Constitutive Expression of Epstein-Barr Virus-Encoded RNAs and Nuclear Antigen During Latency and After Induction of Epstein-Barr Virus Replication

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We examined the fate of two major products of latency as Epstein-Barr virus was induced to replicate. We studied a superinducible clone of HR-1 cells in the presence and absence of induction by phorbol ester, and we analyzed the X50-7 line with and without superinfection by an HR-1 viral variant which disrupts latency. The two methods of induction yielded qualitatively similar results. After induction, there was abundant synthesis of viral transcripts, amplification of viral DNA, and the appearance of many new viral polypeptides. Nonetheless, there were no changes in the cytoplasmic abundance of Epstein-Barr virus-encoded RNAs and no alteration in the level of Epstein-Barr virus nuclear antigen mRNA or polypeptide. Thus, under conditions in which numerous other Epstein-Barr virus gene products are activated, the two major latent gene products are expressed at a constitutive level. Expression of Epstein-Barr virus-encoded RNAs and nuclear antigen must therefore be regulated in a manner completely different from expression of replicative functions.

Within lymphocytes, Epstein-Barr virus (EBV) usually maintains itself in a latent state. During latency, few regions of the viral genome are transcribed, the viral episome is maintained at constant copy number from one cell generation to the next, and no viral progeny are synthesized. Latency can be disrupted experimentally by treatment with phorbol ester (24) or sodium butyrate (12) or by superinfection with certain viral variants (13). Upon induction of viral replication, there is abundant transcription of a large portion of the genome, the appearance of many new intracellular viral polypeptides, amplification of viral DNA, and release of mature virions.

Two virally encoded products of latency which have been unambiguously identified are the EBV-encoded RNAs (EBERs) and the EBV nuclear antigen (EBNA) (11, 15). EBERs are two small RNAs, 164 and 173 bases in length, encoded by the *EcoRI* J fragment, which exist complexed with a host cell protein termed La (17). EBERs are transcribed by RNA polymerase III. Their function is not known. At least one component of the antigen complex serologically defined as EBNA is a 78-kilodalton polypeptide encoded by the left-most 2.9 kilobase pairs of the *BamHI* K fragment (4). EBNA polypeptide contains a stretch of about 200 residues of glycine and alanine (7). The antigenic polypeptide is found on chromosomes at metaphase (5), but its biochemical function is obscure.

The present experiments were designed to determine the fate of these two major products of latency as the virus entered the replicative life cycle. It was hoped that such experiments might eventually provide clues about the function of EBERs and EBNA and also information about regulation of their expression. We took advantage of two experimental systems in which the same cells could be examined during latency and replication. In one system, a superinducible clone of P3JHR-1 cells is studied before and after treatment with 12-O-tetradecanoyl-phorbol 13-acetate (TPA) (8). In the other system, the viral genome ordinarily

tightly latent in a line of human umbilical cord lymphocytes, X50-7, can be induced to replicate in at least 10% of cells by superinfection with a stock of variant EBV which contains defective genomes (13).

MATERIALS AND METHODS

Lymphocytes and virus. The superinducible clone of P3JHR-1 cells, HH514-16 (clone 16), was treated with TPA at 20 ng/ml (14). Virus capable of inducing replication in X50-7 lymphocytes was derived from clone HH543-5 (clone 5) (14). Virus stocks were prepared from filtered culture supernatants. All lymphocytes were cultured in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. Success of induction of replication was monitored by indirect immunofluorescence with human sera with antibodies to early antigen and capsid antigen and fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (IgG).

Isolation and analysis of cytoplasmic RNA. RNA extraction was performed as described (23). Northern blotting of large viral mRNAs was done by the procedure of Thomas (21). Small RNAs were resolved by electrophoresis in 10% acrylamide-7 M urea gels. Gels were blotted to diazobenzyl-oxy-methyl-paper in 50 mM sodium acetate NaOAc (pH 4.8) for 12 to 15 h. Blots were prehybridized and hybridized with nick-translated *EcoRI*-J DNA in hybridization solution containing 1% glycine.

EBNA mRNA by S1 analysis. Initial experiments to define the 5' end of the major EBNA exon were carried out with RNA prepared from COS-1 cells transfected with the plasmid pSV2-gpt-*BamHI*-K or, as a control, pSV2-gpt (16). The probe for the 5' end of the exon was a 540-base-pair (bp) *BamHI*-*SalI* subfragment from the left end of *BamHI*-K, which was cloned into M13-MP 9 phage. Uniformly labeled single-stranded probe for S1 analysis was prepared by in vitro synthesis of a complementary strand with 0.5 µg of single-stranded M13 MP 9 DNA as a template and 2.5 pg of pentadecamer primer (New England Biolabs). The 20-µl reaction mixture included cold dATP, dGTP, and dTTP at 10 µM and 25 mCi of [α -³²P] dCTP (4,000 Ci/mMol; Amersham

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FIG. 1. Induction of viral mRNA. Northern blots of total cytoplasmic RNA from cells induced by phorbol ester or superinfection by an inducing virus. Both methods of induction result in activation of viral message from a variety of regions of the genome. No viral messages are detected from these regions in uninduced cells.

Corp.), 5 U of Klenow fragment of DNA polymerase (Boehringer-Mannheim Chemicals).

S1 nuclease digestion was performed by the procedure of Favaloro et al. (3). Total cytoplasmic RNA (10 μ g) from transfected COS-1 cells or (ca. 100 μ g) from lymphocytes was precipitated with 50 to 100 ng of radio-labeled probe DNA, conditions under which EBV DNA was in excess of EBNA mRNA. Nucleic acid pellets were suspended in 80% formamide–0.4 M NaCl–40 mM PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 6.4)–1 mM EDTA. The solution was heated to 80 to 85°C for 10 min and transferred to a 55°C water bath for 3 h. After hybridization, a 10-fold volume of S1 nuclease buffer (5% glycerol, 100 mM NaCl, 60 mM NaOAc, 4 mM ZnSO₄) containing 2,000 to 4,000 units of S1 nuclease per ml was added. S1 digestions were carried out at 22°C for 1 h.

EBNA polypeptide. Parallel cultures were treated with TPA for 4 days or superinfected for 7 days with clone 5 virus or mock infected with clone 16 virus. EBNA was examined by immunoblotting as described (4). Extracts with 250 μ g of protein were boiled for 5 min in sodium dodecyl sulfate sample buffer and centrifuged briefly, and the supernatant was electrophoresed through 5% stacking and 10% resolving acrylamide gels (10). The gel was electroblotted to nitrocellulose (22) and incubated with a 1:400 dilution of RM, human serum which contained antibody to the EBNA polypeptide, or, as a control in a 1:200 dilution of WC, human serum which lacked antibody to the EBNA polypeptide but contained antibody to the replicative viral proteins (G. Miller, E. Grogan, D. K. Fischer, J. C. Niederman, R. T. Schooley, W. Henle, G. Lenoir, and C.-R. Liu, submitted for publication). The filter was washed several times, incubated in ¹²⁵I-labeled staphylococcal protein A (1.0 μ Ci), washed, dried, and exposed to film with intensifying screens.

RESULTS

Description of latency and viral replication by using different methods of induction. In the absence of phorbol ester, 1% or fewer clone 16 cells express replicative antigens detectable by human sera with antibody to viral capsid

antigen and early antigen. However, 72 h after treatment with TPA, ca. 30% or more of clone 16 cells synthesize viral replicative antigens. In X50-7 cells, the genome is tightly latent, and no viral replicative antigens can be identified. By contrast, at least 10% of X50-7 cells synthesize early antigen and viral capsid antigen by 72 h after superinfection with clone 5 virus (data not shown). By 48 h after treatment with TPA, many new viral mRNAs are made in clone 16 cells; new mRNAs are evident by 28 h after superinfection of X50-7 cells with clone 5 virus (Fig. 1). Within the limitations of the Northern blotting technique, mRNAs induced by TPA which are homologous to *EcoRI*-C are similar in size (Fig. 1). A region of *Bam*HI-K near the exon which encodes EBNA is also transcriptionally active during induction of viral replication in X50-7 cells (Fig. 1; see Fig. 3). Several viral mRNAs homologous to the *Hind*III-*Bam*HI subfragment Cf which represents the right-most 2 kilobase pairs of the *Bam*HI K fragment, are induced; the most prominent is 1 kb.

After TPA treatment of clone 16 cells and superinfection of X50-7 cells, there is amplification of the EBV genome (13, 14). In Fig. 2 is shown an experiment in which X50-7 cells were either superinfected with clone 5 virus or mock infected. Cellular DNA was prepared from samples of both cultures daily, and equal amounts (5 μ g) were digested with *Eco*RI, electrophoresed, blotted, and probed with *Eco*RI-J, the region of the genome which encodes EBERs. It is

FIG. 2. Genome amplification. A Southern blot shows an increase in copy number of viral DNA in cells induced by superinfection with clone 5 virus. For a representative viral DNA sequence, the *Eco*RI J fragment which encodes EBERs was used as a probe. An increase is seen initially due to superinfection, but there is clearly an additional increase thereafter. In this experiment, we were unable to determine whether the increase in viral DNA comes from the superinfecting or the endogenous viral genome.

evident (Fig. 2) that in all samples, there are more copies of the *Eco*RI J fragment in the superinfected than in the uninfected cells. The relatively low level of copies observed after 48 h is due to variability in DNA extraction. Some of these copies may represent the input clone 5 virus, but there also appear to be increased amounts of EBV DNA thereafter in the superinfected culture. Viral DNA also increases in amount after induction of clone 16 cells with phorbol esters (23; unpublished data).

Thus, whether measured by antigen expression, viral mRNA induction, or genome copy number, it is evident that both systems show major differences between the latent and replicative state of the genome. Against this background of biological data we measured the content of the two latent products, EBERs and EBNA.

Abundance of EBERs. The quantities of EBERs in cytoplasm were measured before and after induction of replication in the two systems (Fig. 3). After induction of clone 16 cells by TPA (Fig. 3A and B), there was no apparent change in the abundance of EBERs over the period of 12 to 72 h when there were dramatic increases in the abundance of certain viral mRNAs (Fig. 1). Identical results are seen after induction of X50-7 cells by superinfection with clone 5 virus

in two separate trials (Fig. 3). In the first experiment (Fig. 3A and B), the RNA was prepared at 12 and 48 h after superinfection; in the second experiment (Fig. 3C and D), RNA was prepared at intervals of 1 to 7 days. Once again, there are no differences in the quantities of EBERs detected in induced and uninduced cells.

The base-line level of EBERs varies in the two cell systems under study. The ethidium bromide-stained gels (Fig. 3A and C) indicate that there is approximately the same amount of total RNA in each lane, yet clones of HR-1 cells (clone 16 cells in lanes 1 through 8 of Fig. 3A and B; clone 5 in lane 7 of Fig. 3C and D) seem to contain more EBERs (ca. fivefold) than do X50-7 cells. Although HR-1 cell clones synthesize more EBERs than do X50-7 cells, addition of HR-1 clone 5 virus to X50-7 cells does not cause a change in the level of EBERs in X50-7 cells. BJAB cells, from an EBV genome-negative Burkitt Lymphoma line, do not contain EBERs (Fig. 3C and D).

Transcription of EBNA. We also studied expression of a latent viral mRNA which is transcribed by RNA polymerase II. These experiments became feasible when we mapped the exact boundaries of the exon coding the EBNA polypeptide. Identical results were obtained by S1 analysis of mRNA

FIG. 3. Constitutive expression of EBERs. Identical amounts of total cytoplasmic RNA were loaded in each gel lane as seen by ethidium stain (A and C). Northern blots of these gels demonstrate no change in cytoplasmic abundance of EBERs after induction by phorbol ester (B) or superinfection (D). No hybridization was detected in EBV-negative cell lines (B and D).

FIG. 4. Constitutive expression of EBNA message. S1 nuclease analysis detects the coding exon of the EBNA message in COS-1 cells transfected with pSV2-BamHI-K or in lymphocytes infected with EBV. In lanes 5 through 10, identical amounts of total cytoplasmic RNA were hybridized with the BamHI-SmaI subfragment of BamHI-K. No change in the intensity of the 179-bp S1 digestion product is detected when cells are induced by superinfection or by treatment with phorbol ester. At 72 h, phorbol-treated clone 16 cells begin to synthesize new messages, as seen in lane 9. These induced mRNAs protected the entire probe (540 bp) and 355 bp of the probe. The band seen at 120 bp is not specific.

from EBV-transformed lymphocytes and from two gene transfer systems, L cells and COS-1 cells (Weigel and Miller, manuscript in preparation). The 5' end of this exon protects 179 bases of a 540-bp BamHI-SmaI fragment which is the left end of BamHI-K (Fig. 4). The same 179-bp fragment is protected by mRNA from COS-1 cells transfected with pSV2-BamHI-K (13) (Fig. 4, lane 2) or by mRNA from a lymphoid line, FF500 (lane 4). RNA from COS-1 cells transfected with pSV2-gpt alone does not protect the EBV DNA (Fig. 4, lane 3). The same 179-bp fragment is protected by RNA from X50-7 and clone 16 cells. The intensity of this band, which would increase if there were induction of this mRNA, does not change after treatment with TPA or superinfection. However, 72 h after treatment of clone 16 cells with TPA, two additional protected fragments are seen whose sizes are ca. 540 and 355 bases. We believe these to represent other mRNAs originating upstream of the 540-bp BamHI-SmaI fragment which are induced during viral replication (Weigel and Miller, in preparation). Thus, using the same probe, we find induction of adjacent mRNAs but not of the EBNA mRNA.

EBNA polypeptide during latency and replication. Although the abundance of EBNA mRNAs does not change upon induction of the viral replicative cycle, it is still

possible that the content of EBNA polypeptide increases due to changes in translation of these messages. Therefore, we examined parallel cultures of induced and uninduced cells for the content of the EBNA polypeptide by using the immunoblotting technique (Fig. 5A). In addition to the two induction systems used to study transcription of EBERs and EBNA mRNAs, we also looked at EBNA abundance in Raji cells which had been induced to make early antigens by TPA treatment or by superinfection by clone 5 virus. Within slight errors of extraction, in every pair of cultures, the abundance of EBNA polypeptide was the same in induced and uninduced cells. There is a variation in the abundance and the size of EBNA from one lymphoid line to the next (4, 6, 18).

To demonstrate that other EBV polypeptides were in fact induced, we reacted an identical immunoblot containing the same cell extracts as found in Fig. 5A with a human serum which contains high titers of antibody against EBV replicative proteins but which lacks antibody to the BamHI-K EBNA (Miller et al., submitted for publication) (Fig. 5B). In each pair of cultures, there is a marked increase in the number and abundance of viral specific proteins under conditions in which EBNA polypeptide remains constant.

DISCUSSION

During a time when there is a profound increase in synthesis of new viral mRNAs (Fig. 1), amplification of the viral genome (Fig. 2), and production of many new viral polypeptides (Fig. 5B), EBERs, EBNA transcripts, and EBNA protein remain constant (Fig. 3, 4, and 5A). The results are the same, whether disruption of latency is brought about by phorbol ester or by superinfection with a viral variant containing heterogenous DNA.

The data presented make it likely but do not prove that this constant expression is taking place in every cell of the two lines studied. Less likely, in our opinion, is the possibility that constant expression of EBERs and EBNA after induction of viral replication is the net result of increases in some cells and decreases in others.

Abundance of EBERs. EBER expression during both life cycles of the virus has been studied previously. Hummel et al. concluded that EBERs were increased after TPA induction, but they compared an induced marmoset cell line with an uninduced human neonatal lymphoid line (9). Since EBER concentration can vary among cell lines (Fig. 3), it is essential to compare the same cell before and after induction. Previously, we carried out such studies in a single HR-1 cell clone and reported a slight decrease in EBER abundance after TPA induction (23). However, our earlier results were based on Northern blots of RNA separated on agarose gels and transferred to nitrocellulose filters. This technique is less accurate for small RNAs than resolving by polyacrylamide and blotting to diazobenzylxymethyl-paper (1). It is now clear that EBER concentration in the cytoplasm is unaffected by induction.

To some extent, the level of EBERs seems to be characteristic of each lymphoid line, regardless of the state of activation of the genome. Whether this is due to host cell control or to the number or properties of the latent genomes is not clear. However, addition of virus from a cell line with higher EBER copy number, such as HR-1, to one with lower EBER copy number, such as X50-7, does not affect EBER abundance (Fig. 3D). This result makes it appear unlikely that EBER abundance is simply controlled only by viral genes. EBERs are small RNAs which are complexed with host cell proteins and are transcribed by cellular RNA polymerase III. The other well-studied example of small

FIG. 5. Constitutive expression of EBNA polypeptide. Immunoblots of protein extracted from cells induced with phorbol ester or by superinfection demonstrate no change in the abundance of EBNA after induction (A). The size of EBNA does vary, as expected, among the 3 cell lines (4). An identical immunoblot reacted with a serum having a high titer to viral replication proteins but no anti-EBNA (B) demonstrates the induction of many viral specific proteins after either phorbol treatment or superinfection.

RNAs among DNA viruses are the VA RNAs, which function late in the replicative cycle of adenovirus and are necessary for efficient translation of late viral products (20). There are striking structural similarities between VA RNAs and EBERs, and there is some evidence that EBERs can functionally substitute for VA RNAs (2). However, a major biological difference between the two is that EBERs are present throughout the life cycle of the virus and may well function during latency. However, the ultimate biochemical role of viral small RNAs may prove to be similar.

Abundance of EBNA. These data represent the first analysis of regulation of expression of the EBNA gene located in *Bam*HI-K. The results are unexpected. Under conditions of induction, transcription from the portion of *Bam*HI-K which encodes EBNA is dramatically different from transcription of other regions of *Bam*HI-K which encodes replicative functions as yet unidentified (Fig. 1 and 4). Transcription from the non-EBNA-coding regions of *Bam*HI-K is markedly enhanced by induction stimuli; we have identified by S1 analysis at least two major transcriptional units located 3' to the EBNA gene whose synthesis is induced by TPA and superinfection (Weigel and Miller, in preparation), and there are induced transcripts which enter the *Bam*HI K fragment (Fig. 4) from the 5' end. Furthermore, in four different systems (Fig. 5) in which a variety of new polypeptides is made after induction, EBNA polypeptide abundance is unaltered. The availability of human sera which contain high titers of antibody to the replicative proteins and yet lack antibody to the EBNA polypeptide make this analysis possible. Since abundance of both EBNA transcript and EBNA polypeptide remains constant after induction, it is most likely that regulation of EBNA expression is at the level of RNA synthesis or processing.

Possible mechanisms for distinguishing latent from replicative functions. We do not have enough information to understand the biochemical mechanisms which might underlie differences in expression of latent and replicative functions of the EBV genome. However, the present experiments

provide the first clear example of regulation of expression of two specific genes which represent latent functions. Thus, these experiments provide a basis for further work to explore the mechanism.

What might account for the constitutive expression of the two major latent products? One possibility might be called selective template recognition. There are usually several copies of the EBV genome in the latent state (19). All of these genomes, or possibly a subset, may serve as templates for expression of latent functions. When the genome is activated, some or all of the latent genomes must then serve as templates for transcription of early genes. There may be alterations in newly replicated templates which allow them to be used for transcription of late genes. However, the latent genomes may continue to be responsible for expressing latent function and newly synthesized genomes may not be suitable templates for transcription of latent products. Some such mechanism could account for constitutive expression of EBNA and EBERs even when there is an obvious increase in the number of genomes (Fig. 2). Selective template recognition may operate through genome integration, methylation, association with chromatin, or other changes in configuration.

Different polymerases or transcription factors may be responsible for transcription of latent versus replicative genes. For example, EBNA might be transcribed by a host cell RNA polymerase, whereas transcription of replicative genes may require either a different cell polymerase, a virally encoded or modified RNA polymerase, or viral transcription factors. A prediction of this model is that EBNA transcription is fixed by the amounts or state of activation of host cell RNA polymerases which are not affected by induction stimuli. If this model is correct, polymerase recognition sites of latent and viral replication genes may be different. Another possible mechanism is that the amount of EBNA mRNA and protein found in latency is held constant as the result of posttranscriptional regulation, e.g., RNA processing.

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