuclease analysis of IB-4 cytoplasmic polyadenylated RNA transcribed from the R strand of EBV *Bam*HI fragment X. In the lanes labeled *Xho*I and *Hpa*I, DNA on both sides of the restriction sites was used in the hybridization to RNA. In the lanes labeled *Xho*IL and *Xho*IR, only DNA to the left or right, respectively, was used. Since *Hin*dIII also cleaves the vector, only *Hin*dIII fragment b was used in the lanes labeled *Hin*dIII. The brackets indicate a family of protected DNA segments of different sizes. The blots shown were made from formaldehyde agarose gels. *Bam*HI fragment X contains a partial copy of IR1, shown as IR1(D), and part of U2. The part of IR1 *Pst*I fragment b which is within *Bam*HI fragment X is indicated below the *Bam*HI fragment X restriction endonuclease map. The transition from IR1 to U2 is 123 base pairs to the right of the *Xho*I site shown. The *Hin*dIII fragments a and b of *Bam*HI fragment X used for hybridization to blots of cytoplasmic RNA (see Fig. 7) are shown. For further explanation, see legend to Fig. 4 and 5.

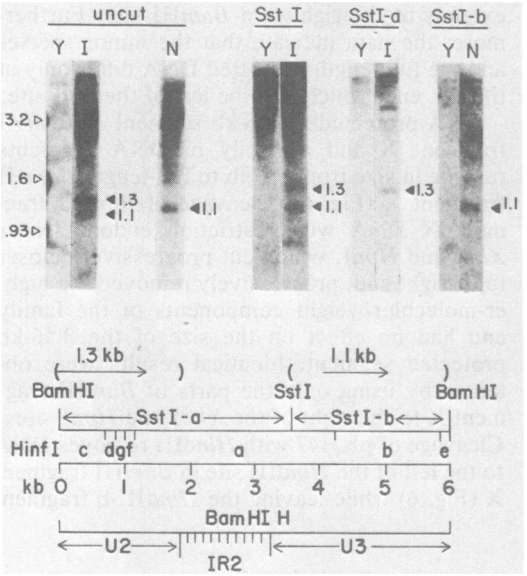

FIG. 7. S1 nuclease analysis of IB4 RNA hybridized to the R strand of EBV *Bam*HI fragment H. Hybridizations with IB4 non-polyadenylated nuclear RNA (N) are included. The blots shown were made from alkaline gels. The *Sst*I fragments a and b and the *Hin*fI fragments c of *Bam*HI fragment H used for hybridization to blots of cytoplasmic RNA (see Fig. 8) are shown. As indicated by the schematic diagram below the restriction maps, *Bam*HI fragment H contains part of U2, all of IR2, and part of U3. For other abbreviations and symbols, see legend to Fig. 4 and 5.

attached to pKH47 homopolymer. RNA-protected DNA segments of 0.56 and 0.9 kb were identified using the R strand of *Hin*dIII-b. These data confirm that the 0.56-kb protected DNA segment is entirely to the right of the *Hin*dIII site within the *Bam*HI-X–*Hin*dIII-b fragment and the family of larger protected DNA segments has a common right end at or near the right end of *Bam*HI fragment X. The data also indicate that the family of protected DNA segments arises because of heterogeneity beginning 1.1 kb from the right end of *Bam*HI fragment X and extending to the left end of *Bam*HI fragment X (Fig. 6).

Cytoplasmic polyadenylated RNA protected 1.3- and 1.1-kb segments of *Bam*HI fragment H DNA (Fig. 7). Use of the *Sst*I fragments a and b of *Bam*HI fragment H in hybridizations with RNA showed that the 1.3-kb protected DNA maps to the left of the *Sst*I site, and the 1.1-kb protected DNA maps to the right (see map of *Bam*HI fragment H in Fig. 7). ^{32}P-labeled *Hin*fI fragment c (the leftmost *Hin*fI fragment of *Bam*HI fragment H) hybridized to the 1.3-kb protected DNA (data not shown), confirming that this protected DNA maps near the left end of *Bam*HI fragment H. Since the *Bam*HI-H–*Sst*-I-b fragment does not hybridize to the 3-kb cytoplasmic polyadenylated RNA (see below) but does identify an abundant 1.1-kb nuclear non-polyadenylated RNA, S1 nuclease analysis was performed with nuclear non-polyadenylated RNA to see whether the 1.1-kb protected DNA observed in Fig. 7 could result from contamination of cytoplasmic RNA with nuclear non-polyadenylated RNA. A 1.1-kb protected DNA resulted (Fig. 7, lane N). S1 nuclease analysis with cytoplasmic polyadenylated RNA which was less contaminated with nuclear RNA revealed only the 1.3-kb protected segment (data not shown). Protection of the R strand of *Bam*HI fragment H by the nuclear non-polyadenylated RNA indicates that this RNA is transcribed from the same strand as the cytoplasmic polyadenylated RNA.

Identification of DNA segments which encode the 3-kb RNA. To determine which DNA segments identified in S1 nuclease analysis encode the 3-kb or other possible cytoplasmic RNAs and which are likely to have been identified because of contamination of the cytoplasmic RNA with nuclear RNA, small fragments of *Bam*HI fragments V, X, and H were hybridized to Northern blots of cytoplasmic polyadenylated or nuclear RNA (see maps of *Bam*HI fragments V, X, and H in Fig. 5 through 7). DNA from the *Hin*fI fragment a of *Bam*HI fragment V hybridized to the 3-kb RNA (Fig. 8); DNA from the

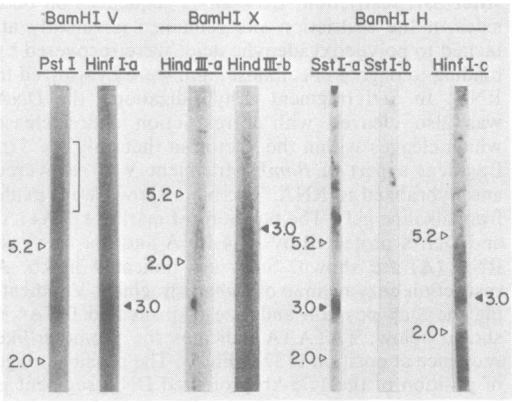

FIG. 8. Hybridization of fragments of *Bam*HI fragments V, X, and H to the 3-kb RNA on blots of IB-4 cytoplasmic polyadenylated RNA. The DNA fragments indicated above the lanes were labeled with ^{32}P and were hybridized to RNA blots. The fragments used are indicated on the maps in Fig. 4 through 6. Ribosomal RNA markers (△) and hybridization of the DNA probes to the 3-kb RNA (▲) are shown. Sizes are indicated in kb. The bracket indicates hybridization of the *Pst*I fragment b probe to nuclear RNAs which contaminated the cytoplasmic RNA preparation.

*Pst*I-b fragment of *Bam*HI fragment V did not, although it hybridized well to nuclear RNA (see Fig. 2) and identifies a small amount of nuclear polyadenylated RNA in the cytoplasmic polyadenylated RNA preparations. Since the *Pst*I fragment b sequences are not represented in the 3-kb cytoplasmic RNA but are represented in nuclear RNA, the full-length and 1.75- to 3.1-kb minor *Bam*HI-V fragments that were protected in the S1 nuclease analyses of DNA (see Fig. 5) cannot be exons of the 3-kb RNA and are likely to be part of contaminating nuclear RNAs. Similarly, the *Hin*dIII fragment b of *Bam*HI fragment X hybridizes to the 3 kb RNA, but the *Hin*dIII fragment a does not (Fig. 8). This indicates that the 0.56-kb segment of RNA transcribed from the *Hin*dIII fragment b (see Fig. 6) is probably part of the 3-kb RNA. The longer protected DNAs of *Bam*HI fragment X, in addition to being heterogeneous, contain the *Hin*dIII fragment a and *Pst*I fragment b sequences of *Bam*HI fragment X. Therefore, they cannot be exons of the 3-kb RNA since the *Hin*dIII-a and *Pst*I-b portions of *Bam*HI fragment X do not hybridize to the 3-kb RNA. Also, as would be expected from the S1 experiments, the leftward *Sst*I fragment a and *Hin*fI fragment c of *Bam*HI fragment H and the part of the *Hin*fI fragment a to the left of IR2 hybridize to the 3-kb RNA, whereas the rightward *Sst*I fragment b and IR2 do not (Fig. 7 and data not shown). *Eco*RI fragment J, which maps to the left of IR1 in U1, does not hybridize to any of the IR1-U2 RNAs.

Reconstruction experiments in which the hybridization of *Bam*HI-X–*Hin*dIII fragment b to the 3-kb RNA was compared with hybridization to known amounts of *Bam*HI-X under similar conditions indicate that there is approximately 0.0038 ng of the *Bam*HI-X part of the 3-kb RNA per 5 μg of IB4 cytoplasmic polyadenylated RNA. This estimate and the polyadenylated RNA yield per cell indicate that there are approximately three copies of the 3-kb RNA per cell.

DISCUSSION

The simplest model (Fig. 1) which would account for the accumulated data describing RNA from IR1 and U2 is that the CCAAT-34 nucleotide-TATAA sequence in IR1 is part of the functional promoter for transcription of the IR1-U2 R strand in latently infected cells. The smallest transcript would start in the last complete copy of IR1, extend through 0.9 kb of IR1, continue through the *Bam*HI site between *Bam*HI fragments V and X into the 0.6 kb of IR1 and 1.4 kb of U2 in *Bam*HI fragment X, and then continue through at least 1.3 kb of *Bam*HI fragment H U2. After polyadenylation at a site about 1.3 kb into *Bam*HI fragment H U2, this transcript would be approximately 5.3 kb. Other polyadenylated RNAs would begin in more leftward copies of IR1 and would vary in size upwards from 5 kb in 3-kb increments. The 1.1-kb U3 non-polyadenylated RNA could be a 3′ cleavage product of the putative primary transcript since it is also transcribed from the R strand of EBV DNA. However, no RNA encoded by IR2 has been detected. The 3-kb cytoplasmic polyadenylated RNA would be spliced from the large IR1-U2 polyadenylated RNAs and consist (3′ to 5′) of a polyadenylate tail (probably less than 0.2 kb), segments of 1.3 and 0.56 kb from *Bam*HI-H and -X U2 which may be continuous, and small exons as yet undetected from the left end of U2 and IR1, totaling 0.9 kb (Fig. 1). Alternatively the 1.75-kb RNA-protected IR1 DNA segment could encode an exon of the 3-kb RNA.

Data in support of this model include the following. (i) There are large nuclear RNAs which differ in length by approximately 3 kb (Fig. 2). IR1 is a 3-kb tandem repeat. S1 nuclease analysis shows that some RNA molecules include at least one full-length copy of IR1 (Fig. 5). (ii) RNA hybridizes only to the R strand of EBV IR1 or U2 DNA, indicating that most or all of the RNA is transcribed from left to right as shown in Fig. 1. (iii) There is a CCAAT-34 nucleotide-TATAAA sequence which is part of a promoter for in vitro transcription of the R strand of IR1 (8; Fig. 4). In latently infected cells, an RNA segment was detected which is complementary to and nearly equal in length to the part of the IR1 R strand 3′ to the CCAAT-34 nucleotide-TATAAA sequence (Fig. 5). This 1.75-kb segment could be the 5′ end of the precursor RNA. The cytoplasmic RNA preparations were contaminated with nuclear RNAs. The discrepancy in size between the 1.9-kb runoff product of in vitro transcription and the 1.75-kb RNA segment detected by S1 nuclease analysis with RNA from latently infected cells could be due to the different types of gels (methyl mercury versus formaldehyde) and different markers (RNA versus DNA size markers). Alternatively, the 1.75-kb RNA segment could be part of a partially or completely spliced RNA. The 1.75-kb segment is unlikely to be an exon of the 3-kb RNA since there are two other RNA-protected DNA segments totaling 1.9 kb (see below), and although the *Bam*HI-V–*Hin*fI-a part of the 1.75-kb DNA segment hybridized to the 3-kb RNA, *Hin*fI fragment e, the rightmost part of the 1.75-kb segment, did not hybridize to the 3-kb RNA (data not shown). This suggests that not all of the IR1 sequence to the right of the promoter becomes part of the 3-kb RNA. (iv) The 3-kb cytoplasmic polyadenylated RNA is a spliced RNA since it is encoded partly by

*Bam*HI-V–*Hin*fI-a and by *Bam*HI-H–*Hin*fI-a, which are 3.5 kb apart in the EBV genome (Fig. 1, 5, and 7), and the intervening *Bam*HI-X–*Hin*dIII-a fragment does not hybridize to the 3-kb RNA (Fig. 1 and 7 and data presented above). The IR1 sequence (8) reveals many potential splice donor and acceptor sites in the part of IR1 to the right of the putative promoter. (v) All IR1 and U2 DNA fragments tested, including the *Bam*HI-V–*Pst*I-b fragment (much of which is included in the *Bam*HI-X–*Hin*dIII-a fragment), hybridize to the large nuclear RNAs, as would be expected if these RNAs are unspliced or partially spliced precursors to the 3-kb cytoplasmic RNA (Fig. 1). (vi) Cytoplasmic polyadenylated RNA is complementary to possibly continuous 0.56- and 1.3-kb segments of the R strand of *Bam*HI fragment X and H U2 DNA. Furthermore, these DNA segments hybridize to the 3-kb cytoplasmic polyadenylated RNA. (vii) The large nuclear RNAs and the 3-kb cytoplasmic RNA do not extend into IR2 or U3; thus, they have similar 3′ ends.

RNAs having common 3′ and variable 5′ domains were detected from IR1 and U2 (Fig. 5 and 6). Since the cytoplasmic preparations in which these RNAs were detected were contaminated with nuclear RNA, these RNAs could be partially spliced precursors to the 3-kb RNA. Alternatively, they could be mature, less-abundant RNAs which are spliced differently from the 3-kb RNA. Partially or anomolously spliced globin precursor RNAs have been detected in the nuclei of Friend erythroleukemia cells (13). The variably spliced EBV IR1-U2 polyadenylated RNAs are, in aggregate, as abundant as the putative fully spliced RNAs. These variably spliced molecules could in aggregate account for the seemingly discrepant finding that there are fewer copies of the 3-kb RNA per latently infected transformed cell than of the RNA specified by the EBV U5 region (van Santen et al., manuscript in preparation), whereas cytoplasmic polyadenylated RNA hybridizes more to the IR1-U2 region than to the U5 region.

The model proposed for generation of the 3-kb EBV RNA is similar to that proposed for the formation of polyoma virus late messenger RNA (26, 47). The 5,560-base pair circular polyoma virus DNA encodes giant RNA molecules which are tandemly repeated whole genomic transcripts (1, 26, 47). Multiple copies of a 57-nucleotide sequence which is present only once in polyoma virus DNA are present at the 5′ end of polyoma late messenger RNAs (26, 47). Each copy of the 57-nucleotide repeat is spliced from a complete genome transcript (47).

Since the P3HR-1 virus is deleted for U2 and IR2 and is unable to initiate growth transformation (7, 17, 18, 24, 38, 40), the IR1-U2 RNA is believed to be important in initiating or maintaining latent infection or growth transformation. The IR1-U2 RNA would, therefore, be expected to be a constant feature of latent growth-transforming infection. However, the U2 region which encodes 1.9 kb of the 3′ end of the IR1-U2 RNA varies extensively among transformation-competent EBV isolates (11, 23).

A low-abundance 2.0-kb nuclear but not cytoplasmic polyadenylated RNA was also identified in this study to be encoded by the right part of *Bam*HI fragment H. A more abundant 1.9-kb polyadenylated early RNA encoded by *Bam*HI fragment H has been previously detected in the cytoplasm of productively infected B95-8 cells (20). A small amount of this RNA may be transcribed and polyadenylated but not transported in latent infection.

ACKNOWLEDGMENTS

We are grateful for the gift of recombinant EBV DNAs from Timothy Dambaugh and Victor Razkowski and for the assistance of Julia Dowling in tissue culture. Initial in vitro transcription experiments with IR1 DNA were done by Timothy Dambaugh and Roberto Weinman.

This research was supported by Public Health Service grants CA 19264 and CA 17281 from the National Institutes of Health and by grant MV 32G from the American Cancer Society. E.K. is a faculty research awardee of the American Cancer Society. V.v.S. was supported by a Public Health Service National Research Service Award CA 09267 from the National Institutes of Health. M.H. is supported by Public Health Service grant AI 07182 from the National Institutes of Health.

LITERATURE CITED

1. **Acheson, N. H., E. Buetti, K. Scherrer, and R. Weil.** 1971. Transcription of the polyoma virus genome: synthesis and cleavage of giant late polyoma-specific RNA. Proc. Natl. Acad. Sci. U.S.A. **68**:2231–2235.
2. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymydylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69**:1408–1412.
3. **Bailey, J., and N. Davidson.** 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. **70**:75–85.
4. **Berger, S., and C. Birkenmeier.** 1979. Inhibition of intractable nucleases with ribonucleoside vanadyl complexes; isolation of RNA from resting lymphocytes. Biochemistry **18**:5143–5149.
5. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
6. **Berk, A. J., and P. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
7. **Bornkamm, G. W., J. Hudewentz, U. K. Freese, and U. Zimber.** 1982. Deletion of the nontransforming Epstein-Barr virus strain P3HR-1 causes fusion of the large internal repeat of the DS_L region. J. Virol. **43**:952–968.
8. **Cheung, A., and E. Kieff.** 1982. Long internal direct repeat in Epstein-Barr virus DNA. J. Virol. **44**:286–294.
9. **Dambaugh, T., C. Beisel, M. Hummel, W. King, S. Fennewald, A. Cheung, M. Heller, N. Raab-Traub, and E. Kieff.** 1980. Epstein-Barr virus DNA VII: molecular cloning and detailed mapping of EBV (B95-8) DNA. Proc. Natl. Acad. Sci. U.S.A. **77**:2999–3003.
10. **Dambaugh, T., E. Kieff, F. K. Nkrumah, and R. J. Biggar.**

1979. Epstein-Barr virus RNA. IV. Viral RNA in Burkitt tumor tissue. Cell **16**:313–322.
11. **Dambaugh, T. R., and E. Kieff.** 1982. Identifiction and nucleotide sequences of two similar tandem direct repeats in Epstein-Barr virus DNA. J. Virol. **44**:823–833.
12. **Davis, R., D. Botstein, and J. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Donaldson, D., A. McNab, G. Rovera, and P. Curtis.** 1982. Nuclear precursor molecules of the two B-globin mRNAs in Friend Erythroleukemia cells. J Biol. Chem. **257**:8655–8660.
14. **Gerber, P., J. Whang-Peng, and J. H. Monroe.** 1969. Transformation and chromosome changes induced by Epstein-Barr virus in normal human leukocyte cultures. Proc. Natl. Acad. Sci. U.S.A. **63**:740–747.
15. **Goodman, H., and R. MacDonald.** 1979. Cloning of hormone genes from a mixture of cDNA molecules. Methods Enzymol. **68**:75–90.
16. **Hayashi, S.** 1980. A cloning vehicle suitable for strand separation. Gene **11**:109–115.
17. **Hayward, S. D., S. G. Lazarowitz, and G. S. Hayward.** 1982. Organization of the Epstein-Barr virus DNA molecule. II. Fine mapping of the boundaries of the internal repeat cluster of B95-8 and identification of additional small tandem repeats adjacent to the HR-1 deletion. J. Virol. **43**:201–212.
18. **Heller, M., T. Dambaugh, and E. Kieff.** 1981. Epstein-Barr Virus DNA. IX. Variation among viral DNAs from producer and nonproducer infected cells. J. Virol. **38**:632–648.
19. **Heller, M., V. van Santen, and E. Kieff.** 1982. Simple repeat sequence in Epstein-Barr virus DNA is transcribed in latent and productive infections. J. Virol. **44**:311–320.
20. **Hummel, M., and E. Kieff.** 1982. Epstein-Barr virus RNA. VIII. Viral RNA in permissively infected B95-8 cells. J. Virol. **43**:262–272.
21. **Kawai, Y., M. Nonoyama, and J. S. Pagano.** 1973. Reassociation kinetics for EBV DNA: nonhomology to mammalian DNA and homology of viral DNA in various diseases. J. Virol. **12**:1006–1012.
22. **Kieff, E., T. Dambaugh, M. Heller, W. King, A. Cheung, V. van Santen, M. Hummel, C. Beisel, S. Fennewald, K. Hennessy, and T. Heineman.** 1982. Biology and chemistry of Epstein-Barr virus. J. Infect. Dis. **146**:506–517.
23. **King, W., T. Dambaugh, M. Heller, J. Dowling, and E. Kieff.** 1982. Epstein-Barr virus DNA. XII. A variable region of the EBV genome is included in the P3HR-1 deletion. J. Virol. **43**:979–986.
24. **King, W., A. L. Thomas-Powell, N. Raab-Traub, M. Hawke, and E. Kieff.** 1980. Epstein-Barr virus RNA. Viral RNA in a restringently infected, growth-transformed cell line. J. Virol. **36**:506–518.
25. **King, W., V. van Santen, and E. Kieff.** 1981. Epstein-Barr virus RNA. VI. Viral RNA in restringently and abortively infected Raji cells. J. Virol. **38**:649–660.
26. **Legon, S., A. J. Flavell, A. Cowie, and R. Kamen.** 1979. Amplification in the leader sequence of late polyoma virus mRNAs. Cell **16**:373–388.
27. **Lehrach, H., D. Diamond, J. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
28. **Lindahl, T., A. Adams, G. Bjursell, G. W. Bornkamm, C. Kaschka-Dierich, and U. Jehn.** 1976. Covalently closed circular duplex DNA of Epstein-Barr virus in human lymphoid cell line. J. Mol. Biol. **102**:511–530.
29. **Manley, J., A. Fire, A. Cano, P. Sharp, and M. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a soluble whole cell extract. Proc. Natl. Acad. Sci. U.S.A. **77**:3855–3859.
30. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
31. **McDonnell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119–146.
32. **Miller, G., J. Robinson, L. Heston, and M. Lipman.** 1974. Differences between laboratory strains of Epstein-Barr virus based on immortalization, abortive infection and interference. Proc. Natl. Acad. Sci. U.S.A. **71**:4006–4010.
33. **Nilsson, K.** 1979. The nature of lymphoid cell lines and their relationship to the virus, p. 225–281. *In* M. Epstein and B. Achong (ed.), The Epstein-Barr virus. Springer-Verlag KG, Berlin.
34. **Nonoyama, M., and J. S. Pagano.** 1972. Separation of Epstein-Barr virus DNA from large chromosomal DNA in non-virus producing cells. Nature (London) New Biol. **238**:169–171.
35. **Orellana, T., and E. Kieff.** 1977. Epstein-Barr virus-specific RNA. II. Analysis of polyadenylated viral RNA in restringent, abortive, and productive infections. J. Virol. **22**:321–330.
36. **Pope, J. H., M. K. Horne, and W. Scott.** 1968. Transformation of fetal human leukocytes in vitro in filtrates of a human leukemic cell line containing herpes-like virus. Int. J. Cancer **3**:857–766.
37. **Powell, A. L. T., W. King, and E. Kieff.** 1979. Epstein-Barr virus-specific RNA. III. Mapping of the DNA encoding viral RNA in restringent infection. J. Virol. **29**:261–274.
38. **Pritchett, R. F., S. D. Hayward, and E. Kieff.** 1975. DNA of Epstein-Barr virus. I. Comparative studies of the DNA of Epstein-Barr virus from HR-1 and B95-8 cells: size, structure, and relatedness. J. Virol. **15**:556–569.
39. **Pritchett, R. F., M. Pedersen, and E. Kieff.** 1976. Complexity of EBV homologous DNA in continuous lymphoblastoid cell lines. Virology **74**:227–231.
40. **Raab-Traub, N., R. Pritchett, and E. Kieff.** 1978. DNA of Epstein-Barr virus. III. Identification of restriction enzyme fragments that contain DNA sequences which differ among strains of Epstein-Barr virus. J. Virol. **27**:388–398.
41. **Ragona, G., I. Ernberg, and G. Klein.** 1980. Induction and biological characterization of the Epstein-Barr virus (EBV) carried by the Jijoye lymphoma line. Virology **101**:553–557.
42. **Reedman, B. M., and G. Klein.** 1973. Cellular localization of an Epstein-Barr virus (EBV)-associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. Int. J. Cancer **11**:499–520.
43. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase. J. Mol. Biol. **113**:237–251.
44. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:502–517.
45. **Svedmyr, E., and M. Jondal.** 1975. Cytotoxic effector cells specific for B cell lines transformed by Epstein-Barr virus are present in patients with infectious mononucleosis. Proc. Natl. Acad. Sci. U.S.A. **72**:1622–1666.
46. **Thomas, P. A.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
47. **Treisman, R.** 1980. Characterization of polyoma late mRNA leader sequences by molecular cloning and DNA sequence analysis. Nucleic Acids Res. **8**:4867–4888.
48. **van Santen, V., A. Cheung, and E. Kieff.** 1981. Epstein-Barr virus RNA. VII. Size and direction of transcription of virus specified cytoplasmic RNA in a cell line transformed by EBV. Proc. Natl. Acad. Sci. U.S.A. **78**:1930–1934.
49. **Wellauer, P., and I. Dawid.** 1973. Secondary structure maps of RNA: processing of HeLa ribosomal RNA. Proc. Natl. Acad. Sci. U.S.A. **70**:2827–2831.
50. **zur Hausen, H., and H. Schulte-Holthausen.** 1970. Presence of EB virus nucleic acid homology in a "virus free" line of Burkitt's tumor cells. Nature (London) **227**:245–248.