Identification of Sequences in Epstein–Barr Virus DNA Required for theExpression of the Second Epstein–Barr Virus-determined Nuclear Antigenin COS-1 Cells

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SUMMARY

The BamHI WYH region of the Epstein–Barr virus (EBV) genome encodes a protein localized to the nucleus of the infected cell, the EBV-determined nuclear antigen EBNA2. We have constructed a series of recombinant vectors that carried the complete EBNA2 gene, or the gene modified so as to contain defined deletions involving presumed exons and regulatory elements of the gene. The recombinant vectors were transfected into COS-1 cells which permit the replication of simian virus 40 origin-containing plasmids to a high copy number, and the transient expression of EBNA2 was analysed. A recombinant plasmid that carried a BglII-NotI subfragment of the BamHI WYH region (nucleotides 44,664 to 50,628) contained all the information necessary for inducing the expression of a full length EBNA2 polypeptide. Moreover, EBV DNA sequences between nucleotides 45,442 and 48,337 could be deleted without interfering with the ability of the vectors to induce EBNA2. On the other hand the loss of the left one-third of the BglII-NotI fragment completely abolished the EBNA2-inducing capacity of the vector. A rightward promoter consensus sequence in the BamHI W part of the BglII-NotI fragment was functional in COS-1 cells expressing EBNA2 and essential for EBV-specific RNA synthesis. The results indicated that transcription of the EBNA2 gene was initiated in the BamHI W fragment, that the transcript was spliced and that all of EBNA2 was encoded within the continuous long open reading frame in the BamHI Y and H fragments.

INTRODUCTION

The lymphotropic Epstein–Barr virus (EBV) is ubiquitous in human populations and is intimately associated with some lymphoproliferative disorders (for review, see Klein & Klein, 1984). It has been firmly established that EBV is the causative agent of infectious mononucleosis. The role of EBV in the development of African Burkitt’s lymphoma (BL) and anaplastic nasopharyngeal carcinoma (NPC) is probably less direct. EBV infection leading to the presence of immortalized cells in the host might be the first in a sequence of events that lead to the development of malignant disease.

EBV is able to transform human B-lymphocytes in vitro into continuously dividing, immortalized cell lines. All EBV genome-carrying cells, including BL and NPC biopsies, contain an EBV-determined nuclear antigen, EBNA, detected by anti-complement immuno-fluorescence (ACIF) with sera from EBV-seropositive donors (Reedman & Klein, 1973). EBNA is, however, heterogeneous. One component of the ACIF-detected nuclear antigen, which has been designated EBNA1, is associated with a group of polypeptides that vary in size between 70K and 92K in different EBV-transformed cells. The exon coding for EBNA1 is contained within the BamHI K fragment of EBV DNA (Dillner et al., 1984; Fisher et al., 1984; Hennessy & Kieff, 1983; Summers et al., 1982). Four other EBV-determined, transformation-specific nuclear proteins, EBNA2, EBNA3, EBNA4 and EBNA5, have been defined by immunological
techniques (Dillner et al., 1986; Hennessy & Kieff, 1983; Hennessy et al., 1985, 1986; Kallin et al., 1986; Sculley et al., 1984; Strnad et al., 1981). DNA-mediated gene transfer experiments have demonstrated that the coding sequence for EBNA2 is within the BamHI WYH region of the EBV genome (Mueller-Lantzsch et al., 1985; Rowe et al., 1985; Rymo et al., 1985). The rightward long open reading frame in the BamHI Y and H fragments (BYRF1; Baer et al., 1984) contains EBNA2-specific sequence information. This has been shown by using antisera to a hybrid protein expressed in Escherichia coli from a part of the BamHI H fragment fused to the lacZ gene in an expression vector (Hennessy & Kieff, 1985). Similarly, rabbit antibodies against chemically synthesized peptides representing different parts of the BYRF1 open reading frame react with the EBNA2 polypeptide (Dillner et al., 1985). From these results it is not clear, however, whether BYRF1 encodes all of EBNA2 or if other parts of the genome, e.g. the neighbouring open reading frames in the BamHI W and Y fragments, are also involved. Recently, Dambaugh et al. (1986) found that a vector construct containing a murine leukaemia virus promoter and a cap site upstream of the BYRF1 open reading frame, induces the expression of a full size EBNA2 in rodent fibroblasts. The results provide strong evidence that EBNA2 is translated from the BYRF1 open reading frame beginning with the potential ATG initiation codon at position 48505.

We have recently demonstrated that mouse cells stably transfected with a series of EBV DNA-containing recombinant vectors, the common denominator of which was the BamHI WYH fragment region, express a nuclear antigen similar in size and immunoreactivity to the EBNA2 polypeptide in EBV-transformed lymphoid cells (Rymo et al., 1985). In the present study we continued the characterization of the putative EBNA2 gene using COS-1 monkey cells. These cells permit the replication of the simian virus 40 (SV40) origin-containing recombinants to a high copy number (Mellon et al., 1981) giving a strong transient expression of the EBNA2 gene. Subfragments of the BamHI WYH region and fragments modified so as to contain deletions involving presumed exons and regulatory elements of the gene were introduced into the cells and EBNA2 expression was analysed at the RNA and polypeptide levels.

METHODS

Cell culture and DNA transfections. COS-1 monkey kidney cells were obtained from Y. Gluzman (Cold Spring Harbor Laboratory, N.Y., U.S.A.). The cells were maintained in Iscove's modification of Dulbecco's medium (Gibco) containing 10% foetal calf serum and were subcultured for 40 h before transfection. DNA transfections were performed by a modification of the technique of McCutchan & Pagano (1968). Cells were trypsORIZED, washed and suspended in a solution containing DEAE-dextran (0-5 mg/ml; mol. wt. 500000, Pharmacia) and the appropriate recombinant vector DNA (107 cells and 10 μg of DNA per ml). The suspension was incubated for 60 min at 37 °C. After the transfection the cells were washed, exposed to 15% (v/v) dimethylsulphoxide for 2 min at 37 °C (Sussman & Milman, 1984), washed again, seeded on culture dishes and then incubated for 4 h at 37 °C in medium containing 100 μM-chloroquine (Luthman & Magnusson, 1983). This treatment increased the efficiency of transfection as judged from the fraction of cells that expressed nuclear antigens after transfection.

For EBNA staining the cells were harvested from monolayers by trypsinizing, washed twice and resuspended in a solution containing 0-8 mM-MgCl2, 1 mM-CaCl2, 30 mM-glycerol pH 7 and 20% (v/v) foetal calf serum. Smears were prepared by air-drying a drop of dense cell suspension on a microscope slide followed by fixing in acetone: methanol (2:1) at -20 °C for 5 min. The cell smears were hydrated in phosphate-buffered saline (PBS) and incubated with EBNA antibody-positive human serum diluted 1:10, for 15 min at 37 °C in a humidified atmosphere. After washing in PBS the smears were incubated for 15 min at 37 °C with serum from EBV-negative donors (diluted 1:10) as the complement sources. Fixed complement was detected by incubation with a suitable enzyme cleavage and purified by agarose gel electrophoresis. The staggered ends of the fragment were repaired by...
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incubation with the large fragment of DNA polymerase I and the four deoxynucleoside triphosphates. The BglII–NotI fragment was then ligated with T4 DNA ligase to BglII linkers (C-A-G-A-T-C-T-G; New England Biolabs) present in 200-fold excess and purified from unbound linker molecules by gel chromatography on AcA22 (LKB). Following digestion with BglII and repurification on an AcA22 column the fragment was ligated into the BglII site of pSV2-gpt/BglII and the mixture was used to transform E. coli HB101. Small scale plasmid preparations were analysed by restriction enzyme cleavage to identify a recombinant plasmid which contained the BglII–NotI fragment in such an orientation that the BamHI W fragment part was next to the SV40 ori-containing segment. This plasmid was designated pEÅ6.

When the plasmid pEÅ6 was propagated in E. coli a spontaneous mutant, pEÅ7, was isolated. The pEÅ7 plasmid contained a 2-2 kilobase pair (kbp) deletion in the fragment (Fig. 1). The precise location of the deletion was determined by DNA sequencing using fragments 5’ end-labelled at the BamHI site at position 48848 with [γ-32P]ATP. The deletion spans from nucleotides 46560 to 48777 (data not shown).

Plasmid pÅ8 was constructed by deleting the PvuII-XhoI subfragment from the BglII–NotI fragment of pEÅ6. Plasmid pÅ6 DNA was digested to completion with PvuII and XhoI and the larger of the two fragments, which contains the pSV2-gpt/BglII vector and both ends of the BglII–NotI fragment, was isolated by agarose gel electrophoresis. The fragment was repaired with the large fragment of DNA polymerase I, ligated with XhoI linkers, recircularized and used to transform E. coli HB101.

Plasmid pÅ9 was obtained by deleting the XhoI–HindIII subfragment from the BglII–NotI fragment of pEÅ6. Plasmid pEÅ6 DNA was cleaved with XhoI and HindIII and the larger of the two fragments was isolated by agarose gel electrophoresis. The deleted plasmid was ligated with XhoI linkers, recircularized and used to transform E. coli HB101.

Plasmids pÅ10 and pÅ11 were constructed in the following manner. PvuII–NotI and the XhoI–NotI subfragments of the BglII–NotI fragment containing BamHI WHY and BamHI YH sequences, respectively (see Fig. 1), were prepared from pÅ6 DNA, ligated to BglII linkers and inserted into the BglII site of the pSV2-gpt/BglII vector as described above. After transformation of E. coli HB101, the recombinant plasmids were characterized by restriction endonuclease mapping. Plasmids containing the inserted fragment in the same orientation as in pÅ6 were chosen for further work.

The plasmids pÅ12 and pÅ13 were obtained by enzymic deletion progressing bidirectionally from the PvuII site in the BamHI W fragment of pÅ6. Ten μg of pÅ6 DNA was linearized by cleavage with PvuII and digested with 1 unit of Bal31 exonuclease (Boehringer Mannheim) for 30 min at 30 °C in a 400 μl volume containing 10 mM-Tris–HCl pH 8.0, 200 mM-NaCl, 12 mM-CaCl2, 12 mM-MgCl2 and 1 mM-EDTA. The reaction was terminated by the addition of 50 μl of 500 mM-EDTA and the mixture was phenol-extracted, ether-extracted and ethanol-precipitated. The shortened DNA was repaired with the large fragment of DNA polymerase I, ligated to synthetic PvuII linkers (C-C-A-G-C-T-G-G; New England Biolabs) purified on AcA22, redigested with PvuII, circularized with T4 DNA ligase and used to transform E. coli HB101. Colonies were screened for the size of the BamHI subfragments of the BglII inserts by small scale plasmid isolation and restriction enzyme cleavage. Selected plasmids were also screened by digestion with BglII and PvuII to determine the size of the fragment that carries the BamHI W promoter sequence and to estimate the extent of enzymic deletion. The endpoints of the deletions in pÅ12 and pÅ13 were resolved at the nucleotide level by DNA sequencing and were 45170 to 46868 and 45442 to 47010, respectively.

Plasmids pÅ14 to pÅ27 were obtained from pÅ9. Plasmid DNA was linearized with XhoI, digested with Bal31 exonuclease, repaired with the large fragment of DNA polymerase I, ligated to XhoI linkers, circularized with T4 DNA ligase and used to transform E. coli HB101. The approximate size of the right XhoI–BamHI fragment of the BamHI Y fragment, which contains the left part of the BYRF1 open reading frame, was determined by restriction enzyme cleavage and electrophoretic analysis. The precise locations of the right endpoints of the deletions in the plasmids pÅ19 to pÅ23 were determined by nucleotide sequence analysis and corresponded to nucleotides 48337, 48362, 48412, 48440 and 48435, respectively.

PAGE and immunoblotting. COS-1 cells were collected 2 to 3 days after transfection and dispersed by sonication at a concentration of 50 × 10⁶ cells/ml in 10 mM-sodium phosphate buffer pH 6.8 containing 2% SDS, 10 mM-mercaptoethanol, and 0.1 mM-PMSF. The cell extract was boiled for 5 min and centrifuged at 15000 g for 10 min. Samples of 50 μl or less were loaded on the polyacrylamide gels. SDS–PAGE was performed in 5% stacking, 10% separating polyacrylamide gels essentially as described by Laemmli (1970). The electrophoretic transfer of proteins to nitrocellulose membranes was performed as described by Burnette (1981). Transfer buffer was 25 mM-Tris–base, 192 mM-glycine, 0.05% SDS and 20% methanol. The electrophoretic transfer was accomplished in a Bio-Rad Trans-Blot apparatus at 6 V/cm for 16 h at 4 °C. The nitrocellulose filter was blocked by incubation for 2 h at 37 °C in a solution containing 10 mM-Tris–HCl pH 7.4, 150 mM-NaCl, 3% (w/v) bovine serum albumin (BSA; fraction V), and 0.25% gelatin (w/v). The filter was incubated in 20 ml of a 1:10 dilution of an EBNA antibody-positive human serum in the blocking buffer. After 2 h of incubation at 37 °C the filter was washed with three changes over about 30 min in 200 ml of 10 mM-Tris–HCl pH 7.5, 150 mM-NaCl, 0.25% NP40, 0.25% sarcosyl. The
filter was then incubated for 1 h at 37 °C with $^{125}$I-labelled Protein A (5 × 10^5 c.p.m./ml) in blocking buffer supplemented with 5 mM-NaI. The filter was washed five times in the washing buffer above and air-dried.

Separation and labelling of cellular RNA and hybridization to DNA fragments. Total cellular RNA was isolated by extraction with guanidinium isothiocyanate and centrifugation through a caesium chloride cushion essentially as described by Chirgwin et al. (1979). Polyadenylated [poly(A) +] RNA was selected for by chromatography on oligodeoxythymidylate-cellulose. The procedures for the labelling of RNA with $^{125}$I and hybridization to electrophoretically separated DNA fragments immobilized on nitrocellulose membranes have been described (Rymo, 1979).

S1 endonuclease analysis. S1 endonuclease analysis of the transcripts was carried out as described previously (Weaver & Weissmann, 1979; Berk & Sharp, 1977; Svensson et al., 1983). Briefly, 5' end-labelled restriction fragments derived from the BgII-NotI fragment were denatured and annealed to 5 μg of poly(A)+ RNA isolated from COS-1 cells transfected with the BgII-NotI fragment. The hybrids were treated with S1 nuclease, precipitated with isopropanol and electrophoresed in a polyacrylamide gel with 7 M-urea alongside sequencing reactions of the corresponding fragment and using restriction enzyme cleavage fragments of pBR322 as size markers.

DNA sequence analysis. DNA sequence analysis was performed by the chemical modification method of Maxam & Gilbert (1980).

RESULTS

Effect of deletions in the putative EBNA2 gene on antigen expression in COS-1 cells

In a previous report we showed that EBNA2 expression can occur in 3T3 mouse fibroblasts stably transformed by selection vectors containing the BamHI W, Y and H fragment regions of B95-8 EBV DNA. To confirm this result and to define more closely the coding and non-coding DNA sequences required for EBNA2 expression we constructed plasmids that carried the complete putative gene for EBNA2 (pEΔA6), or the gene modified so as to contain defined
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Fig. 2. Characterization of EBNA polypeptides induced in COS-1 cells by transfection with EBV DNA-containing recombinant vectors. Extracts were prepared from COS-1 cells transfected with the different vectors indicated in Fig. 1 (pEAA6 to pEAA27) and analysed by SDS-PAGE immunoblotting as described in the text. Extracts of the EBV-positive lymphoid cell line Raji were analysed in lanes 1, 9 and 14. The other extracts analysed were from COS-1 cells transfected with pEAA6 (lanes 2 and 12), pEAA7 (lane 3), pEAA8 (lane 4), pEAA10 (lane 5), pEAA11 (lane 6), pSV2-gpt/BglII (lane 7), no DNA (lane 8), pEAA13 (lane 10), pEAA12 (lane 11) and pEAA9 (lane 13).

deletions (pEAA7 to pEAA24, see Fig. 1). The pEAA6 plasmid carried an EBV DNA fragment which encompassed the major BglII subfragment of the BamHI W fragment, the complete BamHI Y fragment and the left minor part of the BamHI H fragment up to the first NorI repeat. This fragment was referred to as the BglII–NorI fragment. Assuming a rightward direction of EBNA2 transcription the fragment contained three promoter consensus sequences, two long open reading frames, BW11RF1 and BYRF1, corresponding to 380 and 512 amino acid residues respectively, a number of shorter open reading frames, and a polyadenylation signal (Baer et al., 1984; Cheung & Kieff, 1982; Jones et al., 1984; Jones & Griffin, 1983). When plasmid pEAA6 DNA was transfected into COS-1 cells a transient expression of a nuclear antigen was observed in ACIF tests using anti-EBNA-positive human sera which had been shown by immunoblotting to contain antibodies against the EBNA2 polypeptide. Between 20 and 30% of the cells expressed the antigen in most experiments. The nuclear fluorescence of the positive COS-1 cells was generally of about the same intensity as that of the Raji control cells. Protein extracts were prepared from COS-1 cells transfected with the pEAA6 recombinant vector and analysed by SDS-PAGE and immunoblotting. A 90K polypeptide of a somewhat lower electrophoretic mobility than the EBNA2 polypeptide in Raji cells was identified in the cells (Fig. 2).

The pEAA7 plasmid was a spontaneous deletion mutant of pEAA6. the 2.2 kbp deletion was mapped by DNA sequence analysis and had the endpoints 46560 and 48777. The deletion removed the BW11RF1 open reading frame, the promoter sequence in BamHI Y and the first 348 nucleotides of BYRF1 including the first methionine codon. This plasmid did not induce EBNA2 in the transfected cells. However, smaller deletions affecting the same general region of the putative EBNA2 gene except the BYRF1 open reading frame did not interfere with the EBNA2-inducing capacity of the vectors as shown by the results obtained with the plasmids pEAA8 and pEAA9 (Fig. 2, lanes 4 and 13). In pEAA8 the BW11RF1 open reading frame was completely removed and in plasmid pEAA9 a methionine-containing short open reading frame in BamHI Y was deleted.
Fig. 3. Identification of the 5' end of the EBNA2-encoding exon by deletion analysis. A series of deletion mutants, pEAA14 to pEAA27, was isolated in which the deletion in the recombinant vector pEAA9 had been extended by Bal31 digestion progressing from the XhoI site in the BamHI Y fragment. The deletions were defined by cleavage of plasmid DNAs with BamHI and XhoI and electrophoretic analysis of the fragments. Lanes 1 to 14 contain plasmids pEAA27 to pEAA14, lane 15 contains pEAA9. Lane M contains fragments of pBR322 used as size markers, the numbers to the left give the marker lengths in base pairs. The recombinant vectors were tested for their ability to induce the expression of EBNA2 in COS-1 cells by ACIF staining and immunoblotting. ACIF tests were negative for lanes 1 to 6, faintly positive for lanes 7 and 8 and positive for lanes 9 to 15. Immunoblotting gave negative results for lanes 1 to 8 and positive results for lanes 9 to 15.

Are sequences from the BamHI W part of the BglII-NotI-carrying vector necessary for the induction of EBNA2 expression? Two recombinant vectors were constructed that lacked part (pEAA10) or all (pEAA11) of the BamHI W fragment. These vectors did not induce EBNA2 when introduced into COS-1 cells (Fig. 2, lanes 5 and 6), nor did they promote the synthesis of EBV-specific RNA in the cells (data not shown). This suggested that EBNA2-specific transcription in cells transfected with the EBNA2-inducing vectors was initiated mainly from a promoter in the BamHI W fragment and not from the late SV40 promoters in the pSV2-gpt part of the recombinant plasmids. In addition to the BamHI W promoter consensus sequence the deleted vectors had also lost several short open reading frames and potential splice sites. To determine whether the sequences containing these latter elements were dispensable, the pEAA12 and pEAA13 recombinant vectors were constructed. In pEAA13 the deletion removed all methionine-containing open reading frames in the BamHI W fragment. The deletion in pEAA12 extended even closer to the W promoter and further removed several potential splice donor sites. When these vectors were transfected into COS-1 cells, pEAA13 induced the transient expression of a full length EBNA2 polypeptide whereas pEAA12 did not (Fig. 2, lanes 11 and 10).

To define the BamHI Y part of the EBNA2-encoding exon more closely, a series of deletion mutants was isolated in which the deletion in the recombinant vector pEAA9 had been extended by Bal31 digestion. The deletions were defined by cleavage of plasmid DNAs with BamHI and XhoI and electrophoretic analysis of the resulting fragments (Fig. 3). Digestion of pEAA9 DNA yielded two fragments derived from the BamHI Y region, the larger of which was 809 bp and contained the 5' end of the BYRF1 open reading frame (Fig. 3, lane 15). The corresponding fragments in the plasmid series pEAA14 to pEAA27 were progressively shorter, down to a size of
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(a) 1 2 3 4 5 6  (b) 1 2 3 4 5 6

Fig. 4. Identification of RNA sequences complementary to the BamHI WYH region of EBV DNA in pEAA6-transfected COS-1 cells. Plasmid DNAs containing individual cloned fragments of EBV DNA were cleaved with BamHI or BglII, electrophoresed in 0.8% agarose and transferred to nitrocellulose. Lanes 1, BamHI W cloned in pSV2-gpt; lanes 2, BamHI Y cloned in pBR322; lanes 3, BamHI H cloned in pSV2-gpt; lanes 4, the BglII–NotI subfragment (see Fig. 1) cloned in pSV2-gpt/BglII; lanes 5, pSV2-gpt; lanes 6, pBR322. Polyadenylated RNA was prepared from COS-1 cells transfected with pEAA6 and pSV2-gpt DNA labelled with $^{125}$I and hybridized with the separated DNA fragments on membranes (a) and (b) respectively.

approximately 350 bp. This corresponded to a deletion running about 60 bp into the BYRF1 open reading frame which starts at position 48 430. Plasmid DNAs were transfected into COS-1 cells and the transient expression of EBNA2 was analysed by ACIF staining and immunoblotting. The results showed that vector constructs which contained the right part of the BamHI Y fragment from position 48 337, e.g. pEAA19 in which all short methionine-containing open reading frames in BamHI Y 5' to BYRF1 had been deleted, were able to induce the expression of EBNA2 both as ACIF-reacting material and as a 90K polypeptide in immunoblotting experiments (Fig. 3, lanes 9 to 15). Two of the recombinant DNAs, pEAA20 and pEAA21 (Fig. 3, lanes 7 and 8), yielded occasional, but clearly more than background, EBNA-positive cells on ACIF staining (0.5 to 1%) but no EBNA2 band in immunoblotting analysis. The deletion in pEAA21 removed BamHI Y sequences up to position 48 412, thus deleting several possible splice acceptor sites, but did not run into BYRF1. Plasmid DNA from a recombinant vector with a deletion endpoint at position 48 435 or vectors with larger deletions (pEAA22 to pEAA27) did not induce the expression of EBNA2 in COS-1 cells.

EBV-specific RNA synthesis in transfected cells

The BglII–NotI EBV DNA fragment in the recombinant vector pEAA6 represents a region of the EBV genome known to be transcribed in EBV-transformed, non-virus-producing lymphoid cells. The details of the process are, however, not clear. In order to characterize EBV-specific RNA synthesis in COS-1 cells expressing EBNA2, total RNA and poly(A)$^+$ RNA were prepared from transfected cells, labelled with $^{125}$I, and hybridized to nitrocellulose membranes containing electrophoretically separated restriction fragments of recombinant plasmid DNAs. As seen in Fig. 4 the poly(A)$^+$ RNA fraction from pEAA6-transfected cells contained RNA sequences homologous to the BamHI W, Y and H fragments of EBV DNA. Control cells transfected with pSV2-gpt/BglII DNA did not contain similar sequences, and nor did cells
Fig. 5. Characterization of the 5' end of the EBNA2-encoding exon by S1 nuclease mapping. The BYRF1-containing HindIII-BamHI subfragment of the BamHI Y fragment (see map, Fig. 1), 5' end-labeled at the BamHI site, was annealed to total cellular (lane 3) and poly(A)+ (lane 4) RNA from COS-1 cells transfected with pEAA6 DNA. The products were treated with S1 nuclease and the resistant fragments were analyzed by electrophoresis in a 8% polyacrylamide gel containing 8 M-urea. Lanes 1
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transfected with the vectors pEAA10 and pEAA11 which lacked the BamHI W promoter. In similar experiments the BamHI W fragment was cleaved with PvuII and the BamHI Y fragment with HindIII to produce two subfragments of each fragment (see map, Fig. 1). 125I-labelled poly(A)+ RNA extracted from pEAA6-transfected cells hybridized to all four subfragments to about the same extent.

In order to identify the splice acceptor junction at the 5' end of the mRNA exon containing the BYRF1 open reading frame, poly(A)+ RNA from pEAA6-transfected cells was annealed with the right HindIII–BamHI subfragment of the BamHI Y fragment 5' end-labelled at the BamHI site. S1 nuclease digestion and electrophoretic analysis of the hybridization mixture showed that the protected fragment had a size of about 480 bp (Fig. 5). This would correspond to a splice acceptor site at approximately position 48370.

There is a rightward promoter consensus sequence in the BamHI W segment of the BglII–NotI fragment where EBNA2-specific transcription might be initiated (Fig. 1). To determine whether the sequence promoted EBV-specific RNA synthesis in transfected COS-1 cells, S1 nuclease analysis was employed. A 5' end-labelled short subfragment of the BamHI W fragment encompassing the putative promoter sequence was annealed with poly(A)+ RNA from cells transfected with pEAA6 DNA. After S1 nuclease digestion of the hybridization mixture, protected fragments of about 80 bp were found with their end-label distributed around position 45100 in a pattern typical of that of a transcription initiation site (Fig. 6). The promoter sequence was found 25 bp 5' to this position. The results suggested that transcription was initiated here and that the promoter in BamHI W was functional in COS-1 cells.

DISCUSSION

It is now well established that the BamHI WYH region of the EBV genome encodes a protein localized to the nucleus of the EBV-infected cell, which has been designated EBNA2 (Dillner et al., 1985; Hennessy & Kieff, 1985; Mueller-Lantzsch et al., 1985; Rowe et al., 1985; Rymo et al., 1985). It is also clear that the long rightward open reading frame beginning in the BamHI Y fragment of EBV DNA (BYRF1, see Fig. 1) encodes a major part of the EBNA2 protein including its C terminus (Dambaugh et al., 1986; Dillner et al., 1985; Hennessy & Kieff, 1985).

The aim of the present investigation was to further define regions of the EBV genome necessary for the generation of an authentic EBNA2 polypeptide. Our four major findings were the following. (i) A recombinant plasmid, pEAA6, which carried EBV DNA sequences corresponding to the major part of the BamHI W fragment, the complete BamHI Y fragment and the left minor part of the BamHI H fragment (nucleotides 44664 to 50628, see Fig. 1) contained all the information necessary for inducing the expression of a normal length EBNA2 polypeptide after transfection into COS-1 cells. (ii) EBV DNA sequences between nucleotides 45442 and 48337 could be deleted from the BamHI WY region of the recombinant vectors without abolishing their ability to induce EBNA2. The deleted sequences included all short methionine-containing open reading frames in BamHI W 3' to the putative promoter sequence and the long open reading frame that begins in BamHI W (BW11RF1). (iii) The BamHI W

Fig. 5—continued

and 2, no subfragment added. 32P-labelled restriction enzyme cleavage fragments of pRB322 DNA were run as size markers in lane M, the numbers to the left give the fragment lengths in nucleotides. The arrow indicates the protected fragment.

Fig. 6. Mapping of the 5' ends of mRNAs initiated at the BamHI W promoter sequence in pEAA6-transfected COS-1 cells. The strategy of the S1-endonuclease analysis is indicated in (a). A BglII–AvaI subfragment of BamHI W was 5' end-labelled at the AvaI cleavage site (nucleotide 45180) 115 bp from the promoter consensus sequence and annealed with poly(A)+ RNA isolated from COS-1 cells transfected with pEAA6 DNA. S1 nuclease-resistant fragments were analysed by electrophoresis in an 8% polyacrylamide gel containing 8 M-urea (b, lane 1). DNA sequencing reactions on the 5' end-labelled fragment used to form the hybrid were run in parallel on the gel. Lane M contains fragments of pBR322 used as size markers, the numbers to the right give the marker lengths in nucleotides. The nucleotide sequence of the appropriate region of B95-8 EBV DNA is indicated below the gel, nucleotide 45072 marks the position of the deduced promoter sequence (Baer et al., 1984). The arrows indicate the distribution of the 5' ends of the polyadenylated RNAs.
region of EBV DNA between nucleotides 44664 and 45442 contained elements necessary for the expression of EBNA2. (iv) The rightward promoter consensus sequence in BamHI W was functional in COS-1 cells and essential for the synthesis of EBNA2-specific mRNA.

The most plausible interpretation of these results is that all of EBNA2 is encoded within the BYRF1 open reading frame. The same conclusion was reached by Dambaugh et al. (1986). Our data exclude the possibility that one of the short methionine-containing open reading frames in BamHI W or Y encoded the first amino acids of EBNA2.

Where is the transcription of EBNA2-specific RNA initiated? A number of EBV transcripts have recently been identified by cDNA cloning, which contain sequences homologous to parts of the BamHI W and Y fragments in addition to sequences from other genomic regions (Bodescot et al., 1984, 1986; Bodescot & Perricaudet, 1986; Sample et al., 1986; Speck & Strominger, 1985). Most or all of these transcripts seem to encode nuclear polypeptides present in proliferating, non-virus-producing cells (Dillner et al., 1986; Hennessy & Kieff, 1985; Hennessy et al., 1986; Speck & Strominger, 1985). Interestingly, the 5' parts of the transcripts contain exons from the BamHI W and Y regions, which are identical in the different cDNA clones (exons W1, W2, Y1 and Y2; Speck & Strominger, 1985). A number of these clones also contain exons derived from the BamHI C fragment part of the short unique region upstream from the BamHI W exons (Bodescot & Perricaudet, 1986; Bodescot et al., 1986). The EBV genome contains a promoter consensus sequence 30 to 90 bp 5' to the end of these clones. It has been suggested that a family of mRNAs with a common leader sequence is generated by alternative splicing from a giant primary transcript initiated at this promoter or at some other promoter in the BamHI C fragment (Bodescot & Perricaudet, 1986; Bodescot et al., 1986; Speck & Strominger, 1985). However, none of the cDNA clones appears to contain the complete 5' end of the transcript. Thus it is still possible that synthesis of transcripts which belong to this family and do not contain BamHI C exons is initiated in the BamHI W region. A strong promoter consensus sequence has been identified in the BamHI W repeats (Cheung & Kieff, 1982; Jones et al., 1984). This sequence promotes the transcription of the BamHI W fragment by HeLa cell extracts (van Santen et al., 1983). Furthermore, two cDNA clones containing the EBNA2-encoding BYRF1 open reading frame have been found which begin 42 bp 3' to this promoter sequence (Sample et al., 1986). The present investigation demonstrated that the BamHI W promoter was functional in COS-1 cells which had been transfected with BamHI WYH-carrying vectors and transiently expressed EBNA2. When the promoter-containing part of the BamHI W fragment was deleted the recombinant vectors lost their ability to induce EBV-specific RNA synthesis and EBNA2 expression.

Thus our results suggested that the synthesis of EBNA2-specific RNA started at position 45098, 25 nucleotides 3' to the BamHI W promoter sequence. This idea was strengthened by our recent finding of a strong transcription-activating function in the 5' flanking region of the BamHI W promoter (A. Ricksten et al., unpublished results). The results also suggested that the primary transcript was spliced and contained a splice donor site somewhere between positions 45170 and 45442 in the EBV genome, i.e. the left deletion endpoints of our recombinant vectors pEΔA12 and pEΔA13. Only one of eight G-T-containing sequences in this region had any further homology to a splice donor consensus sequence [\(\overline{\text{T}}-\text{A}-\text{G/G-T-}\overline{\text{A}}-\text{G-T}\)], so we consider it most likely that this site was used. This potential donor site at position 45340 corresponded to the splice junction between the W1 and W2 exons found in the BamHI W sequence-containing family of cDNA clones (Bodescot et al., 1984, 1986; Bodescot & Perricaudet, 1986; Sample et al., 1986; Speck & Strominger, 1985). Our results did not exclude the possibility that the transcript was also spliced at a position closer to the initiation site. The W1 exon seemed to be necessary for the generation of an EBNA2-encoding mRNA whereas both the W2 exon and the Y1 and Y2 exons either were not part of the mRNA or were at least dispensable. This was shown by the fact that recombinant vectors where the corresponding sequences had been deleted (e.g. pEΔA8 and pEΔA9) still induced EBNA2. The splice acceptor junction leading into the BYRF1-containing exon should have been found somewhere between positions 48337 and 48435 according to our Bal31 deletion analysis. The S1 nuclease protection experiments suggested that the acceptor site was around position 48370. Examination of possible acceptor sites [\(\overline{\text{T}}\)-\text{N-}\overline{\text{T}}-\text{A}-\text{G/G}\)] in this
region of the EBV genome revealed that the acceptor site at position 48,386 was a likely candidate. This site was also involved in the Y2/Y3 exon splice junction in the T1 cDNA clone isolated by Bodescot et al. (1984), and the T65 and T62 cDNA clones of Sample et al. (1986).

Obviously, the data presented here did not reveal the exact configuration of the EBNA2-encoding message. This will have to await the isolation and characterization of the molecule by other techniques. Our results might, however, be helpful in the identification of the correct mRNA among several transcripts derived from this region of the EBV genome. Although the results of Sample et al. (1986) suggest that the EBNA2-encoding mRNA is polycistronic, our data raise the question of whether the EBNA2-encoding exon might also be present in mRNA molecules in which it is not preceded by other open reading frames. Furthermore, the data support a model for the generation of EBNA2-specific mRNA in EBV-transformed, non-virus-producing lymphoid cells in which transcription is initiated from the strong promoter sequence in one of the BamHI W repeats. It is conceivable that the situation is different in virus-producing cells. Here promoters in the BamHI C fragment region might be employed to initiate RNA synthesis-producing transcripts related to the cDNAs isolated from B95-8 cells by Bodescot & Perricaudet (1986) and Bodescot et al. (1986).

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