Promoter switching in Epstein–Barr virus during the initial stages of infection of B lymphocytes

(mutant viral genomes/S1 nuclease protection/transfection of B lymphocytes/infection time course)

MAXIMILIAN WOISETSCHLAAGER, CHANDRA N. YANDAVA, LISA A. FURMANSKI, JACK L. STROMINGER, AND SAMUEL H. SPECK

Division of Tumor Virology, Dana–Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115

Contributed by Jack L. Strominger, December 26, 1989

ABSTRACT Transcription of the genes encoding the six viral nuclear antigens present in Epstein–Barr virus latently infected lymphocytes can be initiated from one of two promoters (Cp and Wp) mapping near the left end of the viral genome. These promoters are used in a mutually exclusive manner in clonal cell lines established from either Burkitt lymphoma tumors or *in vitro* infection of peripheral B lymphocytes. In this paper the role of Cp and Wp during viral latency is investigated. Cp appears to be the promoter normally employed during established latent infection. Analysis of two cell lines that use Wp revealed a deletion spanning Cp in the endogenous viral genomes, suggesting that cell lines exhibiting Wp activity harbor mutated viral genomes with a nonfunctional Cp. However, in contrast to the preferred usage of Cp exhibited by established Epstein–Barr virus-infected cell lines, Wp was shown to be exclusively utilized during the initial stages of viral infection. In addition to Wp activity, Cp usage was apparent by 6 days post-infection. A model is proposed involving B-lymphocyte differentiation-driven promoter switching during the establishment of viral latency.

Epstein–Barr virus (EBV) infection of B lymphocytes predominantly results in a latent infection in which little virus is produced with a concomitant growth transformation of the lymphocytes. Ten viral genes are known to be expressed during latency, six of which are transcribed from a long complex transcriptional unit that spans the lefthand 100 kilobases (kb) of the viral genome (for review, see ref. 1). The resulting mRNAs all share common 5' exons and contain unique 3' coding exons. Transcription is driven by one of two promoters, Cp or Wp, located near the left end of the viral genome (see Fig. 1). Only one of these promoters is active in any given clonal cell line (2).

In this paper the question of the functional roles of Cp and Wp during viral latency is addressed. We show that (i) when both functional promoters are present on a reporter construct, Cp is preferentially utilized by cell lines that exclusively use *either* Cp or Wp with their endogenous viral genomes; (ii) Wp is inactive in Cp-utilizing cell lines transfected with the same construct; (iii) the region encompassing Cp is deleted from the endogenous viral genomes of two Wp-utilizing lymphoblastoid cell lines (LCLs); (iv) deletion of the region containing Cp from a reporter construct containing a functional Wp induces Wp activity; and (v) Wp is exclusively used during the initial stages of EBV infection of B lymphocytes with activation of Cp apparent by 6 days post-infection. A model is proposed to account for these results.

FIG. 1. Transcriptional organization of the EBV nuclear antigen (EBNA) genes. Regions of the viral genome encoding the EBNA genes are indicated. The outer concentric arc represents the EBNA 1 primary transcript, and the two arrowheads at the 5' end represent transcription initiation at either Cp or Wp. The exploded portion of the diagram illustrates the organization of exons present at the 5' end of all EBNA mRNAs. The proximity of Cp and Wp to the latent origin of replication (*oriP*), which also functions as an EBNA 1-dependent enhancer, is shown. Only the lefthand-most Wp is illustrated. TR, terminal repeats.

MATERIALS AND METHODS

Cells, Transfections, and Chloramphenicol Acetyltransferase (CAT) Assays. X50-7 (3), JY (4), and IB4 (5) are human LCLs transformed with EBV. Jijoye (6) and Clone-13, a subclone of P3HR1 (7), are EBV-positive Burkitt lymphoma cell lines. B95-8 is a spontaneously productive marmoset LCL containing EBV from a human patient. All cell lines were grown at 37°C in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum. DNA transfection and CAT assays were carried out as described (2).

Plasmid Constructions and Site-Directed Mutagenesis. Site-directed mutations in CW1CAT were introduced according to Kunkel (8) using a commercially available mutagenesis kit (Bio-Rad). Cp and Wp promoter mutants were generated by altering the respective CCAAT boxes, thereby creating a new *EcoRI* recognition site, overlined (CP-mut37-mer: 5'-CTT-AGGGGCCGCGAATTCCTTAAGGGCTACATTTTTC-3'; Wp-mut38-mer: 5'-CCTCCCTAGAACTGAGAATTCCT-

GCTGTCTGGCTTAC-3'). Genomic DNA blotting was carried out as described (9) using random primed radiolabeled DNA fragments (Amersham) as probes.

RNA Preparation and S1 Nuclease Analyses. Total cellular RNA was prepared by the method of Auffray and Rougeon (10). Fifty micrograms of RNA was used in S1 nuclease protection analyses with specific oligonucleotides as described (2). The C1-58mer is specific for Cp activity and is described in ref. 2. The W0W1-60mer spans the W0W1 exon splice junction, extends upstream of the cap site, and is therefore diagnostic of Wp activity (W0W1-60mer: 5'-GTC-TCCCCTAGGATTTGTGTGGACTCCTGGCGCTCTGAT-GCGACCAGAAATAGCTGCAGG-3'). Due to the larger amount of input RNA in the assays, the concentration of S1 nuclease was raised to 500 units/ml.

In Vitro Infection of Peripheral Blood Lymphocytes. The EBV-positive Burkitt lymphoma line Akata served as a source for the production of immortalizing virus (11). Cells were resuspended in fresh medium at 1×10^6 cells per ml and virus production was induced by the addition of 1% goat anti-human IgG antibodies (Cappel Laboratories) for 48 hr at 37°C. Viral particles were concentrated 100-fold from cell supernatants by centrifugation at $13,000 \times g$ for 2 hr. Peripheral blood lymphocytes from a healthy donor were enriched by centrifugation in a Ficoll cushion (Pharmacia). One hundred microliters of concentrated Akata virus was added per 2×10^7 cells and incubated for 2 hr at 37°C in the presence of 100 ng of cyclosporin A per ml (Sandoz Pharmaceutical). Afterwards the cells were washed and resuspended in RPMI 1640 medium containing 10% fetal bovine serum and cyclosporin A. Aliquots were taken at various time points for the preparation of total cellular RNA.

RESULTS

Cp Is Preferentially Used when Both Promoters Are Present. To address the question of the activities of Cp and Wp in EBV-infected cell lines, several constructs were generated in which the region from *oriP* to the W1 exon was cloned upstream of the bacterial gene encoding CAT (Fig. 2). To distinguish between Cp and Wp activity, site-directed muta-

tions were introduced that destroyed the CCAAT boxes of Cp and/or Wp. The mutations effectively disrupted promoter function in constructs that contained limited upstream sequences (data not shown). The parent plasmid, CW1CAT, contains both functional promoters. These constructs were assayed in the Cp-utilizing cell lines Jijoye and JY and in the Wp-utilizing cell lines Clone-13 and X50-7 (Fig. 2). In all cases mutation of Wp did not significantly decrease the observed CAT activity, and in the case of the Jijoye cell line there was a reproducible increase in CAT expression. In contrast, mutation of Cp led to a significant decrease of CAT activity in all four cell lines. In the Cp cell lines (Jijoye and JY) the double promoter mutant [CW1CAT(mCp/Wp)] exhibited the same activity as the Cp mutant, further indicating that Wp is *not* functional in these cells. However, in the Wp cell lines (Clone-13 and X50-7) mutation of Cp and Wp did lead to a further decrease in activity compared to the Cp mutant. This effect was most pronounced in the Clone-13 cell line and may reflect Wp activity that was induced by mutation of Cp.

The residual activity observed with the construct in which Cp and Wp are mutated appears to be due to another, previously unrecognized, promoter mapping to the region upstream of Cp (M.W. and S.H.S., unpublished results). Notwithstanding the latter observation, the data indicate that transfection of a reporter construct containing functional Cp and Wp promoters results in preferential utilization of Cp in Cp- and Wp-utilizing cell lines, whereas the exogenous Wp exhibits little activity.

The results obtained with the CAT constructs were confirmed by S1 nuclease protection analyses of RNA isolated from cells transfected with a reporter construct containing both functional promoters. To ensure that all relevant cis-acting elements were likely to be present, a construct was generated in which the human β -globin gene was fused to the Y2 exon (CWY2BG; see Fig. 2). Thus, additional sequences downstream of Wp as well as upstream sequences were present. This construct was transfected into representative Cp (JY) and Wp (X50-7 and Clone-13) cell lines (Fig. 3). No Wp activity was detected with RNA prepared from the JY cell line, indicating that the exogenous construct mimicked

FIG. 2. Organization of the viral promoters involved in transcription of the EBNA genes. The structures of two sets of reporter constructs are shown with respect to a partial viral *Bam*HI restriction map. Vertical brackets indicate the fusion of the W1 exon with the CAT gene (CW1CAT series) or the Y2 exon with the β -globin gene (CWY2BG series). Small horizontal lines denote exons, arrows mark the positions of the promoters under study, and crosses indicate site-directed mutated Cp and/or Wp. The CAT activities of the four CW1CAT plasmids in four different cell lines are shown to the right. The numbers represent the means of at least three independent transfection experiments and are relative to the parent plasmid CW1CAT. JIJ, Jijoye; Cl-13, Clone-13.

FIG. 3. S1 nuclease protection experiments with specific oligonucleotides that are diagnostic for Cp (C1 oligo) or Wp (W0W1 oligo) activity. Cl-13, Clone-13. See Fig. 2 for the structures of transfected β -globin constructs.

the mutually exclusive Cp activity observed with the endogenous viral genome (Fig. 3). However, in the Wp-utilizing cell lines the exogenous construct displayed Cp activity (Fig. 3). This result is in direct contrast to the activity displayed by their endogenous viral genomes and confirms the results obtained with the CAT constructs (see Fig. 2). It should be noted that it was not possible to measure Cp activity of the exogenous construct in the JY cell line or Wp activity of the exogenous construct in the X50-7 cell line due to the activity of the endogenous viral genomes.

Cp Is Deleted in Some Wp-Utilizing Cell Lines. Although it is possible that the activities of Cp and Wp on the transfecting plasmid do not accurately reflect their activities in the intact viral genome, the results from these experiments raise the possibility that the endogenous Cp present in Wp active cell lines might not be functional. To investigate the question of whether mutations in the region of Cp may have occurred in

Wp active cell lines, Southern blots of *Bam*HI-digested genomic DNA from several cell lines were probed with either the entire viral *Bam*HI C fragment or a small fragment containing Cp (Fig. 4). The DNAs from two Cp-utilizing cell lines (JY and B95-8) and three Wp-utilizing cell lines (Clone-13, IB4, and X50-7) were analyzed. The expected size fragment (9.2 kb) was detected with DNA from Clone-13, B95-8, and JY (although the size of the fragment from JY is slightly smaller), when the entire *Bam*HI C fragment was used as a probe (Fig. 4A), but DNA from the IB4 and X50-7 cell lines revealed an $\approx$ 3.5-kb deletion in *Bam*HI C (labeled dC).

When the same DNAs were probed with a labeled Cp fragment, the deleted *Bam*HI C fragment present in IB4 and X50-7 did not hybridize, whereas the *Bam*HI C fragment in the other cell lines was readily detected (Fig. 4B). Thus, the deletion appears to encompass the region of Cp. Notably, the deleted *Bam*HI C fragments present in the IB4 and X50-7 genomes are nearly the same size, suggesting that deletion of specific cis-acting elements may be important for the Wp phenotype. It should be noted that we have identified several polymorphisms that distinguish the IB4 and X50-7 DNAs (data not shown), ruling out the possibility that both DNA samples were isolated from only one of these cell lines.

Deletion of Cp Induces Wp Activity in a Cp Active Cell Line. To address the question of whether the deletion sustained in the IB4 and X50-7 genomes is involved in the observed Wp activity exhibited by their endogenous viral genomes, a similar deletion was introduced into the CWY2BG construct by removal of the internal *Sac* II fragment in *Bam*HI C fragment [CWY2BG(dSacII); see Fig. 2]. This creates a 2.8-kb deletion spanning the region of Cp. As shown in Fig. 3, when transfected into the JY cell line the deleted construct (CWY2BG/dSacII) exhibited Wp activity, whereas the parent construct (CWY2BG) did not.

Wp Is Exclusively Employed Early in EBV Infection of B Lymphocytes. The data presented above suggest that Cp is the preferred promoter for driving transcription in established latently infected cell lines. This raises the question of the role of Wp during viral infection. Clearly the hallmark of EBV infection of B lymphocytes is the partial differentiation of resting or pre-B cells to a continuously proliferating lymphoblastoid state. This suggests a possible role for Wp during the initial stages of infection where it might function to initiate viral gene expression in infected quiescent cells.

To investigate the activity of Wp and Cp during the initial stages of infection, virus was prepared from the Akata cell line (11) by induction with anti-immunoglobulin and used to infect adult peripheral B lymphocytes. Cells were harvested at various time points after infection and RNA was prepared. As shown in Fig. 5, Wp activity could be detected by 18 hr and the level of activity remained relatively constant over the first 6 days after infection. In contrast, no Cp activity could be detected during the first 3 days after infection. However, by 6 days after infection Cp activity was readily apparent. At this time, both promoters appeared to be transcriptionally active. This may reflect ongoing infection events that result in a mixed population of lymphocytes at different stages of infection. The data indicate that Wp activity precedes Cp activity and may represent a programmed progression from the initial stages of infection to established latency.

DISCUSSION

In this paper the activities of Cp and Wp during latency have been investigated. The earlier observation that the usage of these two promoters is mutually exclusive (2) prompted a study of whether this phenomenon is regulated at the level of either cis-acting elements or trans-acting factors. It is clear from the results presented in this paper that introduction of

FIG. 4. Genomic Southern blots of *Bam*HI-digested DNA from five EBV-positive cell lines. Cl-13, Clone-13. (A) The entire viral *Bam*HI C fragment from B95-8 was used as a probe. C defines the wild-type 9.2-kb *Bam*HI C band. Its deleted form is designated as dC. W marks cross-hybridization of the probe with the viral 3.1-kb *Bam*HI W fragment. (B) A similar blot was probed with a 650-base-pair (bp) DNA fragment that contains the region around Cp (EBV coordinates 11,076–11,725).

FIG. 5. Infection time course of adult peripheral lymphocytes infected with Akata virus. Total cellular RNA harvested at the indicated times after infection was subjected to S1 nuclease protection analyses with Cp- and Wp-specific oligonucleotides. RNAs from the EBV-positive cell lines JY (Cp user) and X50-7 (Wp user) served as positive controls.

exogenous constructs containing both functional promoters into latently infected cells results in preferential usage of Cp. This result is consistent with the mutually exclusive behavior of these promoters observed endogenously. The fact that the plasmid Cp was predominantly active regardless of whether the endogenous viral genome is actively transcribing from Cp or Wp suggests that Cp is the promoter normally employed during latency. This implies that cell lines utilizing Wp are mutants, with respect to either the endogenous viral genomes or cellular transcription factors. The latter can seemingly be largely ruled out since the transfected constructs containing both promoters exhibit Cp activity in Wp-utilizing cell lines. However, in these studies we have only assayed promoter usage in two Wp-utilizing cell lines, and further studies with a larger panel of Wp-utilizing cell lines is required.

Analysis of the viral genomes present in the Wp-utilizing LCLs IB4 and X50-7 supports the hypothesis that Wp activity is the result of mutation of the viral genome. Furthermore, deletion of the region containing Cp in the transfected constructs resulted in the induction of Wp activity. This indicates that cis-acting elements in the region of Cp or Cp itself are involved in suppressing Wp activity.

It is important to note that the viral genomes present in the Clone-13 cell line contain Cp, demonstrating that Wp activity does not require deletion of this region of the viral genome. However, the virus present in the Clone-13 cell line (a subclone of the P3HR-1 cell line) is a nonimmortalizing strain that has sustained a major deletion that removed the entire EBNA 2 gene and a portion of the EBNA 4 gene (12). Another BL cell line, Daudi, also harbors a virus with a nearly identical deletion and interestingly this cell line also exclusively uses Wp (2). Thus, the distal lesions in the Clone-13 and Daudi cell lines may be functionally involved in their Wp phenotype. This cannot, however, completely explain the absence of endogenous Cp activity since the Clone-13 cell line exhibited Cp activity with the exogenous plasmid constructs. Thus, appropriate transcription factors for Cp activity are present in these cells. It therefore seems likely that

there are additional lesions in some cis-acting elements involved in controlling Cp and Wp activity.

An analysis of promoter usage during the initial stages of EBV infection revealed that Wp activity clearly precedes Cp activity. This result, in conjunction with the observed preferential utilization of Cp in cells with an established infection, suggests a model in which Wp is designed to function in resting or pre-B cells. It should be noted that Wp is present in multiple copies on the viral genome. It is possible that all of them are transcriptionally active during the initial stages of infection, which may reflect a need for an initial high level of viral gene expression. Subsequently, the viral antigens induce partial differentiation of the infected cells, a process that has been well documented (13). At an appropriate stage, which may correspond to the differentiation state found in LCLs, the requisite transcription factors for driving Cp become available and a switch from Wp to Cp occurs.

The mutually exclusive behavior of Cp and Wp observed in established cell lines would dictate that induction of Cp activity results in a concomitant down-regulation of Wp activity. Two well-characterized examples of developmentally regulated promoter switching have been described. The stage-specific promoter switch in the *Drosophila* alcohol dehydrogenase gene appears to be accomplished by transcriptional interference (14). In contrast, the developmentally regulated expression of the chicken ϵ - and β -globin genes is mediated by a cis-acting stage-specific element (15). In both cases, as in the case of Cp and Wp, the promoters appear to operate in a mutually exclusive fashion and the activity of the promoter situated downstream (proximal) precedes that of the upstream promoter (distal). A comparison of the CAT activities observed with the CW1CAT(mCp) and CW1CAT(mCp/Wp) constructs revealed no differences in Cp active cell lines, which argues against transcriptional interference as the mechanism of silencing Wp. However, the putative activity of a third promoter that appears to map to the region upstream of Cp, and is present in the double mutant construct, could function to suppress Wp activity by this mechanism. In contrast, the double promoter mutant did exhibit lower activity, compared to the Cp mutant, in Wp-utilizing cell lines (particularly in the Clone-13 cell line). This is consistent with a model involving transcriptional interference in suppressing Wp activity in Wp-utilizing cell lines. The discrepancy in the results obtained with these constructs in Cp- and Wp-utilizing cell lines may reflect differences in the availability of transcription factors necessary to drive Wp in these cell lines.

Finally, a possible reservation with the analysis of promoter activity using exogenous plasmid constructs is whether they accurately mimic the regulation exhibited in the intact viral genome. This issue may be further addressed by tagging the C1 and W0 5' exons to allow discrimination between exogenous and endogenous promoter activity (e.g., exogenous Wp activity in X50-7 cells). Furthermore, it would be desirable to assay promoter usage in resting B cells by transfection of these primary cells with the reporter constructs described here. This, however, has been historically difficult and awaits technical improvements.

This work was supported by Grants CA43143 (S.H.S.) and CA47554 (J.L.S.) from the National Institutes of Health, a Leukemia Society Special Fellowship to S.H.S. and a Postdoctoral Fellowship to M.W. by the Fonds zur Foerderung der Wissenschaftlichen Forschung, Vienna.

1. Speck, S. H. & Strominger, J. L. (1989) in *Advances in Viral Oncology*, ed. Klein, G. (Raven, New York), Vol. 8, pp. 133-150.
2. Woisetschlaeger, M., Strominger, J. L. & Speck, S. H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6498-6502.

3. Rowe, D. T., Farrell, P. J. & Miller, G. (1987) *Virology* **156**, 153–162.
4. Terhorst, C., Parkham, P., Mann, D. L. & Strominger, J. L. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 910–914.
5. King, W., Dambaugh, T., Heller, M., Dowling, J. & Kieff, E. (1980) *J. Virol.* **36**, 506–518.
6. Brandsma, J. & Miller, G. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6851–6855.
7. Rabson, M., Gradoville, L., Heston, L. & Miller, G. (1982) *J. Virol.* **44**, 834–844.
8. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488–492.
9. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
10. Auffray, C. & Rougeon, T. (1980) *Eur. J. Biochem.* **107**, 303–314.
11. Takada, K. & Ono, Y. (1989) *J. Virol.* **63**, 445–449.
12. Jeang, K. & Hayward, D. S. (1983) *J. Virol.* **48**, 135–148.
13. Gordon, J. (1989) in *Advances in Viral Oncology*, ed. Klein, G. (Raven, New York), Vol. 8, pp. 173–185.
14. Corbin, V. & Maniatis, T. (1989) *Nature (London)* **337**, 279–282.
15. Choi, O. B. & Engel, J. D. (1988) *Cell* **55**, 17–26.