Epstein-Barr Virus Induces Cellular Transcription Factors to Allow Active Expression of EBER Genes by RNA Polymerase III*

The EBER genes of Epstein-Barr virus (EBV) are transcribed by RNA polymerase (pol) III to produce untranslatable RNAs that are implicated in oncogenesis. These EBER transcripts are the most highly expressed viral gene products in EBV-transformed cells. We have identified changes to the cellular transcription machinery that may contribute to the high levels of EBER RNA. These include phosphorylation of ATF2, which interacts with EBER promoters. A second is induction of TFIIIC, a pol III-specific factor that activates EBER genes; all five subunits of TFIIIC are overexpressed in EBV-positive cells. In addition, EBV induces BDP1, a subunit of the pol III-specific factor TFIIIB. Although BDP1 is the only TFIIIB subunit induced by EBV, its induction is sufficient to stimulate EBER expression in vivo, implying a limiting function. The elevated levels of BDP1 and TFIIIC in EBV-positive cells stimulate production of tRNA, 7SL, and 5S rRNA. Abnormally high expression of these cellular pol III products may contribute to the ability of EBV to enhance growth potential.

EBERs can stimulate DNA and protein synthesis when trans-fection into normal cord blood lymphocytes in the absence of other viral genes (8, 9). Transfection of EBER genes into NIH3T3 cells or EBV-negative BL lines can allow them to form colonies in soft agar (10, 11). Furthermore, the EBER-transformed BL cells can produce tumors in mice (12–14). Because EBER transcripts are not translated, these remarkable discoveries provided the first evidence of oncogenic RNA. The massive expression of EBER genes is a striking feature of EBV-transformed cells, yet little is known about how it is achieved. Several observations suggest that it is not simply due to inherent strength of the EBER promoters but instead requires EBV-induced changes to the cellular environment. For example, stable transfection of EBER1 alone allowed maximal expression of ~10^5 transcripts/cell, whereas latent infection with the EBV genome can result in ~10^7 EBER1 transcripts/cell (11, 15). However, significant EBER expression only becomes apparent 36 h after infection, following the appearance of other EBV latent gene products (16). Furthermore, when latently infected cells switch to lytic viral replication, EBER gene transcription decreases dramatically (17). These data point to a strong influence of trans-acting factors in controlling the EBERs. We present evidence that the high levels of EBER expression in latently infected tumor cells reflect changes to the host transcription machinery. Thus, the pol III-specific transcription factors TFIIIC and BDP1 are both overexpressed in EBV-positive cells of lymphoid or epithelial origin. This is associated with a specific increase in levels of some pol III products. We show that induction of TFIIIC may be mediated, in part, by ATF-2, which undergoes an activating phosphorylation in response to EBV. ATF-2 also interacts with the EBER genes, which may further enhance their transcription. These combined effects can explain the high levels of EBER RNA that are diagnostic of EBV-associated tumors (5, 6).

MATERIALS AND METHODS

Cell Culture and Extraction—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen). Honel, Ad/AH, and Akata cells were cultured in RPMI (Invitrogen). All media were supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. G418 (300 µg/ml) was included in the media of EBV-
positive HeLa, Ad/AH, and Akata cells. Whole cell protein extracts were prepared as previously described (18).

**Antibodies and Western Blotting**—Western blotting was carried out as previously described (18). Ab7 against TFIIIC220 was a generous gift from Dr. Arnie Berk (19). We have described previously antibodies 4286 against TFIIIC110 (20), 128 against BRF1 (21), and 2663 against BDP1 (21). Antibody 3238 against TFIIIC102 was raised by immunizing rabbits with synthetic peptide MSGFSPLIDYLEGK (human TFIIIC102 residues 1–15) coupled to keyhole limpet hemocyanin. Antibody 1898–64 was prepared by affinity purification of anti-serum 1898 against TFIIIC90 (22) using synthetic peptide GMGNADDEQQEETGSC (human TFIIIC90 residues 613–627). Antibodies 58C9 against TBP, M-19 against TAF, 48, L-19 against TFIIIC63, N-96 against total ATF-2, F-1 against ATF2 phosphorylated at Thr-71, and C-11 against actin were from Santa Cruz Biotechnology.

**Transfection Assays**—HeLa cells were transfected in Opti-MEM medium (Invitrogen) using Lipofectamine (Invitrogen) transfection reagent. Cells were incubated for 6 h in transfection mixture and then in fresh medium for an additional 40 h before harvesting. For luciferase assays, extracts were made in passive lysis buffer (Promega) according to the manufacturer’s instructions. Luciferase levels were then quantified using a Luminoskan Ascent Luminometer (Labsystems) using the dual-luciferase assay kit (Promega). Firefly luciferase activity was normalized against Renilla luciferase activity from cotransfected plasmid Ubi-Renilla (31).

For RT-PCR analysis, RNA was extracted 48 h after transfection using TRI reagent (Sigma) according to the manufacturer’s instructions. Protein for Western blots was extracted as previously described (18).

**Chromatin Immunoprecipitation**—ChIP assays were performed as previously described (26) using antibodies Ab7 against TFIIIC220, 3238 against TFIIIC102, 1H4 against EBNA1 (27), M-19 against TAF,48, H-79 against c-Jun, and N-96 against ATF-2 (Santa Cruz Biotechnology). Amplification of the EBER1 gene region used the same primers and cycling parameters as above. Primers and PCR conditions have been described for ARPP P0, tRNA, and 5S rRNA genes (24, 26). Primers 5′-GTTTGCAGTTCCCCTGGTTAC-3′ and 5′-GACCACGAGTGTACCT-3′ were used to give a 143-bp product; cycling parameters were 95 °C for 150 s, followed by 25 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s and then 5 min at 72 °C.

**Promoter Constructs and Mutagenesis**—The TFIIIC220 promoters above were used to amplify from HeLa genomic DNA a fragment that was cloned into pGEM-T easy vector (Promega) to give pGEM-220. Reporter construct pGGL-220 was then made by digesting pGEM-220 with Spel and Ncol and subcloning the resultant fragment into pGL3-Basic vector (Promega) treated with Ncol and Nhel. pGGL-220 contains a 261-bp fragment extending from 167 bp upstream of the predicted TFIIIC220 transcription start site to 94 bp downstream.

PCR mutagenesis (28) was used to introduce substitutions into the CRE of the TFIIIC220 promoter in pGEM-220, with 5′-CGGGAGATGTGGATCATGCGCGC-3′ and 5′-GCGGCATTGACACACTCCCCG-3′ overlapping oligonucleotides and Sp6 and 5′-GGGCCGTATCTTCTTCATAGCC-3′ flanking primers. The fragment was then treated with Spel and Ncol restriction endonucleases and cloned into Nhel- and Ncol-digested pGL3-Basic vector to give pGGL220mt.

**RESULTS**

**EBV Can Stimulate Expression of Cellular pol III Transcripts and TFIIIC in HeLa Cells**—Several DNA tumor viruses have been found to stimulate transcription of cellular pol III templates (reviewed by Ref. 33). To test whether this is also the case...
for EBV, we used RT-PCR to assay expression of pol III transcripts. Representatives were examined of each of the three types of promoter arrangement that are used by pol III (Fig. 1A). 5S rRNA genes have type 1 promoters comprising A- and C-blocks embedded within the transcribed region; tRNA genes have type 2 promoters composed of A- and B-blocks that are also located within the transcribed region; 7SK, MRP, and U6 snRNA genes have type 3 promoters that involve TATA boxes and proximal sequence elements that are located upstream of the transcription start site (33–35). EBER gene promoters can be considered a hybrid, with internal A- and B-blocks that are typical of type 2 promoters, as well as upstream motifs, including a TATA box, that contribute to transcription (29, 36, 37). The cellular 7SL genes have an upstream promoter arrangement similar to the EBER genes, as well as important internal promoter sequences (36, 38–40). Levels of 5S rRNA, tRNA, and 7SL RNA were found to be significantly elevated in EBV-positive HeLa cells when normalized to the control mRNA encoding acidic ribosomal phosphoprotein P0 (ARPP P0), a pol II product (Fig. 1B). This suggests that EBV, like several other DNA tumor viruses, may stimulate pol III transcription. However, none of the type 3 promoter products examined (U6, 7SK, and MRP RNA) showed evidence of induction. Thus, the stimulatory effect of EBV on cellular class III gene expression shows clear selectivity.

TFIIIC is a pol III-specific transcription factor that is required by type 1 and 2 promoters, but not by type 3 (33–35). Because EBV induces types 1 and 2, but not type 3, we examined whether it is regulating TFIIIC. Western blotting demonstrated that all five subunits of TFIIIC are expressed at elevated levels in HeLa cells infected with EBV, whereas actin appears unchanged (Fig. 1C). The mRNA encoding the TFIIIC220 subunit is overexpressed in the EBV-positive HeLa cells (Fig. 1D), but there is little consistent change in levels of the mRNAs for the other four subunits of TFIIIC (two distinct transcripts are detected from the TFIIIC102 gene, probably due to alternative splicing). We conclude that EBV can induce TFIIIC and that this may, under some circumstances, involve a level of post-transcriptional control that has not been reported previously.

EBV Induces TFIIIC Expression in Several Cell Types—In addition to HeLa, we examined whether EBV can activate the pol III machinery in other cell types. Two models of EBV-in-
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A

|   | Ad/AH | Hone1 |
|---|-------|-------|
| EBV | +     | +     |
| 5S  |       |       |
| tRNA |       |       |
| 7SL |       |       |
| U6  |       |       |
| MRP |       |       |
| ARPP |   | P0    |

B

|   | Ad/AH | Hone1 |
|---|-------|-------|
| EBV | -     | +     |
| TFIIC220 |     |       |
| TFIIC110 |     |       |
| TFIIC102 |     |       |
| TFIIC90  |     |       |
| TFIIC63 |     |       |
| ARPP | P0    |       |

C

|   | Akata |
|---|-------|
| EBV | +     |
| 5S  |       |
| tRNA |       |
| 7SL |       |
| U6  |       |
| MRP |       |
| ARPP | P0    |

D

|   | Akata |
|---|-------|
| EBV | +     |
| TFIIC220 |     |
| TFIIC110 |     |
| TFIIC102 |     |
| TFIIC90  |     |
| TFIIC63 |     |
| ARPP | P0    |

FIGURE 2. EBV can induce expression of TFIIC and endogenous pol III products in Ad/AH, Hone1, and Akata cells. A, RT-PCR analysis of the indicated transcripts in matched EBV-negative (lane 1) and EBV-positive (lane 2) Ad/AH and Hone1 cells. B, RT-PCR analysis of the indicated TFIIC mRNAs in matched EBV-negative (lane 1) and EBV-positive (lane 2) Ad/AH and Hone1 cells. C, RT-PCR analysis of the indicated transcripts in matched EBV-negative (lane 1) and EBV-positive (lane 2) Ad/AH and Hone1 cells. D, RT-PCR analysis of the indicated TFIIC mRNAs in matched EBV-negative (lane 1) and EBV-positive (lane 2) Akata cells.

Reduced carcinomas were tested, the Hone1 cell line, which was isolated from an NPC, and the Ad/AH line, which is derived from an adenocarcinoma of the nasopharynx (41). As in HeLa cells, EBV infection of Ad/AH and Hone1 cells stimulates expression of 5S rRNA, tRNA, and 7SL RNA, but not 7SK or MRP RNA (Fig. 2A). U6 RNA levels increase slightly in Ad/AH cells, but not in Hone1. Viral induction of pol III transcription therefore shows specificity. In both these cell lines, it is accompanied by a clear increase in the mRNAs encoding all five subunits of TFIIC (Fig. 2B). Elevated TFIIC expression may therefore be a common feature of EBV-infected carcinoma cells.

As a model of Burkitt lymphoma, we used the Japanese BL-derived Akata line along side a matched EBV-negative subclone that was isolated from parental Akata cells by limiting dilution (42). As in the carcinoma models, expression of 5S rRNA, 7SL RNA, and tRNA is elevated specifically in EBV-positive Akata cells when compared with the EBV-negative derivative, whereas type III promoters show no evidence of activation (Fig. 2C). Similarly, the virally infected Akata cells overexpress mRNAs encoding the five TFIIC subunits (Fig. 2D). EBV can therefore induce TFIIC expression and endogenous pol III products in cells of disparate origin, representing virally induced lymphomas and carcinomas.

TFIIC Binds to EBER Genes in Vivo—The DNA sequences recognized by TFIIC are the A- and B-block internal promoter elements found in most pol III-transcribed genes, including tRNA and EBER genes (33, 35). However, as mentioned above, EBER promoters also have upstream elements that are important for transcription (29, 43). Deletion of the EBER2 B-block was found to ablate expression both in vitro and in vivo (29), but interpretation of this result is complicated by its location within the transcribed region, as loss of expression might reflect destabilization of the mutated transcript. Indeed, genomic footprinting revealed little evidence for TFIIC occupancy at EBER1 or EBER2 (37). We therefore considered it important to establish whether EBER genes utilize TFIIC in vivo. To this end, we carried out ChiPs to assay occupancy in EBV-infected HeLa cells (Fig. 3A). As positive control, we confirmed that the viral EBNA1 protein is bound in the vicinity of the EBER locus, as expected due to the proximity of its oriP recognition site within the viral genome (43). Clear evidence for the presence of TFIIC was obtained using antibodies against TFIIC220 and TFIIC102. An antibody against the pol I factor TAFI48 provided a negative control. Additional evidence of specificity was provided by the use of EBV-negative HeLa cells, which lack EBER genes and therefore give no signal. These data provide evidence that TFIIC does indeed interact with the EBER genes in vivo. In contrast, TFIIC was not detected at the pol II-transcribed acidic ribosomal phosphoprotein P0 gene (Fig. 3B).

The ChiP assay was also used to assess whether EBV infection changes the amount of TFIIC that is bound to endogenous class III genes. This revealed that TFIIC occupancy of chromosomal tRNA genes is significantly elevated in the EBV-positive cells (Fig. 3A). As expected, neither EBNA1 nor TAFI48 were detected at these genes. We conclude that the elevated expression of TFIIC following EBV infection can increase its occupancy of cellular target genes.

ATF2 Activation May Increase Transcription of the EBER and TFIIC220 Genes—In addition to the internal promoter that provides a binding site for TFIIC, both the EBER genes also have a consensus ATF recognition site (CRE) located ~50 bp upstream of the transcription start site (Fig. 4A). Deletion or point mutation of these sequences compromises expression of EBER1 and EBER2 in HeLa and BL cells (29, 43). We observed a similar effect in extracts of the Hone1 NPC line. Thus, point mutation of the CRE caused a marked reduction in EBER2 transcription relative to the wild type (Fig. 4B). This was the case for
both EBV-positive and EBV-negative Hone1 cell extracts. Fig. 4B also shows that extracts from EBV-positive cells give elevated pol III transcription relative to matched extracts from EBV-negative cells. This difference is not restricted to the EBER2 gene but is also seen with other pol III templates, including the adenoviral VA1 gene, which has a TFIIIC-dependent type 2 promoter (Fig. 4C). The relative weakness of the EBER2 promoter is apparent, giving much less transcription than the powerful VA1 template (Fig. 4C).

The occupancy of the EBER CRE motifs has been confirmed in vivo by genomic footprinting (37). However, the footprint did not show which CRE-binding protein(s) occupies these motifs. Several members of the AP-1 and ATF families have been shown to interact with CRE sequences (44). We found by ChIP that ATF2 associates with the EBER genes, but not with SS rRNA genes (Fig. 4D). Only background amplification was observed with negative control antibody against TAF48. Our data do not address the full constellation of factors that may bind the CRE in EBER promoters but do provide evidence for the presence of ATF2 in vivo.

ATF2 can be activated by mitogen-activated protein kinases that phosphorylate residue Thr-71 within its transactivation domain (45, 46). EBV has been shown to activate mitogen-activated protein kinase pathways and trigger hyperphosphorylation and activation of ATF2 (1, 47–50). Consistent with this, we found elevated levels of Thr-71-phosphorylated ATF2 in the EBV-positive cells (Fig. 4E). This did not reflect a consistent increase in the total level of ATF2 (Fig. 4F). Phosphorylation-mediated activation of ATF2 may therefore contribute to the overexpression of EBER genes.

A perfect consensus CRE sequence is also found 81–88 bp upstream of the human TFIIIC220 gene initiation site (Fig. 4A). This motif is conserved in other mammals but not in chickens or zebrafish. Electrophoretic mobility shift assay analysis indicated that this is a high affinity binding site that forms several complexes with HeLa cell proteins (Fig. 5A). Sequence specificity was demonstrated by efficient competition with unlabeled probe, but not with a B-block promoter sequence. Point mutation of the CRE motif (TGACGTCA -> TGGTGTC) abolished competition. The pattern of bands obtained with the TFIIIC220 sequence was identical to that seen using the paradigm CRE from the somatostatin gene promoter (51). However, competition indicated that complex formation occurs preferentially on the former, presumably due to some influence of flanking sequences. As expected from these data, ChIP analysis showed that ATF2 associates with the TFIIIC220 promoter in HeLa cells (Fig. 5B). This interaction is specific, because little or no ATF2 was detected in the vicinity of the TFIIIC110 promoter. With a c-Jun antibody, we obtained weak amplification of the TFIIIC220 promoter that was marginally above the background obtained with the TAF48 negative control; this suggests that c-Jun may be present, but is clearly not conclusive.

Because EBV stimulates phosphorylation of ATF2, which is associated with its activation, we tested whether the TFIIIC220 promoter might be induced through its CRE sequence. To this end, we subcloned into the pGL3 luciferase reporter a fragment that extends from 167 bp upstream to 94 bp downstream of the TFIIIC220 transcription start site. Expression of this construct, pGL220, was elevated in EBV-positive HeLa cells compared with EBV-negative cells after normalization for transfection efficiency (Fig. 5C). To test whether the CRE was responsible for this effect, we constructed pGL220mt, in which the CRE carries the 2-bp substitution that compromises protein binding in electrophoretic mobility shift assays. In contrast to the wild-type pGL220 reporter, the mutant pGL220mt is not activated by EBV (Fig. 5C). We conclude that the human TFIIIC220 promoter contains a functional CRE that mediates transcriptional induction in response to EBV. This response may be mediated through phosphorylation of ATF2 and may contribute to the elevated TFIIIC220 levels in EBV-positive cells.

**EBER Gene Transcription Can Be Stimulated by Raising the Level of TFIIIC or TFIIIB**—The data above show that TFIIIC associates with EBER genes in vivo and is expressed at elevated levels in EBV-positive cells. We therefore tested whether raising the level of TFIIIC can be sufficient to stimulate EBER gene transcription. For this purpose, fractions containing partially purified TFIIIC or pol III were titrated into Hone1 cell extracts (Fig. 6A). We also tested partially purified TFIIIB, another pol III-specific factor. Transcription of the EBER2 gene was stimulated in a dose-dependent manner by the TFIIIC fraction, and this response showed specificity, because little or no activation...
was obtained using the pol III fraction. Control experiments confirmed the activity of the pol III (data not shown); its failure to stimulate transcription suggests that pol III is in relative excess in Hone1 cell extracts as seen previously with other cell types (52–54). Similar results were obtained with extracts of HeLa cells (data not shown).

EBER2 expression can also be stimulated using highly purified TFIIIC generated by DNA affinity chromatography on a column carrying the B-block promoter sequence of the adenovirus VA1 gene (Fig. 6B). These data support the contention that an increase in TFIIIC levels can contribute to elevated EBER gene transcription. However, definitive proof will require the use of recombinant factor.

Bdp1 Induction by EBV Can Increase EBER Transcription—In addition to TFIIIC fractions, partially purified TFIIIB also stimulates EBER2 expression (Fig. 6A). We therefore examined whether TFIIIB, like TFIIIC, is targeted by EBV. TFIIIB has three subunits, BDP1, BRF1, and TBP (34, 35). Western blotting showed that introduction of EBV into HeLa, Hone1, Ad/AH, or Akata cells caused no increase in the expression of TBP or BRF1 (Fig. 7A). Indeed, BRF1 levels were somewhat decreased by the virus in some cases. However, the level of BDP1 protein was clearly elevated by EBV in all four of the cell lines. Although this increase was reflected at the mRNA level in HeLa and Akata cells, this was not the case in Ad/AH or Hone1, where a specific decrease in BDP1 (and BRF1) mRNAs was found (Fig. 7B). This suggests that, under certain circumstances, EBV can induce expression of BDP1 at the protein level as observed above with TFIIIC.

We tested directly whether the selective increase in BDP1 seen consistently in response to EBV is sufficient to stimulate EBER gene transcription. To this end, HeLa cells were transfected with a vector encoding hemagglutinin-tagged BDP1, along with the EBER1 gene and a green fluorescent protein reporter transcribed from the cytomegalovirus promoter. EBER1 RNA levels were increased significantly by overexpression of BDP1, a specific effect not seen with the cotransfected green fluorescent protein control (Fig. 7C). Therefore, the elevated BDP1 concentration observed in EBV-positive cells may indeed contribute to EBER induction.

**DISCUSSION**

High level expression of EBER genes is a feature of most EBV-associated lymphomas and carcinomas. Our data suggest that several distinct changes to the cellular transcription machinery contribute to this phenomenon. The EBER promoters contain an upstream CRE recognition site for ATF2 that makes a significant contribution to their activity (Fig. 4 and
EBV infection can lead to phosphorylation and activation of ATF2, which is likely to stimulate EBER transcription. In addition, we found a high affinity binding site for ATF2 that is conserved in the TFIIIC220 promoter. Like the EBER genes, TFIIIC220 expression may be increased through phosphorylation-mediated ATF2 activation. The EBV-positive cell lines we examined also overexpress the other four subunits of TFIIIC. This appears to involve distinct mechanisms of induction, as convincing matches to the CRE are not found at the other TFIIIC promoters and ATF2 was not detected at the TFIIIC110 promoter. Indeed, in some cases the induction occurs at the protein level, apparently without an associated increase in TFIIIC mRNA. We have confirmed that TFIIIC interacts with EBER genes in vivo and have in vitro evidence that elevated TFIIIC levels can increase EBER transcription. Our attempts to overproduce TFIIIC in vivo have so far failed to alter significantly the concentration of this factor at its target genes (55), so we have yet to prove that induction of TFIIIC has functional consequences in living cells, although this does seem highly likely. The TFIIIB subunit BDP1 is also overproduced in EBV-positive cell lines, and we have been able to confirm that this can stimulate transcription of the EBER genes in vivo. The combined effects of these various molecular changes may be sufficient to ensure that EBER RNA is expressed at exceedingly high levels.

The changes identified above can also be expected to impact on other pol III-transcribed genes. Indeed, we have found elevated levels of tRNA, 5S rRNA, and 7SL RNA in each of the four EBV-infected cell lines examined. In contrast, little or no consistent induction was found for MRP, 7SK, and U6 snRNAs, which are transcribed from type 3 promoters. Although type 3 promoters do not use TFIIIC, they do require BDP1 and might therefore be expected to respond to the increase in BDP1 levels that is trig-
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![Diagram of pol III transcription factors: Pol III, TFIIIB, TFIIIC]

**Figure 6.** Transcription of EBER2 can be stimulated using fractions containing TFIIIB or TFIIIC. A, in vitro transcription assay using 250 ng of EBER2 plasmid and 20 μg of Hone1 cell extract supplemented with 1 or 2 μl of A25(1.0) fraction containing TFIIIB (lanes 5 and 6, respectively), 1 or 2 μl of A25(0.15) fraction containing TFIIIC (lanes 8 and 9, respectively). B, in vitro transcription assay using 250 ng of EBER2 plasmid and 20 μg of HeLa cell extract supplemented with 1 or 2 μl of affinity-purified TFIIIC (lanes 2 and 3, respectively).

BDP1 is not a limiting factor for the transcription of U6 and MRP, 7SK, and U6 genes in the cell lines we have studied. This is consistent with the fact that MRP and 7SK RNA levels did not correlate with fluctuations in Bdp1 expression in a series of cervical carcinoma biopsies (25). Type 3 promoters require a complex factor called SNAPc or PTF that is not used by other pol III-transcribed genes (35). Perhaps SNAPc availability is limiting in the cell lines we have examined, so that the observed increase in BDP1 has little impact on type 3 promoters.

We were surprised to find a decrease in BRF1 expression in the EBV-positive Ad/AH, Hone1, and Akata cells. BRF1 has been shown to be limiting for type 2 promoters in HeLa cells and murine fibroblasts, as well as in Saccharomyces cerevisiae (55–57). However, BRF1 may be in relative excess in some cell types, perhaps including those used in the current study; under such circumstances, a small reduction in BRF1 levels might have little impact on transcriptional output if some other factor remains limiting.

Induction of TFIIIC by EBV appears complex. In each of the four cell lines examined, expression of the TFIIIC220 subunit involves an increase in the corresponding mRNA, an effect that may be mediated by the CRE in its proximal promoter. However, the remaining subunits show a curious mix, with mRNA induction in some cell lines but not in others despite elevated protein levels. For example, TFIIIC63 mRNA increases in response to EBV in Akata, Ad/AH, and Hone1 cells but apparently not in HeLa cells; nevertheless, TFIIIC63 protein is still overexpressed in HeLa cells. The molecular basis of this variable behavior is unclear. We are not aware of any previous reports that TFIIIC expression can be regulated in the absence of a corresponding change in mRNA. One possibility is that subunit turnover decreases in EBV-positive cells. However, direct measurements of stability in cycloheximide-treated cells provided no clear evidence that the virus is influencing turn-over (data not shown). Perhaps EBV can control TFIIIC expression at the translational level.

The relevance of these findings to human disease is suggested by a study that used cDNA microarrays and laser capture microdissection to examine mRNA expression in NPC biopsies (58). Only 1.2% of the 7200 cDNAs on the microarray showed consistent overexpression in NPCs relative to normal nasopharyngeal epithelium, but TFIIIC90 was found in this group (58). We also have preliminary evidence from immunostaining of biopsies that TFIIIC is expressed at elevated levels in NPCs. Previous work found that TFIIIC is frequently overexpressed at both the mRNA and protein levels in human ovarian carcinomas (24). In contrast, no consistent elevation of TFIIIC mRNA was detected in cervical carcinoma biopsies (25). The cervical study, however, was entirely at the RNA level and did not examine protein expression. Because we now have an indication that
TFIIIC production might be subject to post-transcriptional control, the possibility remains that TFIIIC might in fact be overexpressed in cervical cancers too despite the lack of induction of its mRNAs. Whether or not this turns out to be the case, the accumulating data clearly implicate TFIIIC deregulation in the molecular pathology of human cancer.

Has an increase in cellular pol III products been selected for during EBV evolution or has it evolved as a side effect of EBER gene activation? Support for the former possibility comes from the fact that several other DNA tumor viruses also induce expression of tRNA and 5S rRNA genes, including adenovirus, hepatitis B virus, SV40, polyomavirus, and human papillomavirus (reviewed in Ref. 33). Whereas adenovirus, like EBV, requires pol III to transcribe some of its own genes, this is not the case for these other viruses. Furthermore, as mentioned above, pol III transcripts are also overexpressed in ovarian carcinomas, which are not linked with a transforming virus. The consistent and selective overexpression of pol III in EBV-positive malignancies has been known for many years and indeed is used routinely for diagnostic purposes. Our perception of the importance of these genes has grown recently with the discovery of their unexpected transforming capacity. Under such circumstances, it is clearly valuable to ascertain how EBV transcription is controlled, as such knowledge has the potential to identify novel targets for therapeutic intervention. In addressing this issue, we have uncovered additional effects of EBV on cellular gene expression that had not been recognized previously. Such effects may contribute significantly to the abnormal behavior of EBV-infected cells.

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