Transcriptional activity across the Epstein–Barr virus genome in Raji cells during latency and after induction of an abortive lytic cycle

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We have studied the relative rate of transcription across the Epstein–Barr virus genome in the Burkitt’s lymphoma cell line Raji by nuclear run-on analysis during latency and after induction of an abortive lytic cycle with 12-O-tetradecanoylphorbol 13-acetate (TPA) and 5-iodo-2'-deoxyuridine (IUdR). During latency the entire, or almost the entire, viral genome was found to be transcriptionally active to a low or intermediate extent, with some variation in activity along the genome. The fragment with the highest transcriptional activity was EcoRI J, which contains the genes encoding the small nuclear RNAs EBER1 and -2, transcribed predominantly by RNA polymerase III. An intermediate level of transcription was observed between positions 10 and 138 (kb), with areas of slightly higher activity on the large internal repeats and the left duplicated region (DL). The remaining part of the viral genome, between position 138 and the termini, and the termini and position 10 (kb) (with the exception of the EcoRI J fragment), showed very little transcriptional activity, except for the intermediately active regions carrying the right-hand oriLyt (DR) and the terminal repeats. Upon induction of the viral genome with TPA and IUdR, the viral genome was transcriptionally active at a rate at least tenfold that seen during latency. Polymerases were not equally distributed along the genome after induction; the highest density was found in regions 48 to 58 kb, 82 to 84 kb, 102 to 104 kb, 118 to 122 kb and 142 to 145 kb of the viral genome. High transcriptional activity correlated with distinct transcription units in some cases, i.e. BamHI H1LF1 (DL), BamHI MLF1, BamHI ZLF1/BamHI RLF1 and BamHI X (thymidine kinase), but not in others (BamHI H2). Besides initiation of transcription, other regulatory processes such as stabilization and processing of primary transcripts may also contribute to regulation of virus gene expression. Addition of cycloheximide completely abolished the transcriptional activation of the genome mediated by TPA and IUdR.

Introduction

Epstein–Barr virus (EBV), a lymphotropic human herpesvirus, is known to be the causative agent of infectious mononucleosis, and is closely associated with Burkitt’s lymphoma (BL) and nasopharyngeal carcinoma. EBV immortalizes human B lymphocytes in tissue culture. The viral genome persists in multiple, predominantly episomal, copies (Lindahl et al., 1976) and is maintained in a state of latency in which virus production either does not occur or, if it does, only at a very low rate. The viral genome of the non-producer BL line Raji, which was used in this study, has two deletions which are thought to be responsible for the inability of this EBV strain to produce infectious viral particles (Polack et al., 1984a). In Raji cells, spontaneous induction of an abortive lytic cycle occurs in less than 0-01% of cells (zur Hausen et al., 1978).

During latency, only a limited number of regions of the EBV genome are expressed. These are the genes encoding six nuclear (EBNA1, EBNA2, EBNA-LP and EBNA3A, -3B and -3C) and three membrane antigens, the latent membrane protein (LMP), and the terminal proteins, TP1 and TP2 (alternatively designated LMP1, and LMP2a and -b) (for review see Kieff & Liebowitz, 1990). Some, and possibly all, of these genes are expected to contribute to the immortalization process. EBNA1 binds to oriP, the episomal origin of replication, and is required for maintenance of episomes (Yates et al., 1984; Rawlins et al., 1985); EBNA2 enhances serum independence in rodent cells (Dambaugh et al., 1986) and induces expression of cellular genes, such as those encoding CD21, CD23 and other surface markers (Wang et al., 1987; Cordier et al., 1990; Abbot et al., 1990). Recent investigations have shown that the EBNA2A product is also involved in the control of the expression
of virus genes like LMP and TP (Fahraeus et al., 1990; Ghosh & Kieff, 1990; Zimber-Strobl et al., 1991). In RAT-1 cells, LMP can cause morphological transformation and render the cells tumorigenic (Wang et al., 1985). The TP transcripts, which cross the fused termini of the circularized EBV genome (Laux et al., 1988b, 1989; Sample et al., 1989), can encode two related membrane proteins in latently infected lymphocytes.

The state of virus latency can be overcome in vitro by either superinfection of cells with strain P3HR-1 (Yajima & Nonoyama, 1976; Heston et al., 1982; Rabson et al., 1983; Miller et al., 1984, 1985), or chemical induction with 12-O-tetradecanoylphorbol 13-acetate (TPA), 5-iodo-2′-deoxyuridine (IUdR) or other agents (zur Hausen, 1982; Rabson et al., 1983; Miller et al., 1984, 1985). Transcriptional activation of the trans-activator BZLF1 is strongly suspected to be the key event in the lytic cascade (Countryman & Miller, 1985; Chevallier-Greco et al., 1986; 1989; Hardwick et al., 1988; Farrell et al., 1989; Urier et al., 1989; Flemington & Speck, 1990a, b); other known trans-activators, such as BRLF1 and BMLF1, participate in this reaction chain (Lieberman et al., 1986; Oguro et al., 1987; Hardwick et al., 1988; Chevallier-Greco et al., 1989; Marschall et al., 1989; Takada & Ono, 1989). Induction of the lytic cycle leads to the expression of a complex spectrum of RNAs (Hummel & Kieff, 1982a, b; Laux et al., 1988a) and irreversibly to the death of the cell. Thus, EBV-induced cell proliferation requires maintenance of viral latency as an important step in immortalization.

Recently, we have described the pattern of EBV RNAs detectable after activation of an abortive lytic cycle in Raji cells (Laux et al., 1988a). Now, we have analysed the relative rate of transcription across the EBV genome during the state of latency, and after induction with TPA and IUdR. After treatment with TPA and IUdR, 30 to 45% of cells were induced compared to 8 to 12% after treatment with TPA alone.

The relative transcription rate was examined by a nuclear run-on synthesis reaction with nuclei isolated from Raji cells. The extracted run-on RNAs were hybridized to cloned DNA fragments covering the entire EBV genome and immobilized on nylon membranes (Polack et al., 1984b).

We have shown that the entire, or almost the entire, EBV genome is transcriptionally active to a low or intermediate extent during latency. A region of about 130 kb to the right-hand side of oriP, which includes the EBNA genes, is uniformly transcriptionally active, whereas the remaining part, with the exception of some particularly active regions such as the EBER genes, the second oriLyt and the terminal repeats, has significantly lower transcriptional activity. Upon induction, transcription increases by at least tenfold.

Methods

Cells. The EBV-positive BL cell line Raji (Pulvertaft, 1965) and the EBV-negative line BL-41 were grown in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated foetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (280 μg/ml), at 37 °C in 5% CO2. Cells were diluted twice weekly 1:2 to 1:3 with fresh medium. Induction was performed with 20 ng/ml TPA (Sigma) and 50 μg/ml IUdR for 4 to 48 h, and, in some cases, cycloheximide (CHI) (Sigma) was used at a concentration of 50 μg/ml. The degree of spontaneous induction and activation of the lytic cycle by TPA and IUdR was measured 48 h after treatment by immunofluorescence (Henle & Henle, 1966) with serum containing antibodies against early viral proteins, and was 0.01%, and 30 to 45%, respectively, with some variation in different experiments.

Nuclear run-on transcription. The nuclei were prepared as described by Greenberg & Ziff (1984), and modified by Eick & Bornkamm (1986). Cells were washed once in ice-cold PBS, and pellets of 1 x 106 cells were resuspended in 10 mm-Tris-HCl pH 7.4, 10 mm-NaCl, 3 mM-MgCl2, 0.5% (v/v) NP40 and incubated on ice for 5 min. After centrifugation at 50 g, the supernatant was discarded and the nuclear pellet washed by resuspension in 10 ml of the same buffer followed by a second centrifugation. The nuclei were then resuspended in storage buffer [50 mm-Tris-HCl pH 8.3, 5 mm-MgCl2, 0.1 mm-EDTA, 40% (v/v) glycerol] and frozen in liquid nitrogen in 100 μl aliquots, which corresponds to the nuclei of 107 cells. For nuclear run-on RNA synthesis, the nuclei were mixed with 100 μl of reaction buffer [10 mm-Tris-HCl pH 7.5, 5 mm-MgCl2, 300 mm-KCl, 0.5 mm each of ATP, CTP, GTP and 150 μCi [α-32P]UTP (800 Ci/mmol; Amersham)] and incubated for 30 min at 28 °C. To distinguish between RNA polymerase II and III transcription, α-amanitin was added to a final concentration of 4 μM, which is sufficient to block RNA polymerase II activity but not that of RNA polymerases I and III. Subsequently, DNase I was added to a final concentration of 10 μg/ml and the incubation continued for 5 min at 30 °C. After the addition of 20 μl 10% SDS, 10 μl 0.5 mm-EDTA pH 8.0, 10 μl proteinase K (5 mg/ml, preincubated at 37 °C for 1 h) and 200 μl STE buffer (0.5%, SDS, 100 mm-Tris-HCl pH 7.5, 50 mm-EDTA), the samples were incubated for 1 h at 37 °C. Nuclear transcripts were separated from unincorporated nucleotides on a Sephadex G50 column equilibrated with 10 mm-Tris-HCl pH 7.5, 1 mm-EDTA and 1% SDS.

DNA clones. Cosmid and plasmid clones of EBV strain M-ABA covering the entire EBV genome have been described by Polack et al. (1984b). The pseudo-β-actin gene (Moos & Gallwitz, 1983) and the cloning vector pHC79 served as positive and negative controls, respectively.

Southern blot hybridization. Cosmid and plasmid DNA clones were digested with appropriate restriction endonucleases and the resulting fragments separated by electrophoresis on 1% agarose gels in TBE buffer (50 mm-Tris–HCl, 1 mm-EDTA, 50 mm-boric acid). The manufacturer's protocol was used with minor modifications for Southern blotting of gels onto Zeta-Probe filters (Bio-Rad). Hybridization was performed at 65 °C according to the method of Church & Gilbert (1984). After hybridization the filters were rinsed several times for 30 min each at 55 °C in 0.1 x SSC, 1% SDS, twice for 5 min in 2 x SSC and once in 2 x SSC containing 10 μg/ml R N Ase A (boiled for 2 min before use) for 15 min at 20 °C. The filters were air-dried and exposed to a Kodak X-Omat AR film using a Dupont Cronex Lightning Plus Intensifying Screen.

Autoradiograms were evaluated in a semi-quantitative fashion by densitometric scanning on a 2222-020 UltraScan XL Laser Densitometer.
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Results and Discussion

EBV transcription during latency

To investigate the relative transcription rate of the EBV genome during latency, nuclear run-on synthesis reactions were performed with nuclei of uninduced Raji cells. The extracted $^{32}$P-labelled RNA was hybridized to EBV DNA fragments immobilized on nylon membranes. For a semi-quantitative evaluation, autoradiograms were analysed by densitometric scanning, and the resulting values normalized with regard to size and thymidine content of a given DNA fragment (Fig. 3a). As a specificity control, nuclear run-on RNA was synthesized in nuclei of the EBV-negative BL line BL-41 and hybridized to a filter containing EBV DNA fragments. As shown in Fig. 1 (e, f, lanes 7), BL-41 run-on RNA hybridized only to a $\beta$-actin probe which served as a positive control. Hybridization with labelled nuclear run-on RNA from Raji cells allowed visualization of a number of different EBV DNA fragments (Fig. 1a). Transcription was sensitive to $\alpha$-amanitin, except for the region which is known to encode EBER1 and EBER2 (EcoRI J) (Fig. 1b, lane 1). The EBERs are transcribed predominantly by RNA polymerase III and presumably, to a lesser extent, by RNA polymerase II (Howe & Shu, 1989). The minor reduction in EBER transcription observed in the presence of 4 $\mu$M-$\alpha$-amanitin most likely resulted from inhibition of RNA polymerase II activity. The densitometric evaluation of the autoradiograms is shown in Fig. 3(a); it is apparent that the region transcribed by RNA polymerase III is the region of the viral genome with the highest transcriptional activity during latency. However, it is possible that this activity was overestimated owing to the presence of repeat sequences (Farrell, 1989). Repeated sequences may also have prevented quantitative evaluation of other parts of
the genome. A relatively high density of polymerases, although not as high as on EcoRI J, was observed in the region between positions 10 and 55 kb. This region encompasses the large internal repeats, the EBNA-LP and EBNA2 genes, and the left duplicated region (DL) carrying the left-hand lytic origin of replication. Transcriptional activity was intermediate in the region between positions 55 and 140 kb, and very low in the remaining part of the viral genome, with the exception of the right duplicated region (DR) and the terminal repeats. These regions, which carry the right-hand oriLyt and the TP transcription unit, respectively, showed intermediate transcriptional activity. Transcription of this region might again be detected at higher rates because of the presence of repeated sequences in these regions.

Transcription extended far beyond the EBNA1 open reading frame (ORF), which is supposed to be the last gene of a large transcription unit which is initiated at one of two promoters, one being located close to the plasmid origin of replication, the other within the large internal repeats. The EBNA1 transcription unit does not appear to differ from cellular genes like β-globin or c-myc, with primary transcripts elongated up to several kilobases downstream of the polyadenylation site (Hofer & Darnell, 1981; Eick, 1990). The distribution of RNA polymerases on this large transcription unit is consistent with the view that the different mRNAs encoding the various nuclear antigens are generated by alternative splicing.

A remarkable feature is the unequal distribution of RNA polymerases (i) along the transcription unit encoding the various nuclear antigens and (ii) along the viral genome in general. A higher density of polymerases on the large internal repeats probably reflects a relatively high rate of initiation within or in front of the repeats and a gradual decrease in the number of polymerases behind the EBNA2 gene. Additional variations may be due to new initiation events downstream of the first promoter and/or additional transcription of the antisense strand. Both phenomena have been shown to contribute to an unequal distribution of polymerases along the c-myc gene (Bentley & Groudine, 1986; Nepveu & Marcu, 1986; Nepveu et al., 1987; Eick et al., 1990). Furthermore, owing to the limitations of the experimental system and the problems related to quantitative evaluation of the results, which will be discussed in more detail below, not all variations are necessarily significant.

Another important point is that the rate of transcription is not reflected at the RNA level. The mRNA encoding LMP is the most abundant polyadenylated transcript expressed in Raji cells during latency (Laux et al., 1988a, b), although the transcriptional activity of the LMP gene is extremely low (Fig. 3a, map coordinates 168965 to 169207). This points to an important contribution of post-transcriptional regulatory mechanisms for the regulation of LMP expression. Studying the EBV-immortalized cell line IB4, Sample & Kieff (1990) made the same observation and came to essentially the same conclusion. Again, similarly to IB4 cells and other EBV-positive lymphoblastoid cell lines, DL and DR, which carry the lytic origins of replication, show significant transcriptional activity in uninduced Raji cells in the absence of appreciable amounts of DR and DL transcripts (Metzenberg, 1989; Sample & Kieff, 1990). Transcription of DR and DL (Fig. 3a, located in a 12 kb B95-8 deletion at map coordinate 152012, and map coordinates 50003 to 52817, respectively) may either be due to a very low level of spontaneous induction or reflect transcription which is not necessarily followed by processing and stabilization of RNA. Since even low amounts of DL and DR RNA would have been detected on Northern blots containing poly(A)+ RNA, we assume that both oriLyt-carrying regions are transcriptionally active at a low level during latency.

Transcription after induction of the viral genome with TPA and IUdR

Induction of an abortive cycle with TPA and IUdR resulted in a dramatic increase in the transcriptional activity of the viral genome in Raji cells. Fig. 2 shows Southern blot filters with EBV fragments representing the entire viral genome hybridized to 32P-labelled nuclear run-on RNA synthesized between 8 and 32 h after the addition of TPA and IUdR. The nuclear run-on RNA hybridized strongly to most of the fragments, suggesting that large parts of the viral genome were transcriptionally activated. The only region which was transcribed at a low rate upon induction was the part of BamHI Nhet which is located in the short unique region (map coordinates 0 to 3955) (Fig. 2a, DNA fragment BamHI N). EcoRI J, encoding EBER1 and -2, was transcribed at a high rate after induction. RNA synthesized in the presence of α-amanitin hybridized only weakly to the various EBV DNA fragments, except for EcoRI J, indicating that the induced genes were transcribed by RNA polymerase II (Fig. 1c, d, lanes 1). Also, in the presence of α-amanitin in combination with TPA and IUdR, the rate of transcription of the EBER genes was slightly decreased compared to that seen with TPA and IUdR treatment alone. As stated above, this is probably caused by the inhibition of RNA polymerase II, which is, to a minor extent, also responsible for transcription of these genes (Howe & Shu, 1989). A semi-quantitative evaluation of a similar induction series (TPA and IUdR treatment for between 4 and 48 h, only shown for pM966-20, Fig. 2f) obtained by densitometric
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Fig. 2. Transcriptional activity across the EBV genome after induction with TPA and IUDR for different periods of time. Southern blots of cloned M-ABA EBV DNA digested as in Fig. 1 except cmB-14, digested with BglII (a to f, cmMSalI-A, cmB-14, cmM302-23, cmM301-99, cmM302-21 and pM966-20), were hybridized with nuclear run-on RNA from nuclei prepared from Raji cells induced with TPA and IUDR for 0, 8, 12, 16, 20, 24 and 32 h (lanes 1 to 7, a to e) or 0, 4, 16, 24 and 48 h (lanes 1 to 5, f). Lanes were cut out of different autoradiograms and grouped to show the time course of transcriptional induction. Panels (a) to (e) were taken from one induction series, whereas panel (f) originates from a different experiment. The autoradiograms are flanked by stained gels showing the bands generated by digestion of cosmid or plasmid DNAs with the respective restriction endonucleases. The letters to the left of each panel refer to the restriction fragments generated. EBV restriction fragments which are only partly present within the cloned DNA are marked with a triangle. (g) The BamHI map of the entire EBV genome, and restriction maps of the different cosmid and plasmid clones. The positions indicated in kb refer to the map coordinates of the B95-8 EBV sequence (Baer et al., 1984). The lettering of restriction fragments is according to Polack et al. (1984b).

scanning of autoradiograms is shown in Fig. 3(b). This quantification has to be taken with some caution, however, because the response of the film to $^{32}$P is not linear. In particular, to evaluate faint bands autoradiograms had to be used for scanning which were overexposed with regard to bands yielding strong signals.

Since hybridization had to be normalized for fragment size, the activity of large fragments with moderate or high transcriptional activity might be underestimated. Despite these apparent limitations, the semi-quantitative evaluation is reliable for small fragments and regions of the genome with moderate or low transcriptional activity.
Fig. 3. (a) Transcriptional activity across the EBV genome in Raji nuclei. Semi-quantitative densitometric evaluation of the level of transcription on the EBV genome in uninduced Raji cells (■), compared to that in cells 48 h after induction with TPA and IUdR (□). Autoradiograms of a similar series of Southern blots, as shown in Fig. 2 (data not shown, except for pM966-20 which is shown in Fig. 2), were scanned densitometrically. The densitometric results were normalized for the size and the thymidine content of each DNA fragment. The discrepancy between the transcriptional activity and the amount of RNA transcribed from a particular region is particularly evident for fragment BamHI H2, encompassing part of the EBNA2 ORF (Fig. 3a, map coordinates 48429 to 49964). We have shown previously that the level of EBNA2 RNA is not altered upon induction, whereas transcriptional activity across BamHI H2 increases dramatically (Laux et al., 1988a). The polymerase density is apparently much higher on BamHI H2 than on BamHI Y (Fig. 3a, map coordinates 48848 to 49848 and 47007 to 48847, respectively). However, the difference between BamHI H2 and the adjacent BamHI H1 fragment (Fig. 3a, map coordinates 49849 to 54853) probably is not significant and is an artefact of densitometric scanning, because the activity of small fragments with relatively high transcriptional activity is substantially overestimated compared to that of large fragments with similar activity.

The high transcriptional activity of the BamHI H2 fragment may therefore be a consequence of the high rate of leftward transcription of the BHLF1 gene, which is located on the adjacent BamHI H1 fragment. The RNA polymerases apparently do not stop at the polyadenylation signal located in BamHI H1, but proceed into the adjacent fragment. Hybridization of run-on RNA to single-stranded probes could help to clarify this point.

An example of a fragment which is highly transcriptionally active in induced Raji cells although it carries a late ORF (BRLF1) is BgII j (Fig. 3a, map coordinates 119047 to 120343). This fragment is located within a region of the viral genome with high transcriptional activity in induced Raji cells and it is not clear whether BgII j belongs to a larger early primary transcription unit, as suggested for BamHI H2. Alternatively, regulation at the level of stabilization and processing of primary transcripts might also contribute to regulation of viral gene expression during the lytic or abortive cycle.

With regard to the importance of BZLF1 for induction of the lytic cycle, we were interested in whether BamHI Z, BamHI R and BamHI M are transcriptionally activated in an ordered fashion upon induction. As shown in Fig. 3(b), transcriptional activity on the fragments increased simultaneously, with activation of BamHI Z and M first detectable 4 h after induction. Induction of BamHI Z, R and M by TPA and IUdR was totally abolished in the presence of 50 μg/ml CH (Fig.
indicating that these early genes are regulated exclusively at a transcriptional level. In CH-treated cells, the transcriptional activity of the whole viral genome decreased with time (data not shown). The fact that neither so-called immediate early RNAs (Laux et al., 1988a) nor enhanced transcription of the EBV genome can be detected after induction with TPA and IUdR in the presence of CH, can be explained by positive autoregulation by the trans-acting factor BZLF1 (Utier et al., 1988a) nor enhanced transcription of the EBV genome derived from the EBV P3HR-1 promoter in B-membrane cells.

We thank D. Eick for helpful discussion, G. U. Ryffel for advice in densitometric scanning and M. Dietl for typing the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB31, Medizinische Virologie: Tumorentstehung und -entwicklung) and Fonds der chemischen Industrie.

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(Received 20 November 1990; Accepted 3 June 1991)