JOURNAL OF VIROLOGY, May 1992, p. 2837–2845
0022-538X/92/052837-09$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 66, No. 5

Regulation of the Epstein-Barr Virus DNA Polymerase Gene

FRANK B. FURNARI,[1,2] MELISSA D. ADAMS,[2] AND JOSEPH S. PAGANO[1,2,3]*

Departments of Microbiology and Immunology[1] and Medicine[3] and UNC Lineberger Comprehensive Cancer Center,[2] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Received 2 December 1991/Accepted 12 February 1992

The gene (*pol*) encoding the Epstein-Barr virus (EBV) DNA polymerase is a member of the "early" class of viral genes which are expressed shortly after activation of latent virus infection. First, mRNA from the EBV-producing cell line, B95-8, treated with 12-*O*-tetradecanoylphorbol-13-acetate and sodium butyrate to induce lytic replication and expression of this gene was analyzed. Northern (RNA) analysis revealed a message of 3.7 kb found only in induced cells. 5′ mapping of *pol* mRNA by S1 nuclease and primer extension analyses indicates that transcription initiates at tightly clustered sites within a G+C-rich region 126 bp upstream of the open reading frame. The same initiation region was identified in two other EBV-infected cell lines, P3HR1 and Raji, after induction. Second, a 1.29-kb genomic fragment containing this region, when cloned upstream of the chloramphenicol acetyltransferase reporter gene, demonstrated promoter activity in lymphoid cells cotransfected with pEBV-RZ, a genomic expression construct that includes genes for the EBV immediate-early transactivator proteins, BZLF-1 and BRLF-1. Within the upstream 1.29-kb sequence, two regions of 140 bp and 101 bp appear to be needed for promoter activity. These results demonstrate that unlike most EBV genes studied thus far, the *pol* gene contains multiple transcriptional start sites. The upstream regulatory region of the promoter for the *pol* gene does not contain canonical promoter elements such as TATA and CAAT boxes and, furthermore, is not constitutively active but requires transactivation by two or more viral proteins.

Infection with the Epstein-Barr virus (EBV) results in either latent or productive infection. In latent infection, the episomal form of the genome and restricted expression of the viral genes are the hallmarks. The episomes are replicated by the host cell DNA polymerase, resulting in orderly duplication of limited copy numbers of episomes without viral replication and without cellular destruction. In contrast, in productive EBV infection, large numbers of linear genomes are replicated and encapsidated, mediated by expression of immediate-early, early, and late viral genes, which include a virally encoded DNA polymerase and its cofactors. Thus, there are fundamental differences between the virus-cell relations in latent and productive infections.

In the latent state, restriction of viral gene expression includes suppression of the expression of viral DNA polymerase, which has not been detected (17, 37, 39). However, expression of the gene can be induced by nucleoside analogs such as iododeoxyuridine, phorbol esters, and superinfection and other agents (12, 17, 26, 37, 41). The mechanism whereby viral DNA polymerase expression is controlled and induced is central to understanding not only how latency is disrupted and replication is activated, but it is also relevant to how latency is maintained. The regulation of expression at the level of transcription of the EBV DNA polymerase gene, clearly a crucial aspect of the interplay between productive and latent infection, has not been examined.

The EBV DNA polymerase is distinguishable from host cell α and β polymerase by its salt-stimulated activity and sensitivity to inhibitors of viral DNA synthesis, such as nucleoside and pyrophosphate analogs (11, 12); the latter property is the basis for sensitivity of EBV replication to several antiviral drugs. The coding sequence (the fifth leftward open reading frame [ORF] of the *Bam*HI A fragment [BALF-5]) for the polymerase has recently been cloned and expressed in vitro (29, 36) and in *Escherichia coli* (45a), and the properties of the enzyme are under study. In the Raji cell line, salt-stimulated DNA polymerase activity is not spontaneously expressed. However, after 12-*O*-tetradecanoylphorbol-13-acetate (TPA) exposure, such activity can be detected (41). In virus-producing cell lines such as P3HR1 and B95-8, salt-stimulated DNA polymerase activity is already present at appreciable levels before stimulation with TPA. These levels can be substantially increased by treatment with TPA through the conversion of latently infected cells that comprise the majority of the population to virus-producing cells (12, 35).

Most investigations of gene regulation in EBV have centered on maintenance of latency and disruption of this state by the expression of immediate-early genes encoding BZLF-1 (Z) and BRLF-1 (R) (9, 10, 20–22, 27, 48). However, there has been no attention to understanding events governing the progression of the lytic cycle to the involvement of the polymerase gene. In virus-producing cell lines, polymerase activity and virus production are increased after stimulation with TPA, induction by surface immunoglobulin cross-linking, or treatment with various other viral inducers, depending on the cell line (12, 37, 41, 47).

In this report, expression of this early gene is characterized by mapping transcriptional initiation and by analyzing the promoter activity of the gene. The data indicate that unlike most EBV genes studied thus far, this gene contains multiple transcriptional start sites and that its promoter does not contain a canonical TATA box. The promoter, while it is constitutively inactive, can be transactivated in lymphoid cells by cotransfection with a construct that expresses immediate-early gene products.

MATERIALS AND METHODS

Cell lines. B95-8 (38) is an EBV-positive marmoset B-cell line grown in RPMI medium supplemented with 8% fetal calf

* Corresponding author.

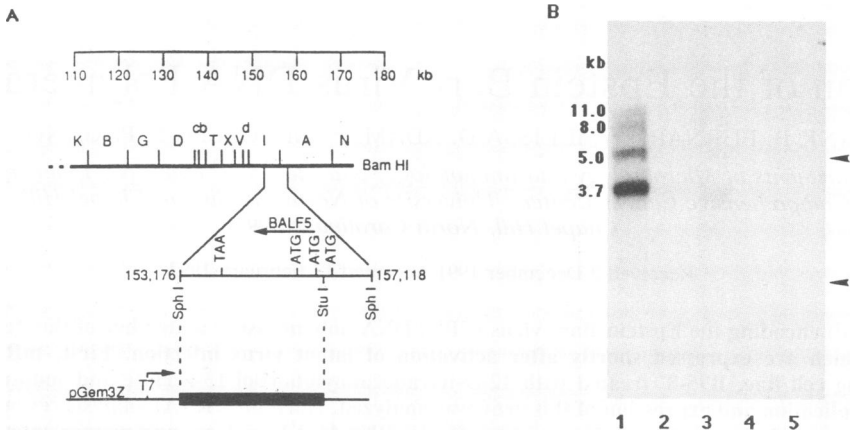

FIG. 1. Schematic diagram of the DNA *pol* gene and Northern blot (RNA) analysis. (A) B95-8 genome location of *pol* ORF (BALF-5) in *Bam*HI I/A is, as shown, flanked by *Sph*I sites at positions 153,176 and 157,118. The fragment *Sph*I at position 153,176 to *Stu*I at position 156,695 was subcloned into pGEM3Z and linearized with *Hin*dIII to generate runoff antisense *pol* transcripts by using T7 RNA polymerase. (B) The probe diagrammed in panel A was hybridized to RNA from B95-8 cells induced with TPA and sodium butyrate. Lanes: 1, 5 μg of poly(A)$^+$; 2, 20 μg of poly(A)$^-$; 3, 20 μg of total RNA, induced with TPA; 4, 20 μg of total RNA, uninduced; 5, 5 μg of poly(A)$^+$ from EBV-negative Jurkat cells. Arrowheads indicate 28S and 18S rRNA positions.

serum. P3HR1 (21) and Raji (44) are EBV-positive cell lines derived from Burkitt's lymphomas and grown in RPMI medium supplemented with 10% fetal calf serum. Jurkat is an EBV-negative T-cell line maintained in RPMI medium supplemented with 10% fetal calf serum. All cells were maintained at 37°C in a 5% CO_2 environment.

Induction of virus replication. The EBV lytic cycle was induced by incubating B95-8 cells with TPA and sodium butyrate for 48 h at 30 ng/ml and 5 mM (final concentrations), respectively. Virus production was monitored by indirect immunofluorescence for EA/VCA antigens by using high-titer nasopharyngeal carcinoma patients' sera and fluorescein isothiocyanate-conjugated goat antihuman antibody.

Transfections and chloramphenicol acetyltransferase (CAT) assays. Plasmid DNA was amplified from *E. coli* and purified over two sequential cesium chloride gradients. For each sample, 10 μg of reporter and 10 μg of transactivator plasmid DNA were electroporated into 10^7 cells at 1,500 V (40), with the University of Wisconsin Zapper electroporation unit. Cells were suspended in 10 ml of RPMI 1640 medium supplemented with 10% fetal calf serum and incubated for 48 h at 37°C in 5% CO_2. Extracts of transfected cells were prepared by washing cell pellets twice in phosphate-buffered saline solution, suspending them in 200 μl of 0.25 M Tris-HCl (pH 7.5), and freeze-thawing four times. Reactions were carried out by incubating each sample with acetyl coenzyme A and [^{14}C]chloramphenicol at 37°C for 1 h (18). Reaction products were separated by thin-layer chromatography, visualized by autoradiography, and quantitated by scintillation counting. Percent acetylation was calculated as the ratio of acetylated reaction products to the entire sample.

RNA isolation and Northern analysis. Total cellular RNA from B95-8, P3HR1, and Raji cells was prepared 48 h postinduction, and polyadenylated RNA was selected (1, 45). For Northern (RNA) analysis, 20 μg of total cellular RNA or 5 μg of poly(A)$^+$ RNA was denatured for 15 min at 65°C in the presence of formaldehyde and resolved on a 1% agarose gel containing 0.66 M formaldehyde, 20 mM 3-(*N*-morpholino)propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA. Following electrophoresis, RNA was blotted onto nitrocellulose filters, fixed by heat, and prehybridized for 2 h at 59°C in prehybridization solution containing 50% formamide, 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM sodium phosphate (pH 7.0), 2.5× Denhardt's solution, and 200 μg of salmon-sperm DNA per ml. Hybridization was performed in the same solution at 59°C. Filters were washed at 65°C in 0.1× SSC–0.1% sodium dodecyl sulfate and treated with RNase A (13).

S1 nuclease and primer extension analyses. For S1 nuclease protection assays of viral RNA, mRNA was prepared, as described above, from TPA-induced and uninduced B95-8, P3HR1, and Raji cells. S1 probes were prepared by subcloning EBV fragment *Sph*I (at position 153,176) to *Stu*I (at position 156,695) into phagemid pBS+ (Stratagene) and generating single-stranded DNA template by using helper phage M13K07. A 39-base ^{32}P-end-labelled primer (positions 156,801 to 156,840) was annealed to the template and extended with Klenow enzyme. After digestion with *Sph*I, the strands were separated on a denaturing polyacrylamide-urea gel, and the appropriate size band was recovered. Five micrograms of poly(A)$^+$ was hybridized for 12 h with 5×10^4 cpm of the end-labelled probe (see Fig. 2) in 30 μl of 40 mM PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (pH 6.4)]–1 mM EDTA (pH 8.0)–0.4 M NaCl–80% formamide at 45°C. S1 digestion was carried out by addition of 800 U of S1 nuclease in 300 μl of 0.28 M NaCl–0.05 M sodium acetate (pH 4.5)–4.5 mM $ZnSO_4$–20 μg of single-stranded calf-thymus DNA per ml. Reactions were terminated by addition of 80 μl of a mixture containing 4 mM ammonium acetate and 50 mM EDTA (pH 8.0). Products were resolved on 7 M urea–6% polyacrylamide gels and visualized by autoradiography.

For S1 nuclease protection assays performed on CAT RNA, an internally labelled single-stranded DNA probe complementary to plasmid pPolCAT was made. Single-stranded template DNA was prepared as described above and annealed to a 20-base primer that hybridized from 133 to 153 bases downstream of the RNA start site region (see Fig. 5). The primer was extended and labelled with Klenow enzyme and [^{32}P]dCTP. After digestion with *Sph*I, the probe was isolated as described above. Polyadenylated RNA from Raji cells was prepared 48 h posttransfection, and 5 μg was

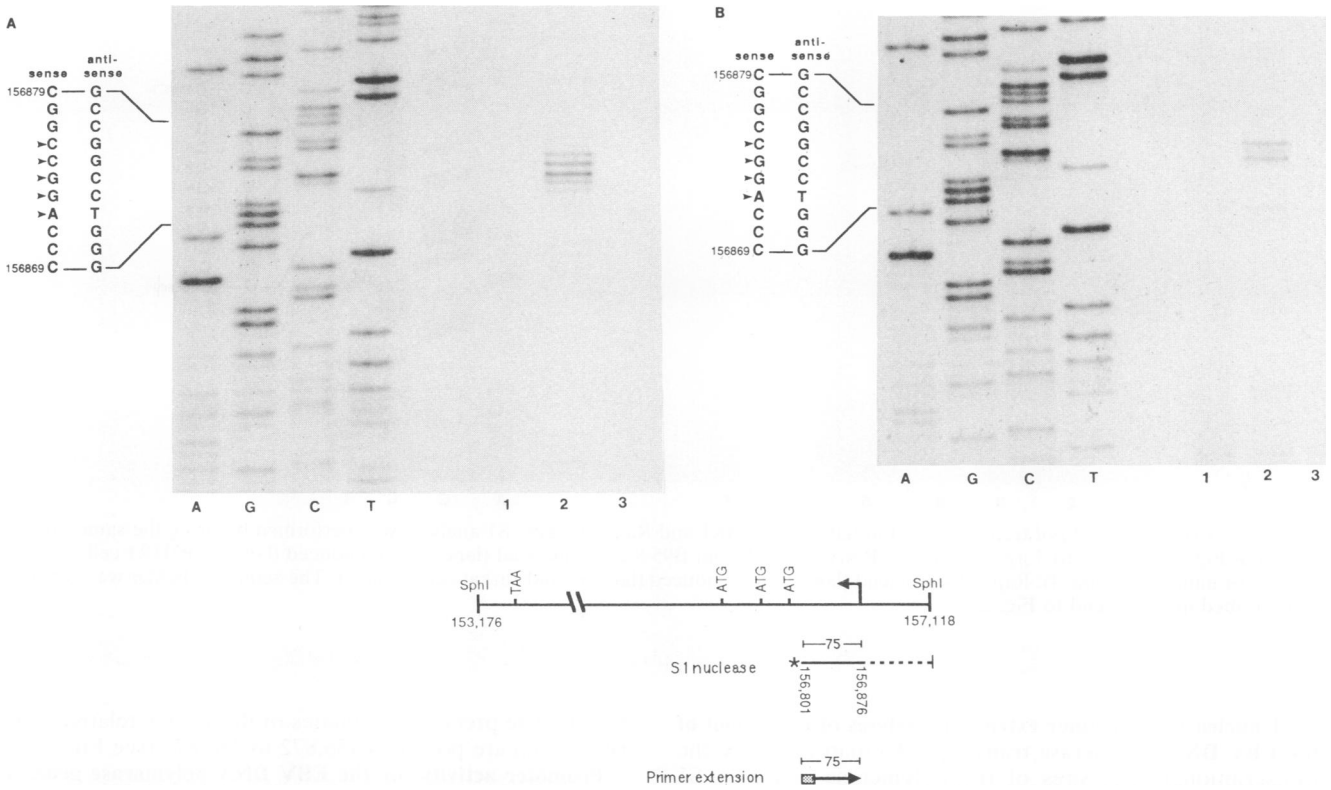

FIG. 2. S1 nuclease and primer extension analyses of the 5′ end of EBV DNA *pol* mRNA. (A) For S1 analysis, the ^{32}P-end-labelled probe was annealed to 5 μg of poly(A)$^+$ RNA isolated from B95-8, uninduced (lane 1) or induced (lane 2), or Jurkat cells (lane 3). (B) For primer extension analysis, the ^{32}P-end-labelled oligonucleotide was annealed to 5 μg of poly(A)$^+$ RNA isolated from B95-8 cells, uninduced (lane 1) or induced (lane 2), or Jurkat cells (lane 3). Hybridized mixtures were either treated with S1 nuclease or reverse transcribed with avian myeloblastosis virus reverse transcriptase, and products were resolved as described in Materials and Methods. The sequence ladder was generated by using the same oligonucleotide primer and single-stranded DNA from a genomic *pol* subclone shown in Fig. 1A to determine precisely the site of transcription initiation. S1 probe (positions 156,801 to 157,118) and oligonucleotide primer (positions 156,801 to 156,840) used in mapping are as diagrammed.

hybridized at 30°C to 10^5 cpm of the labelled probe. S1 digestion was carried out by addition of 100 U of S1 nuclease under conditions described above.

For primer extension analysis, 5×10^4 cpm of ^{32}P-end-labelled primer (see Fig. 2) was annealed as described above, precipitated, and resuspended in 30 μl of reverse transcriptase buffer containing 50 mM Tris (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, and 1 mM dNTPs. Reactions were carried out with 30 U of avian myeloblastosis virus reverse transcriptase at 42°C for 1 h, and products were resolved as described for S1 analysis.

Plasmid constructs. pBS.CAT was constructed by isolating the 1.7-kb *Bgl*II-*Bam*HI CAT gene of pCAT3M (32) and cloning it into the *Bam*HI site of pBS+ (Stratagene). Subsequently, pPolCAT was constructed by isolating a 1.29-kb *Bal*I restriction fragment (EBV coordinates from positions 156,859 to 158,149) and cloning it into the *Hin*cII site directly upstream of CAT. Constructs containing deletions of this fragment are described below.

RESULTS

Induction of EBV DNA polymerase expression in B95-8 cells. To optimize for EBV DNA polymerase expression, EBV-producing B95-8 cells were induced with TPA and sodium butyrate so that a high percentage of cells entered the lytic phase. Virus production was monitored over the course of 72 h by indirect immunofluorescence with the use of sera which contained high titers of EBV antibodies from patients with nasopharyngeal carcinoma. With this induction method, the percentage of cells positive for replicative antigens (EA/VCA) (30 to 35%) was greatest between 48 and 72 h (data not shown).

RNA from the 48-h point was used for a Northern analysis performed with strand-specific ^{32}P-labelled RNA probe antisense to the polymerase ORF (4). This probe was prepared from a genomic fragment encompassing the coding region of the polymerase gene corresponding to the positions from 153,176 to 156,695 subcloned into pGem3Z (Promega) (Fig. 1A). The expected minimal size of the polymerase mRNA according to the length of the coding region is ~3 kb, and a message of 3.7 kb was detected with this probe only in induced cells (Fig. 1B, lanes 1 and 3). Although approximately 5% of uninduced B95-8 cells were spontaneously producing virus, the sensitivity of the probe did not permit detection of polymerase message in untreated cells. In this induction, larger transcripts of 5, 8 and ~11 kb were also detected (Fig. 1, lane 1). These transcripts could represent read-through messages from an upstream ORF, in particular, BALF-4, which terminates 63 nucleotides downstream of the first *pol* ATG, or unprocessed *pol* mRNA.

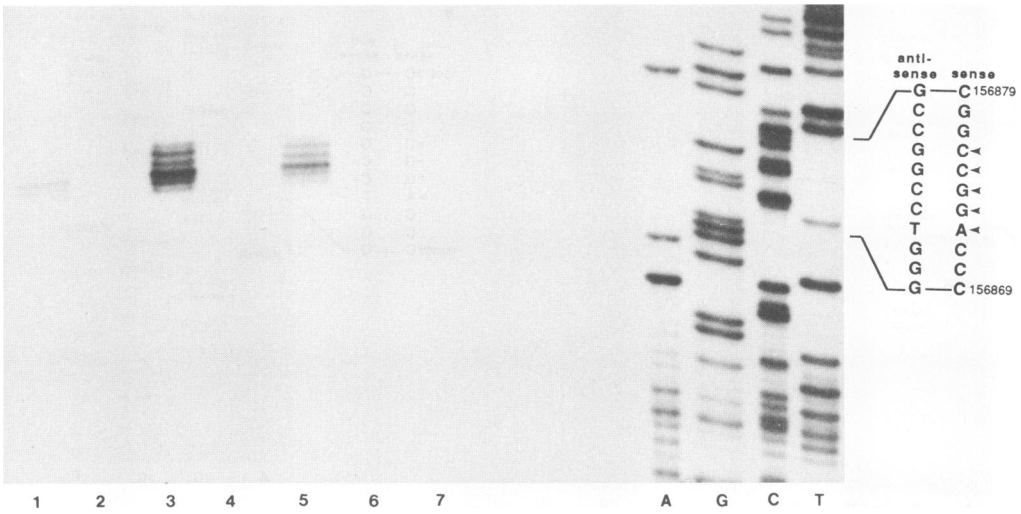

FIG. 3. Comparison of *pol* transcriptional initiation in P3HR1 and Raji cell lines. S1 analysis was performed by using the same probe as shown in Fig. 2 annealed to 5 μg of poly(A)$^+$ RNA isolated from B95-8 cells, induced (lane 1) or uninduced (lane 2); P3HR1 cells, induced (lane 3) or uninduced (lane 4); Raji cells, induced (lane 5) or uninduced (lane 6); and Jurkat cells (lane 7). The sequence ladder was generated as described in the legend to Fig. 2.

S1 nuclease and primer extension analyses of the 5′ end of the EBV DNA polymerase transcript. To map precisely the transcriptional start sites of the polymerase gene, an S1 nuclease protection assay was performed. A ^{32}P-end-labelled single-stranded DNA probe (coordinates from positions 156,801 to 157,118) antisense to polymerase mRNA was synthesized and annealed to polyadenylated RNA from induced cells at the 48-h point (Fig. 2). A sequence ladder corresponding to the probe was used to identify the points of discontinuity between probe and RNA. Protection of probe was not detected with mRNA from uninduced B95-8 cells or from Jurkat cells, an EBV-negative T-cell line (Fig. 2A, lanes 1 and 3). Other S1 analyses were performed with longer probes extending into the ORF downstream of the first in-frame ATG (coordinate at 156,746), all of which identified the same tight cluster of start sites (data not shown).

As a confirmation for the S1 nuclease analyses results, primer extension analysis was performed with the same end-labelled oligonucleotide (coordinates from positions 156,801 to 156,840) used to synthesize the S1 probe (Fig. 2). As shown in the corresponding sequence ladder, the primer extension results agreed with the S1 data, identifying the identical transcriptional initiation region (Fig. 2B) and making it unlikely that the S1 nuclease assay was recognizing an RNA splice junction.

The start sites in TPA-induced P3HR1 cells and Raji cells were also determined by S1 nuclease analyses by using the same probe as that used for Fig. 2. The *pol* transcriptional start sites in the two additional lines were essentially the same as those in B95-8 cells in both this and the previous analysis (Fig. 3).

Sequencing of cDNA clones generated from B95-8 TPA-induced RNA as well as RNA-based polymerase chain reaction analyses all further confirmed the same region of clustered start sites (data not shown).

Thus, transcription of the EBV DNA polymerase gene initiates 126 bp directly upstream of the ORF. The results indicate that multiple start sites are used within a G+C-rich region. The precise coordinates of these sites relative to the B95-8 map are positions 156,872 to 156,877 (see Fig. 7).

Promoter activity for the EBV DNA polymerase gene. To assay for promoter activity within this mapped region, a 1.29-kb genomic fragment corresponding to *Bal*I sites 156,859 to 158,149 was cloned upstream of the CAT gene in pBS+ and designated pPolCAT (Fig. 4). The EBV fragment in the construct was sequenced and had neither a TATA box nor a CAAT box; however, there appeared to be an SP1 motif (GGGCGG) within the transcriptional initiation region between coordinates 156,878 and 156,883 (see Fig. 7). When

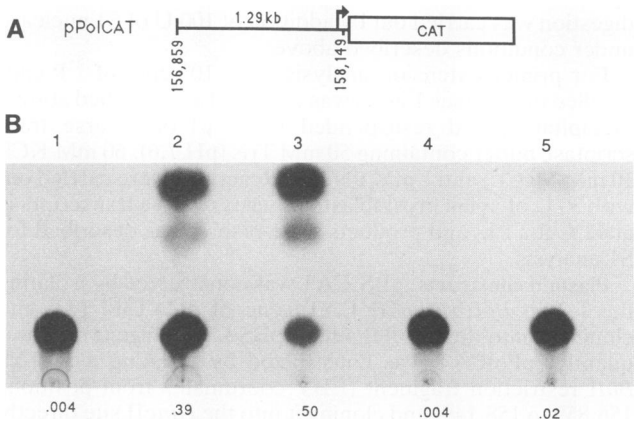

FIG. 4. Promoter activity for the EBV DNA *pol* gene in Raji cells. (A) Schematic representation of pPolCAT showing genomic *Bal*I fragment (coordinates 156,859 to 158,149) cloned upstream of the CAT gene in pBSCAT. (B) pPolCAT was transfected into Raji cells, a latently infected cell line, and assayed for CAT activity. Lanes: 1, pPolCAT; 2, pPolCAT cotransfected with pEBV-RZ; 3, positive-control plasmid, RSVCAT; 4, negative-control plasmid, pBSCAT vector; 5, pBSCAT cotransfected with pEBV-RZ. The percent conversion of chloramphenicol to its acetylated products is indicated beneath each lane.

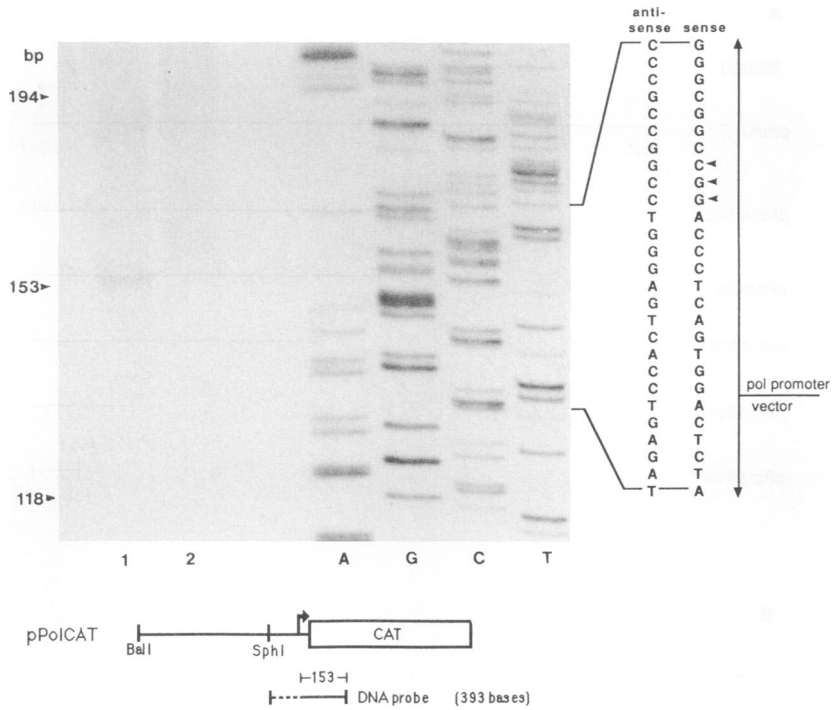

FIG. 5. Confirmation of transcriptional start site in the pPolCAT reporter construct by S1 nuclease protection analysis. Raji cells were transfected with pPolCAT and either pEBV-RZ (lane 1) or pHD101-3 (lane 2). Poly $(A)^+$ RNA was isolated from the transfected cells and hybridized to the single-stranded DNA probe. The sequence ladder was synthesized by using the same oligonucleotide primer to generate the single-stranded DNA probe from pPolCAT.

pPolCAT was transfected into latently infected Raji cells, promoter activity could not be detected (Fig. 4, lane 1). However, the promoter could be activated by cotransfection with a genomic clone (pEBV-RZ) (lane 2) that expresses the EBV immediate-early transactivators, Z and R (22). Thus, the promoter was constitutively inactive in the Raji lymphoid cell line. This result is consistent with the latent nature of this cell line and the undetectable polymerase activity in uninduced cells (17, 41).

S1 nuclease analysis of pPolCAT RNA from transfected Raji cells. To confirm that the same sites for *pol* message initiation were being used in the promoter construct, S1 nuclease analyses on CAT RNA derived from transient transfections were performed. pPolCAT was cotransfected into Raji cells with pEBV-RZ or its parent vector, pHD1013, which lacks the genomic fragment encoding BZLF-1 and BRLF-1. Polyadenylated RNA was harvested 48 h posttransfection and hybridized to a ^{32}P-labelled single-stranded DNA probe (Fig. 5). After digestion with S1 nuclease, a protected DNA fragment of 153 bases was expected for the *pol* promoter according to the previously determined RNA start site region (Fig. 2 and 3). The results demonstrate that in pPolCAT, pEBV-RZ-cotransfected Raji cells, the RNA start sites are the same as those identified for EBV-infected cells.

Analyses of promoter activity of pPolCAT 5′ deletions. To begin to identify the elements responsible for promoter activity, a series of nested 5′ deletions of the pPolCAT parent construct was made by restriction enzyme digestion (Fig. 6). These constructs were transfected into Raji cells either with or without the pEBV-RZ expression construct. pPolCAT and pPolΔHinc gave similar results, but the pPolΔBgl construct disclosed a significant reduction in CAT activity. Further deletions had little effect except for pPolΔAva, which was devoid of promoter activity despite the fact that this construct preserves a Z-responsive element as well as a possible SP1 consensus site (Fig. 7). These results indicate that the 140-bp region between *Hin*cII and *Bgl*II (positions −853 to −713) and the 101-bp region between *Sph*I and *Ava*I (−239 to −138) contain *cis*-acting elements that contribute to promoter activity. Additional deletions of the pPolCAT parent construct from the 3′ end removing the *Sph*I-to-*Bal*I fragment (−239 to +16) and the *Ava*I-to-*Bal*I fragment (−138 to +16) were made. These deletions, which removed the mRNA initiation region, resulted in total loss of promoter activity when cotransfected into Raji cells with pEBV-RZ (data not shown).

DISCUSSION

In this report, the EBV DNA polymerase transcriptional initiation region was mapped in TPA and sodium butyrate-induced virus-producing B95-8 cells. With the use of S1 nuclease analysis and primer-extension assays, transcription was shown to initiate from a small cluster of bases directly upstream of the ORF. The region containing these start sites spans map coordinates from positions 156,872 to 156,877 of the B95-8 genome and lacks both a canonical TATA box and a CAAT box, a characteristic associated with several housekeeping genes (3, 50) and some nonhousekeeping genes (5, 23). Such genes may be regulated via a number of factors and sequence motifs (2, 15, 46).

Furthermore, this region is G+C rich and contains a potential SP1 site overlapping the most-5′ RNA start site. The proximity of this transcription factor binding site could play a part in positioning transcriptional initiation, as with the TATA-less promoter of the mouse thymidylate synthase

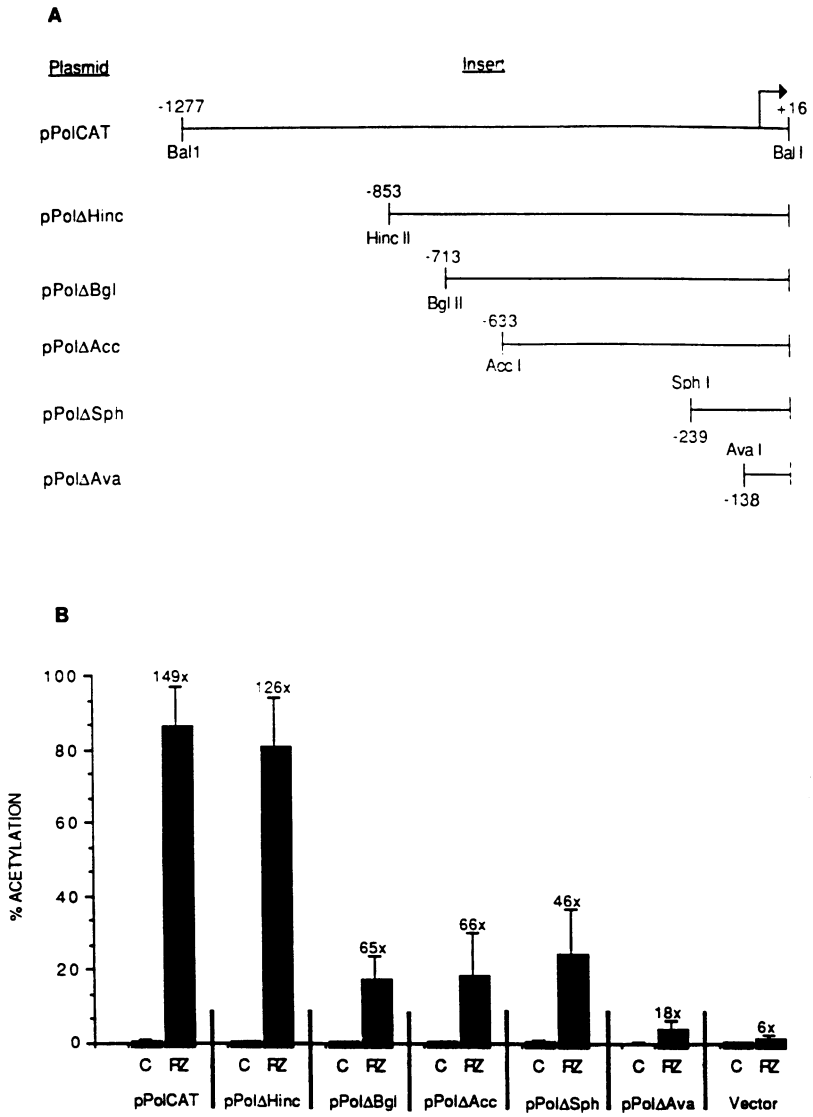

FIG. 6. Schematic representation of deletion constructs of the *pol* promoter region and CAT activity in Raji cells. (A) Nested deletions of the parent promoter construct, pPolCAT, extending from positions −1277 to +3 relative to the major RNA start site, were made by digestion with the restriction enzymes shown and linked to the CAT gene in the promoterless vector pBSCAT. (B) Deletion constructs were transfected into Raji cells, and CAT assay results are presented graphically. The percent conversion of chloramphenicol to its acetylated products is indicated. Numbers above the bars represent fold transactivation of each construct with pEBV-RZ (RZ) over constitutive activity with pHD1013 (C). The numbers shown represent the average fold increase from five experiments. Standard deviation is shown for each sample.

gene (25). Transient assays with a plasmid construct containing a 1.29-kb fragment cloned upstream of the CAT reporter gene demonstrated promoter activity when cotransfected into EBV-positive cells with a genomic clone that expressed the EBV immediate-early transactivators, R and Z. By using S1 nuclease analysis, we mapped the pPolCAT transcriptional start site region from these transient transfections and have confirmed it to be the same as that of the endogenous *pol* message. The same start sites are used in two other EBV cell lines tested, P3HR1 and Raji. It will be interesting to determine whether there are additional start sites that can be distinguished kinetically, as in the case of herpes simplex virus type 1 DNA *pol* transcription (6). However, such studies are likely to be difficult with EBV because they require a synchronous infection system. In addition, removal of the start site region from promoter-CAT constructs results in total loss of promoter activity and, therefore, does not disclose alternate upstream start sites in the constructs tested.

As EBV *pol* message is found only in cells actively producing virus, it was necessary to induce an EBV cell line to a high percentage of cells in the lytic cycle. This induction was achieved with TPA and sodium butyrate treatment of B95-8 cells. Northern analysis demonstrated a polymerase-specific transcript of 3.7 kb along with other sequence-related transcripts of 5, 8, and 11 kb detected with the polymerase-specific probe. Since it is clear from the data that the 5′ end of the polymerase transcript is not spliced and

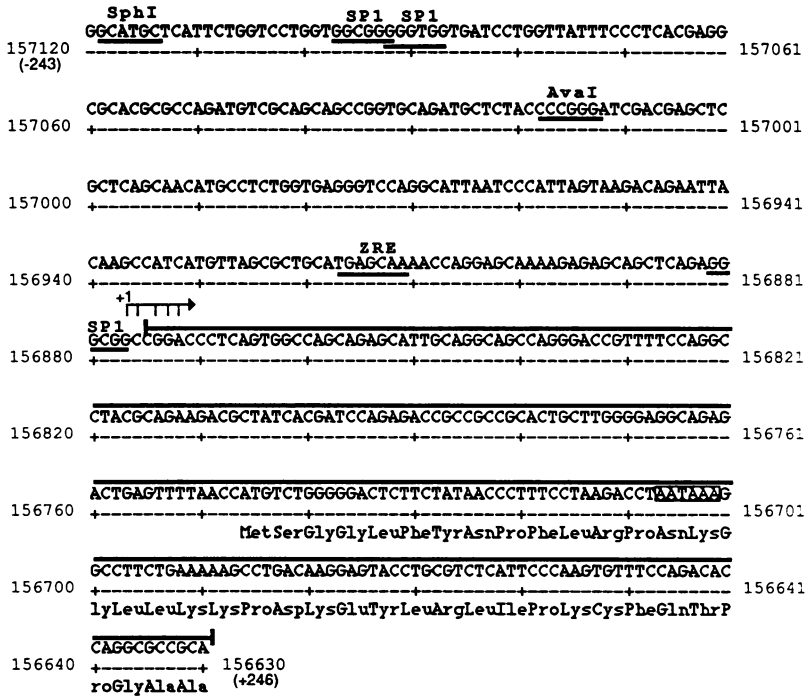

FIG. 7. Features of the DNA sequence in the 5′ region of the EBV DNA polymerase gene. Sequence elements with potential relevance for the expression of *pol* are shown between positions −243 and +245. Potential binding sites for SP1 and ZRE are depicted. The predicted hairpin, which includes the initiation codon for the ORF, is indicated by a horizontal line over the sequence. The polyadenylation signal which is occluded for *pol* expression is boxed.

since functional protein is made from translation of in vitro-made transcripts of the genomic fragment containing the ORF (36), the large forms of polymerase-related transcripts suggested that there may be 3′ processing of RNA to produce mature message. Indeed, examination of the EBV B95-8 genomic sequence does not reveal a polyadenylation signal within 3 kb downstream of the stop codon for the ORF, indicating the likelihood of a splicing event. The processing of the 3′ end of this message is of some interest; its role in regulation of expression is currently being investigated. More likely, the large transcripts represent read-through messages transcribed from the upstream ORF, BALF-4, which encodes glycoprotein B. A BALF-4-specific probe detects 5.0-, 8.0-, and 11.0-kb transcripts from the same induced RNA (data not shown). The existence of such read-through transcripts has been proposed for the similarly positioned glycoprotein B ORF of human cytomegalovirus (30).

Although many enzymatic domains are conserved among the EBV DNA polymerase-coding region and the homologous coding regions in other herpesviruses, the lack of a TATA box in the noncoding regulatory region seems unique to EBV among herpesviruses (30, 52). This characteristic is evidently shared by a gene for another EBV replicative enzyme, the DNase gene (8), which may suggest the possibility of a mechanism for coordinate regulation of these genes.

It is not obvious that Z and R act on the polymerase promoter directly, as has been demonstrated for TATA-containing EBV promoters. Different factors may be involved in regulation. Examination of the *pol* promoter sequence does not reveal a canonical R-responsive element ($GTGCCN_7GTGGAC$ or $GTCCN_6GTGGTG$ [19]) but does reveal a canonical Z-responsive element 40 bp upstream of the transcriptional start site region [TG(A/T)G(C/T)(C/A)A (16, 33, 34, 49)]. However, this site does not fall within the *cis*-acting regions defined so far that are important for promoter activity (Fig. 6). An indication that this promoter requires other transactivators in addition to R and Z is its negligible activity when cDNA constructs that express R and Z are cotransfected into EBV-negative cells. The R- and Z-expressing genomic clone used in this work contains portions of additional ORFs and is able to transactivate the *pol* promoter in EBV-negative lymphoid cells (26a) (data not shown). Another EBV gene product in the activation cascade is BMLF-1, which is activated by R and Z (7). However, BMLF-1 is unlikely to act directly on the *pol* promoter since it is a posttranscriptional transactivator (7, 28). The role of BMLF-1 in this system is being investigated.

Yet another interesting feature is one common to the EBV DNA polymerase and the herpes simplex virus DNA polymerase, namely, the potentially complicated secondary structure in the 5′ region of their transcripts. A ΔG of −71 kcal (−296 kJ)/mol has been reported for the herpes simplex virus type 1 *pol* transcript from positions +155 to +308 (52), and a similar stability of −60 kcal (−25 kJ)/mol is predicted for the EBV *pol* transcript from positions +2 to +245 (relative to the transcriptional initiation region, Fig. 7) by using the GCG FOLD program (14). Predicted hairpins of similar stability have been shown to impair the translation of thymidine kinase gene or human immunodeficiency virus transcripts (31, 42, 43). The consequence of such secondary structure in vivo is not known; however, synthetic transcripts from genomic clones deleting most of this region are more efficiently translated in rabbit reticulocyte lysates (29, 36). In addition, promoter constructs containing this region

show decreased CAT activity, possibly due to a translational block (unpublished results).

Furthermore, 37 bp downstream of the AUG codon, there is a functional polyadenylation signal used during late gene expression of BALF-4 (Fig. 7). This signal must be ignored during transcription of the *pol* gene itself. This inference is suggested by the proximity of this signal to the 5′ end of the message. Occlusion of polyadenylation signals near the 5′ caps of spleen necrosis virus and human immunodeficiency virus has been reported (24, 51); the signals function only when placed more than 500 bp downstream of their promoters. This observation indicates that not only transcriptional mechanisms but also posttranscriptional and translational mechanisms may govern expression of the EBV DNA polymerase.

ACKNOWLEDGMENTS

We thank Shannon Kenney for reviewing the manuscript and for providing the EBV-RZ plasmid, Nancy Raab-Traub for helpful discussions, and Beth Furnari and Julie Wilson for excellent technical assistance.

This work was supported by Public Service grant 5-P01-CA19014-14 from the National Institutes of Health.

REFERENCES

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69:**1048.
2. **Ayer, D. A., and W. S. Dynan.** 1988. Simian virus 40 major late promoter: a novel tripartite structure that includes intragenic sequences. Mol. Cell. Biol. **8:**2021–2033.
3. **Azizkhan, J. C., J. Vaughn, R. J. Christy, and J. H. Hamlin.** 1986. Nucleotide sequence and nuclease hypersensitivity of the Chinese hamster dihydrofolate reductase gene promoter region. Biochemistry **25:**6228–6236.
4. **Baer, R., A. T. Bankier, M. D. Beggin, P. L. Deininger, P. L. Farrell, T. J. Gibson, G. Hartfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310:**207–211.
5. **Blasband, A. J., K. T. Rogers, X. R. Chen, J. C. Azizkhan, and D. C. Lee.** 1990. Characterization of the rat transforming growth factor alpha gene and identification of promoter sequences. Mol. Cell. Biol. **10:**2111–2121.
6. **Bludau, H., and K. Freese.** 1991. Analysis of the HSV-1 strain 17 DNA polymerase gene reveals the expression of four different classes of pol transcripts. Virology **183:**505–518.
7. **Buisson, M., E. Manet, M.-C. Trescol-Biemont, H. Gruffat, B. Durand, and A. Sergeant.** 1989. The Epstein-Barr virus (EBV) early protein EB2 is a posttranscriptional activator expressed under the control of EBV transcription factors EB1 and R. J. Virol. **63:**5276–5284.
8. **Chen, M. R., T. Y. Hsu, J. Y. Chen, and C. S. Yang.** 1990. Molecular characterization of a cDNA clone encoding the Epstein-Barr virus (EBV) DNase. J. Virol. Methods **29:**127–141.
9. **Chevallier-Greco, A., E. Manet, P. Chavrier, C. Monsnier, J. Daillie, and A. Sergeant.** 1986. Both Epstein-Barr virus (EBV) encoded trans-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. EMBO J. **5:**3233–3249.
10. **Countryman, J., and G. Miller.** 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc. Natl. Acad. Sci. USA **82:**4085–4089.
11. **Datta, A. K., B. M. Colby, J. E. Shaw, and J. S. Pagano.** 1980. Acyclovir inhibition of Epstein-Barr virus replication. Proc. Natl. Acad. Sci. USA **77:**5163–5166.
12. **Datta, A. K., R. J. Feighny, and J. S. Pagano.** 1980. Induction of Epstein-Barr virus-associated DNA polymerase by 12-*O*-tetradecanoylphorbol-13-acetate. J. Biol. Chem. **255:**5120–5125.
13. **DeLeon, D. V., K. H. Cox, L. M. Angerer, and R. C. Angerer.** 1983. Most early-variant histone mRNA is contained in the pronucleus of sea urchin eggs. Dev. Biol. **100:**197–206.
14. **Devereux, J., P. Haeberil, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
15. **Dynan, W. S., and R. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell **35:**79–87.
16. **Farrell, P. J., D. T. Rowe, C. M. Rooney, and T. Kouzarides.** 1989. Epstein-Barr virus BZLF1 transactivator specifically binds to a consensus AP-1 site and is related to c-fos. EMBO J. **8:**127–132.
17. **Feighny, R. J., B. E. Henry II, A. K. Datta, and J. S. Pagano.** 1980. Induction of DNA polymerase activity after superinfection of Raji cells with Epstein-Barr virus. Virology **107:**415–423.
18. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
19. **Gruffat, H., E. Manet, A. Rigolet, and A. Sergeant.** 1990. The enhancer factor R of Epstein-Barr virus (EBV) is a sequence-specific DNA binding protein. Nucleic Acids Res. **18:**6835–6843.
20. **Hardwick, J. M., P. M. Lieberman, and S. Hayward.** 1988. A new Epstein-Barr virus transactivator, R, induces expression of a cytoplasmic early antigen. J. Virol. **62:**2274–2284.
21. **Hinuma, Y., M. Konn, J. Yamaguchi, D. Wudarski, J. Blakeslee, and J. Grace.** 1967. Immunofluorescence and herpes-type virus particles in the P3HR-1 Burkitt lymphoma cell line. J. Virol. **1:**1045–1051.
22. **Holley-Guthrie, E. A., E. B. Quinlivan, E. C. Mar, and S. Kenney.** 1990. The Epstein-Barr virus (EBV) BMLF1 promoter for early antigen (EA-D) is regulated by the EBV transactivators, BRLF1 and BZLF1, in a cell-specific manner. J Virol. **64:**3753–3759.
23. **Ishii, S., J. T. Kadonaga, R. Tijian, J. N. Brady, G. Merlino, and I. Pastan.** 1986. Binding of the Sp1 transcription factor by the human Harvey ras 1 proto-oncogene promoter. Science **232:**1410–1413.
24. **Iwasaki, K., and H. M. Temin.** 1990. The efficiency of RNA 3′-end formation is determined by the distance between the cap site and the poly(A) site in spleen necrosis virus. Genes Dev. **4:**2299–2307.
25. **Jolliff, K., Y. Li, and L. F. Johnson.** 1991. Multiple protein-DNA interactions in the TATAA-less mouse thymidylate synthase promoter. Nucleic Acids Res. **19:**2267–2274.
26. **Kallin, B., L. Sternas, A. K. Saemundssen, J. Luka, H. Jornvall, B. Eriksson, P.-Z. Tao, M. T. Nilsson, and G. Klein.** 1985. Purification of Epstein-Barr virus DNA polymerase from P3HR-1 cells. J. Virol. **54:**561–568.
27. **Kenney, S., J. Kamine, E. Holley-Guthrie, J. C. Lin, E. C. Mar, and J. Pagano.** 1989. The Epstein-Barr virus (EBV) BZLF1 immediate-early gene product differentially affects latent versus productive EBV promoters. J. Virol. **63:**1729–1736.
28. **Kenney, S., J. Kamine, E. Holley-Guthrie, E. C. Mar, J. C. Lin, D. Markovitz, and J. S. Pagano.** 1989. The EBV immediate-early gene product, BMLF1, acts in *trans* by a posttranscriptional mechanism which is reporter gene dependent. J. Virol. **63:**3870–3877.
29. **Kiehl, A., and D. I. Dorsky.** 1991. Cooperation of DNA polymerase and EA-D (BMLF1) in vitro and colocalization in nuclei of infected cells. Virology **184:**330–340.
30. **Kouzarides, T., A. T. Bankier, S. C. Satchwell, K. Weston, P. Tomlinson, and B. G. Barrell.** 1987. Sequence and transcription analysis of the human cytomegalovirus DNA polymerase gene. J. Virol. **61:**125–133.
31. **Kozak, M.** 1986. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proc. Natl. Acad. Sci. USA **83:**2850–2854.
32. **Laimins, L. A., P. Gruss, R. Pozzatti, and G. Khoury.** 1984. Characterization of enhancer elements in the long terminal repeat of Moloney murine sarcoma virus. J. Virol. **49:**183–189.

33. **Lieberman, P. M., and A. J. Berk.** 1990. In vitro transcriptional activation, dimerization, and DNA binding specificity of Epstein-Barr virus Zta protein. J. Virol. **64:**2560–2568.
34. **Lieberman, P. M., J. M. Hardwick, J. Sample, G. S. Hayward, and S. D. Hayward.** 1990. The Zta-transactivator involved in induction of lytic cycle gene expression in Epstein-Barr virus-infected lymphocytes binds to both AP-1 and ZRE sites in target promoter and enhancer regions. J. Virol. **64:**1143–1155.
35. **Lin, J. C., J. E. Shaw, M. C. Smith, and J. S. Pagano.** 1979. Effect of 12-*O*-tetradecanoyl-phorbol-13-acetate on the replication of Epstein-Barr virus. I. Characterization of viral DNA. Virology **99:**183–187.
36. **Lin, J. C., N. D. Sista, F. Besencon, J. Kamine, and J. S. Pagano.** 1991. Identification and functional characterization of Epstein-Barr virus DNA polymerase by in vitro transcription-translation of a cloned gene. J. Virol. **65:**2728–2731.
37. **Luka, J., B. Kallin, and G. Klein.** 1979. Induction of Epstein-Barr virus (EBV) cycle in latently infected cells by n-butyrate. Virology **94:**228–231.
38. **Miller, G., T. Shope, H. Lisco, D. Stitt, and M. Lipman.** 1972. Epstein-Barr virus: transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. Proc. Natl. Acad. Sci. USA **69:**383–387.
39. **Miller, R. L., R. Glaser, and F. Rapp.** 1977. Studies of an Epstein-Barr virus-induced DNA polymerase. Virology **76:**494–502.
40. **Neumann, E., M. Schaefer-Ridder, Y. Wang, and P. H. Hofschneider.** 1982. Gene transfers into mouse lymphoma cells by electroporation in high electric fields. EMBO J. **1:**841–845.
41. **Nutter, L. M., S. P. Grill, J.-S. Li, R.-S. Tan, and Y.-C. Cheng.** 1987. Induction of virus enzymes by phorbol esters and *n*-butyrate in Epstein-Barr virus genome-carrying Raji cells. Cancer Res. **47:**4407–4412.
42. **Parkin, N. T., E. A. Cohen, A. Darveau, C. Rosen, W. Haseltine, and N. Sonenberg.** 1988. Mutational analysis of the 5′ noncoding region of human immunodeficiency virus type 1: effects of secondary structure on translation. EMBO J. **7:**2831–2837.
43. **Pelletier, J., and N. Sonenberg.** 1985. Insertion mutagenesis to increase secondary structure within the 5′ noncoding region of a eucaryotic mRNA reduces translational efficiency. Cell **40:**515–526.
44. **Pulvertaft, R. J. V.** 1965. A study of malignant tumours in Nigeria by short term tissue culture. J. Clin. Pathol. **18:**261–273.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, vol. 3, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45a. **Sista, N., et al.** Unpublished data.
46. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57:**103–113.
47. **Takada, K., and Y. Ono.** 1989. Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. J. Virol. **63:**445–449.
48. **Takada, K., S. Shimizu, S. Sakuma, and Y. Ono.** 1986. *trans* activation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV DNA fragment. J. Virol. **57:**1016–1022.
49. **Urier, G., M. Buisson, M. Chambard, and A. Sergeant.** 1989. The Epstein-Barr virus early protein EB1 activates transcription from different responsive elements including AP1 binding sites. EMBO J. **8:**1447–1453.
50. **Valerio, D., M. G. C. Duyvesteyn, B. M. M. Dekker, G. Weeda, T. M. Berkvens, L. Vander Voorn, and A. J. Van der Eb.** 1985. Adenosine deaminase: characterization and expression of a gene with a remarkable promoter. EMBO J. **4:**437–443.
51. **Weichs an der Glon, C., J. Monks, and N. J. Proudfoot.** 1991. Occlusion of the HIV poly(A) site. Genes Dev. **5:**244–253.
52. **Yager, D. R., and D. M. Coen.** 1988. Analysis of the transcript of the herpes simplex virus DNA polymerase gene provides evidence that polymerase expression is inefficient at the level of translation. J. Virol. **62:**2007–2015.