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Deletion Mutants That Affect Expression of Epstein-Barr Virus Nuclear Antigen in COS-1 Cells after Gene Transfer with Simian Virus 40 Vectors Containing Portions of the *Bam*HI K Fragment

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We have identified sequences that affect the efficient expression of Epstein-Barr virus nuclear antigen (EBNA 1) when the structural portion of its gene, found within the 2.9-kilobase-pair *Bam*HI/*Hin*dIII fragment called IIf, is expressed from a simian virus 40 vector. A set of nested deletions at the *Bam*HI end of the fragment was constructed by using BAL 31 digestion, the addition of linkers, and ligation into pSVOd. The mutants were tested for their ability to express antigen in COS-1 monkey cells by using indirect immunofluorescence and immunoblotting. Deletion endpoints were determined by DNA sequencing of the 5′ ends of the mutants. The deletion mutants could be subclassified into four groups based on their ability to express EBNA polypeptide. Mutants that retain more than 106 base pairs upstream from the start of the open reading frame in IIf exhibit antigen expression indistinguishable from that of wild type. Mutants that invade the structural gene by 1,115 or more bases destroy antigen expression. Mutants that alter the splice acceptor site or invade the open reading frame by a short distance make antigen at a markedly lower frequency. There are three mutants, whose deletions map at −78, −70, and −44 base pairs upstream of the open reading frame, that make reduced levels of EBNA. Since these three mutants differ in the extent to which EBNA expression is impaired, the data suggest that there are several critical regions upstream of the open reading frame that regulate EBNA expression in COS-1 cells. It is not known whether these regulatory sequences, which would be located in an intron in the intact genome, play any role in the expression of EBNA in infected lymphocytes.

Epstein-Barr virus (EBV) nuclear antigen (EBNA), an antigen system found in the nucleus of cells immortalized by Epstein-Barr virus, is expressed during the latent phase of the virus life cycle, as well as during virus replication. It is now apparent that EBNA consists of at least two different components (13, 21), one of which is encoded by the leftmost 2.9 kilobase pairs (kbp) of the *Bam*HI K fragment of EBV DNA that has been shown to induce EBNA in gene transfer systems (7, 10, 25). Furthermore, this region of the EBV genome carries a variable number of repeating units, which together are designated internal repeat 3 (IR3) (12). Variation in the size of IR3 DNA correlates with variation in the size of the EBNA polypeptide (7, 12).

In lymphocytes latently infected by EBV, there is a 3.7-kilobase (kb) mRNA that is homologous to *Bam*HI-K (11, 29). The size of the exon in IIf encoding the structural protein of EBNA is 2.0 kb (11). The exact boundaries of this exon have been mapped by S1 nuclease analysis of mRNAs made in lymphocytes and in two eucaryotic gene transfer systems (29). This exon begins 8 base pairs (bp) upstream of a 1,923-bp open reading frame designated BKRF3 and ends 30 bp after the polyadenylation signal (2, 29). Other noncoding exon(s) are evidently present in the complete gene, but these have not been identified. A remarkable facet of EBNA transcription is that the abundance of the mRNA encoding EBNA remains constant during viral latency and replication (28). The mechanisms underlying this regulation of expression of EBNA are not understood.

EBNA is the most abundant viral polypeptide found in latently infected lymphocytes. The protein expressed in lymphocytes is the same size as that seen in various eucaryotic gene transfer systems containing the 2.0-kbp exon (7). A plasmid mutant with a 600-bp deletion in IR3 induces a truncated EBNA in COS-1 cells (20). On polyacrylamide gels, EBNA migrates with an apparent mass of 78 kilodaltons, while the size predicted from the open reading frame is 56 kilodaltons. EBNA is a highly basic protein, as seen in the two-dimensional electrophoresis of EBV intracellular polypeptides; furthermore, the polypeptide predicted from the DNA sequence is also highly basic (2; D. K. Fischer, unpublished data).

IR3, encoding a repeating polymer composed of glycine and alanine, has been expressed as a fusion protein in bacteria (13). Rabbit antibodies raised to the fusion protein expressed in bacteria react with EBNA in lymphocytes (13). Furthermore, antibodies raised in rabbits to a synthetic peptide containing sequences from IR3 also detect EBNA (6).

The *Bam*HI-K polypeptide is the component of EBNA that binds to cellular metaphase chromosomes (9). It has been proposed that EBNA plays a role in immortalization either directly, by encoding a product that influences cell growth, or indirectly, by helping to maintain the EBV genome as an episome. Recently, evidence has been presented that the *Bam*HI-K EBNA is required for the maintenance of the extrachromosomal DNA carrying the EBV origin designated OriP (31).

EBNA encoded by *Bam*HI-K may also be an important antigen in the immunosurveillance of EBV infection. Certain patients with chronic, active EBV infection specifically fail to recognize the EBNA polypeptide encoded by *Bam*HI-K,

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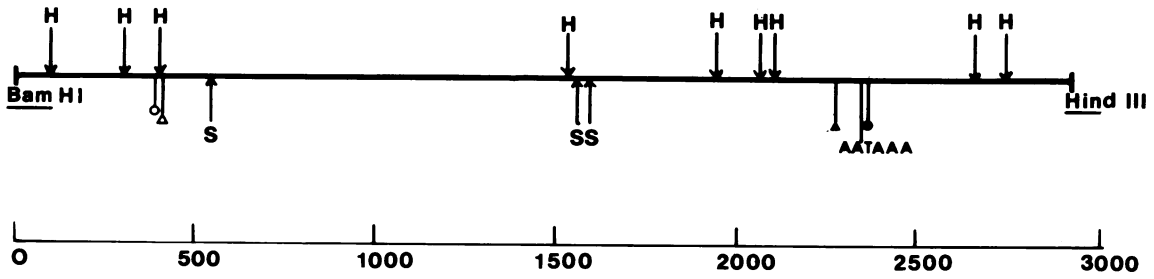

FIG. 1. Map of the *Bam*HI/*Hin*dIII Ilf fragment (Ilf) showing the *Hin*fI (↓) and *Sma*I (↑) sites. Symbols: △, location of start of the open reading frame; ▲, end of the open reading frame; ○, 5′ end of the exon; ●, 3′ end of the exon.

even though their sera contain antibodies to other EBV polypeptides, including EBNA 2 (21).

Ideally, studies of the biologic role and regulation of the expression of EBNA 1 should be carried out by reintroducing mutated EBNA 1 genes into the background of an intact genome. Such experiments are not yet feasible in the current state of EBV genetics. However, since the same splice acceptor site is used and identical 5′ ends of EBNA 1-encoding exons are found in lymphocytes and in two gene transfer systems, we felt that DNA sequences that influence EBNA expression might be identified by using gene transfer techniques (29). In this report, we describe the effect of deletions of DNA that invade the Ilf fragment 5′ to the major coding exon. This set of deletion mutants was cloned on a simian virus 40 vector, and EBNA 1 expression was analyzed in a heterologous system, COS-1 monkey cells.

MATERIALS AND METHODS

Cell line. COS-1 cells (8) were grown in Dulbecco modified Eagle medium containing 5% fetal bovine serum supplemented with penicillin, streptomycin, and amphotericin B.

Construction of mutants. We began with the clones pBRIlf and pSVOd (19). We first linearized 10 μg of pBRIlf with *Bam*HI, extracted with phenol, and precipitated with ethanol. The DNA was then suspended in 25 μl of water and 25 μl of 2× BAL 31 buffer (2× BAL 31 buffer is 0.04 M Tris hydrochloride, 1.2 M sodium chloride, 0.025 M magnesium chloride, 0.025 M calcium chloride, 0.002 M EDTA [pH 7.2]) and preincubated at 30°C for 3 min. Then 3.5 U of BAL 31A (Boehringer-Mannheim Biochemicals) was added, and the digestion was continued at 30°C for 15 min. The reaction was stopped by adding 20 μl of 0.5 M EDTA, 20 μl of 4 M sodium acetate, and 60 μl of Tris-EDTA buffer (0.01 M Tris, 0.001 M EDTA [pH 7.2]); the DNA was extracted with phenol and precipitated with ethanol.

The synthetic oligonucleotide 5′-CGGATCCG-3′, containing a *Bam*HI site was purchased from New England BioLabs, Inc. These *Bam*HI linkers were phosphorylated in the presence of [γ-^{32}P]ATP by T4 polynucleotide kinase. Kinase linkers were separated from unincorporated ATP by passage over a P4 column and were eluted in 0.1 M ammonium acetate. Fractions containing the radioactive peak were pooled, frozen, lyophilized to dryness, suspended in 100 μl of water, frozen, and relyophilized.

Linkers were ligated to the BAL 31A-treated DNA. The resected Ilf fragments were excised from pBRIlf by using *Hin*dIII and an excess of *Bam*HI (see Fig. 2). The resected DNA was ligated into *Bam*HI/*Hin*dIII-cleaved pSVOd and then transfected into HB101. Colonies that were tetracycline sensitive were screened for the presence of deleted Ilf sequences in pSVOd by analysis of minilysate DNA on Southern blots (4, 24).

Mutant 46′, which contains 8 bp less than mutant 46, was constructed by partial *Hin*fI cleavage. Clone 66 DNA was digested with *Hin*fI in the presence of 85 μg of ethidium

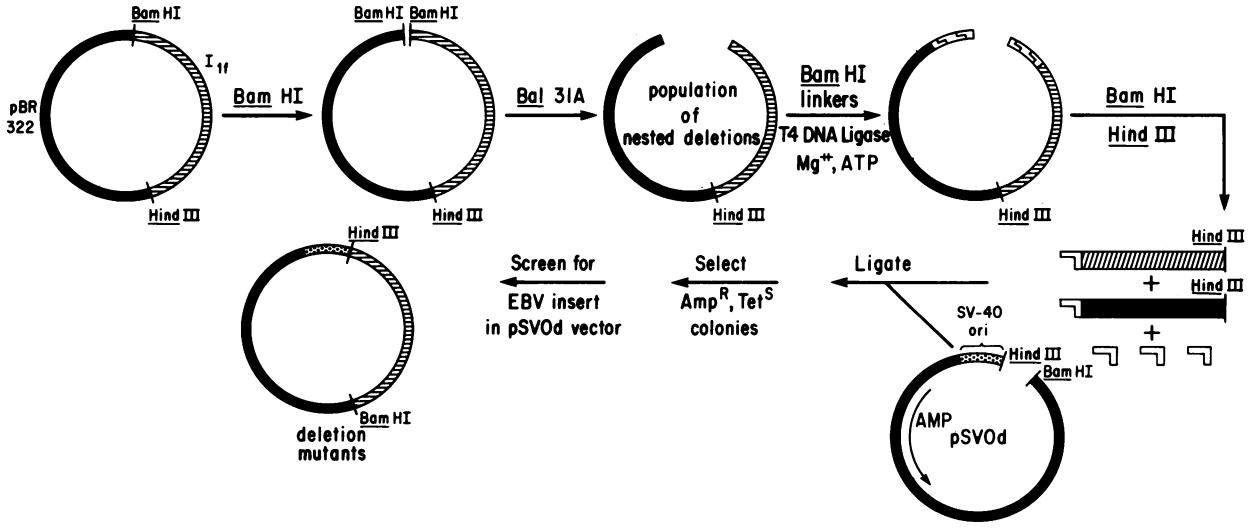

FIG. 2. Construction of deletion mutants from the *Bam*HI end of the *Bam*HI/*Hin*dIII Ilf fragment and cloning in pSVOd.

bromide per ml. The ends generated by *Hin*fI were filled in by using the Klenow fragment of DNA polymerase I. *Bam*HI linkers were added; the DNA was digested with *Bam*HI and *Hin*dIII and ligated into pSVOd.

Restriction mapping. Mutants that appeared to have deletions on the basis of Southern blotting of 0.75% agarose gels were analyzed further by using *Bam*HI/*Sma*I and *Bam*HI/*Hin*fI digests (Fig. 1). The products were run on 8% polyacrylamide gels (30:1) that were stained in 0.5 μg of ethidium bromide per ml and photographed.

Transfection. COS-1 cells were transfected by using the DEAE-dextran method and then chloroquine (17, 18). When transfected with minilysate DNA, the cells, grown in 10-mm well, received about 0.5 μg of DNA as judged by ethidium bromide-stained gels. When DNA that had been purified on CsCl gradients was used, the cells received 1 μg of DNA.

Antigen expression. We tested the ability of the mutants to express EBNA both by indirect anti-immunoglobulin and anti-C3 immunofluorescence. The cover slips were harvested 3 days after transfection. They were washed once in phosphate-buffered saline and fixed in methanol (anti-C3) or acetone-methanol, 2:1 (anti-immunoglobulin) for 10 min. The cover slips were then reacted with a human serum (either RM or GG49) known to have a high titer to the *Bam*HI-K EBNA. A fresh frozen human serum (LH) lacking antibodies to EBV antigens was the source of complement. The second antibody in the anti-C3 test was fluorescein-conjugated rabbit anti-human C3 (Dako, Inc.) and in the anti-immunoglobulin test was sheep anti-human immunoglobulin (Wellcome Research Laboratories).

Immunoblotting. COS-1 cells, grown on 100-mm plates, were transfected with 5 μg of plasmid per plate. After 3 days, the monolayer was washed once with phosphate-buffered saline, lysed in 0.5 ml of electrophoresis sample buffer (0.625 M Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, 10% glycerol, 0.029 M bromphenol blue) and sonicated for 15 s. Extracts were then analyzed by using the discontinuous gel electrophoresis technique (15). The resolving gel was 10%, and the stacking gel was 5% acrylamide. Proteins were transferred to nitrocellulose filters that were then blocked in 0.25% gelatin in Tris-buffered saline (0.9% NaCl, 10 mM Tris hydrochloride [pH 7.5]) overnight (5, 26). Blots were incubated with a 1:200 dilution of RM serum in phosphate-buffered saline for 4 h. The filters were washed three times, 10 min per wash, in 0.05% Tween 20 in Tris-buffered saline and then incubated with 1 μCi of ^{125}I-labeled protein A (Amersham Corp.) in phosphate-buffered saline for 4 h. The filters were washed again and autoradiographed on Kodak XAR-5 film.

DNA sequencing. To locate the endpoints of the deletions precisely, we subcloned the *Bam*HI/*Sma*I subfragment of 10 selected mutants in M13mp8. The fragments were then sequenced by using a pentadecamer primer (New England BioLabs) and the dideoxy chain termination method (22). The endpoints were determined by comparison with the sequence of the 552-bp *Bam*HI/*Sma*I subfragment of IIf from EBV strain FF41 (Fig. 1). To obtain the sequence of this fragment, 200 ng of gel-purified IIf was digested with *Hin*fI, and the ends were filled in with the Klenow fragment of DNA polymerase I. This reaction, in 50 μl, was run for 10 min at room temperature and stopped by the addition of 5 μl of 3 M sodium acetate and 5 μl of 0.25 M EDTA. The DNA was extracted with phenol and coprecipitated in ethanol with M13mp8 replicative form cut with *Sma*I. The DNAs were ligated, transfected into JM103, and plated by using standard techniques. Clear plaques were screened for appropriate inserts by the minilysate procedure. The four *Hin*fI subfragments of the 552-bp *Bam*HI/*Sma*I fragment were obtained in both orientations and both strands were sequenced.

TABLE 1. Effect of deletions on antigen expression in COS-1 cells

JJ401 clone no.	Approx no. of bp removed[a]	Antigen expression[b]
29	0	++
54	104	++
64	175	++
19	187	++
2	196	++
148	198	++
97	203	++
66	209	++
99	211	++
104	211	++
67	213	++
126	214	++
9	214	++
164	219	++
75	220	++
80	220	++
38	225	++
45	225	++
30	236	++
81	270	++
46	295	+
201	300	+
275	360	±
68	377	±
296	378	±
34	400	±
115	820	±
94	820	±
111	850	±
145	870	±
144	930	±
105	1,010	±
33	1,080	±
154	1,100	±
57	1,120	±
5	1,140	±
87	1,240	±
57	1,500	−
4	1,534	−
100	1,605	−

[a] Estimated from electrophoresis on 0.75% agarose gels. The EBNA exon begins 376 bp from the *Bam*HI site.

[b] ++, Wild-type expression, nuclear and cytoplasmic antigens; +, lower frequency of positive cells, qualitatively similar to wild-type expression; ±, very low frequency of positive cells, qualitatively different expression, strictly nuclear antigen; −, no detectable antigen.

RESULTS

Construction, isolation, and preliminary screening of deletion mutants. A set of nested deletion mutants, lacking sequences at the *Bam*HI end of IIf were inserted into the vector pSVOd so that the capacity of the mutants to express EBNA could be studied in COS-1 cells (Fig. 2 describes the cloning procedure). Minilysate DNAs from 40 clones that contained a deleted IIf fragment, as judged by Southern blots of 0.75% agarose gels, were tested for their ability to induce EBNA in COS-1 cells (Table 1). A total of 20 mutants, which

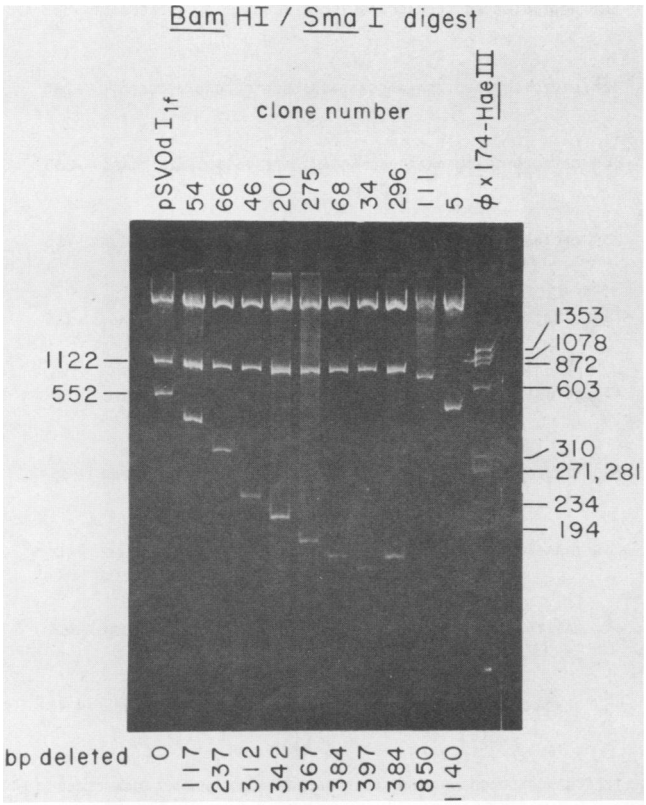

FIG. 3. Size of 10 selected mutants with deletions in the 5′ end of the Ilf fragment. Shown is an 8% polyacrylamide gel stained with ethidium bromide.

removed approximately 270 bp or less from the *Bam*HI end, exhibited antigen expression indistinguishable from that of the wild-type undeleted fragment. The remaining 20 mutants, which had deletions of 295 bp or more, could be subdivided into three groups based on the frequency, intensity, and location of antigen. There were two mutants, with deletions of about 295 and 300 bp, that induced a lower frequency of antigen-positive cells, but the intensity and distribution of antigen were similar to those of the wild type. A total of 15 mutants, which removed from 360 to 1,240 bp, induced a very low frequency of antigen-positive cells; furthermore, antigen induced by these mutants was exclusively nuclear and often quite faint. Finally, three mutants with large deletions of 1,500 bp or more were unable to induce antigen. From the total group, we selected 12 mutants that expressed antigen to various degrees for further study.

Characterization of selected mutants. A more precise estimate of deletion sizes was made by digestion of the mutant DNA with *Bam*HI and *Sma*I and electrophoresis of the products in 10% polyacrylamide (Fig. 3). Of the selected mutants, ten invaded the 552-bp *Bam*HI/*Sma*I fragment; the other 2 mutants deleted this fragment completely and entered the 1,100-bp internal *Sma*I fragment (Fig. 1).

These 12 mutants were retested for antigen expression in COS-1 cells by using DNA purified on CsCl gradients (Fig. 4). Antigen produced by clones 54, 66, and 81 with deletions of 117, 237, and 281 bp, respectively, was identical to that of the wild type; a high frequency of antigen was found in both nucleus and cytoplasm (Fig. 4A). Mutants 46, 46′, and 201 induced bright antigen, distributed within the cell as in the wild type, but in fewer cells (Fig. 4B). The remaining six

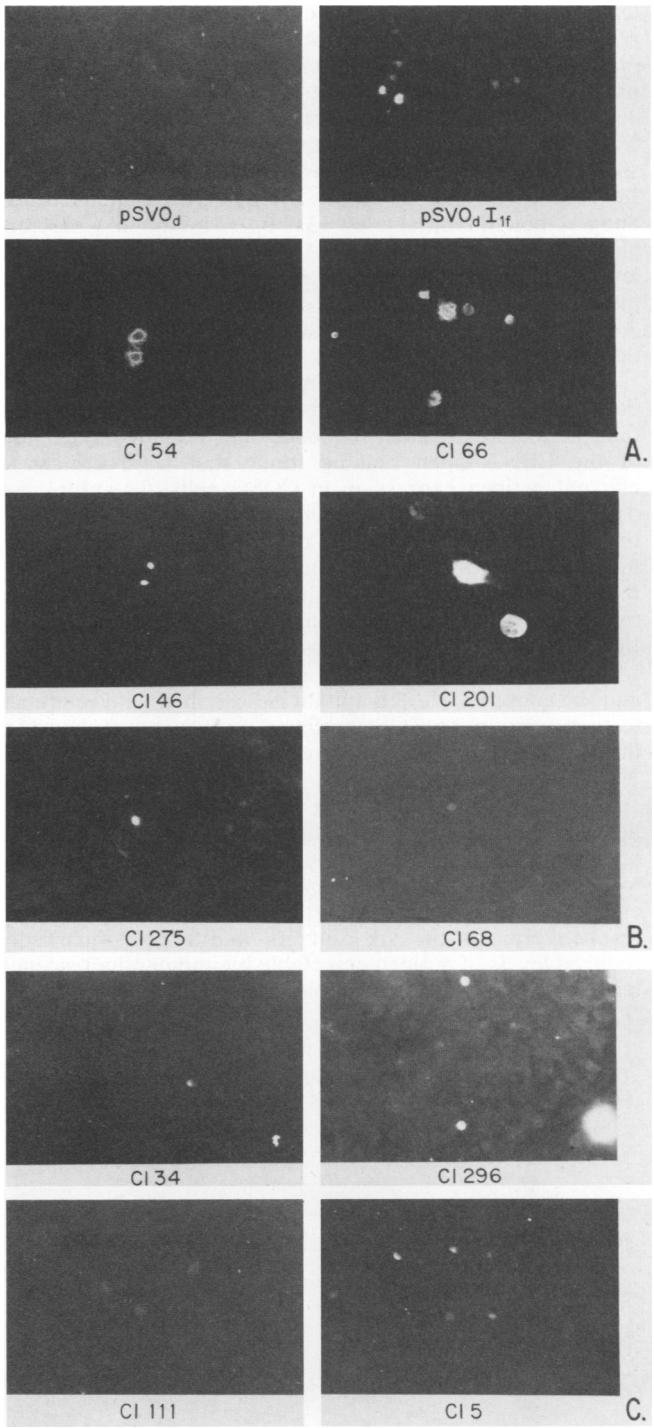

FIG. 4. Expression of antigen detectable by immunofluorescence in COS-1 cells by wild-type Ilf and 10 selected mutants. All photographs at original magnification of ×100, except clone 201, which was at ×250. Data not shown for clones 46′ and 81.

clones caused the appearance of antigen that was exclusively nuclear and often, but not invariably, faint (Fig. 4C). These clones always induced far fewer antigen-positive cells.

Antigen detection by immunoblot. The results from immunofluorescence assays showed changes in the number and intensity of antigen-positive cells. To learn whether such

changes correlated with the level of the *Bam*HI-K EBNA polypeptide, Western immunoblots were done. Once again, mutants 54 and 66 induced wild-type levels of EBNA expression (Fig. 5), as did mutant 81 (data not shown). Mutants 46 and 201 reproducibly exhibited, in several separate transfections, a reduction in the amount of EBNA synthesized, but the protein made was of the expected size (Fig. 5). In these trials, mutant 201 (−342 bp) always induced less antigen than did mutant 46 (−312 bp). Mutant 46′ (−320 bp) also induced a full-sized EBNA 1 polypeptide; however, the level of antigen produced was intermediate between those of mutants 46 and 201 (data not shown). Mutants from which 367 bp or more were deleted failed to produce a polypeptide detectable by immunoblotting.

Location of deletion endpoints by DNA sequencing. The data obtained in antigen expression experiments suggested that the mutants defined sequences upstream from the start of the EBNA open reading frame that were critical to efficient antigen expression in COS-1 cells. Therefore, we used DNA sequencing to determine the exact endpoints of the deletions and to examine the regions that were important. We also sequenced both strands of the 552-bp *Bam*HI/*Sma*I subfragment of IIf from EBV strain FF41 (Fig. 6). This sequence is identical to that in the B95-8 strain (2). For a description of the results of DNA sequence analysis, we assign the A of the initiator methionine as position +1, and the endpoint of each mutant is described with reference to that position. In COS-1 cells as well as in lymphocytes, the 5′ end of the exon encoding the EBNA polypeptide is at −8 (29) (Fig. 7).

Mutants 54, 66, and 81 with wild-type antigen expression lie −265, −172, and −106 bp upstream from the start of the open reading frame. Mutants 46, 46′, and 201, which are able to make a full-sized EBNA polypeptide, but which have decreased levels of expression, lie at −78, −70, and −44 bp respectively. Of the six mutants that express markedly reduced levels of antigen detectable by immunofluorescence but undetectable by immunoblotting, five invade the exon.

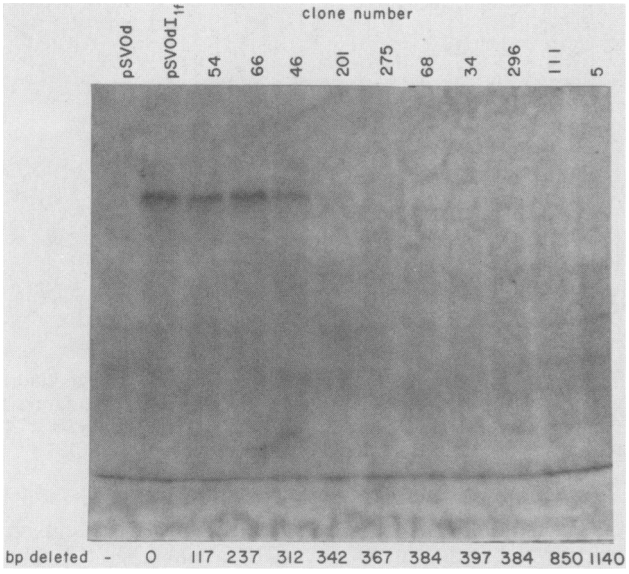

FIG. 5. Detection by immunoblotting of the EBNA polypeptide made in COS-1 cells by IIf and 10 selected deletion mutants. The number of base pairs deleted was determined by polyacrylamide gel electrophoresis (Fig. 3). Data for mutants 46′ and 81 not shown.

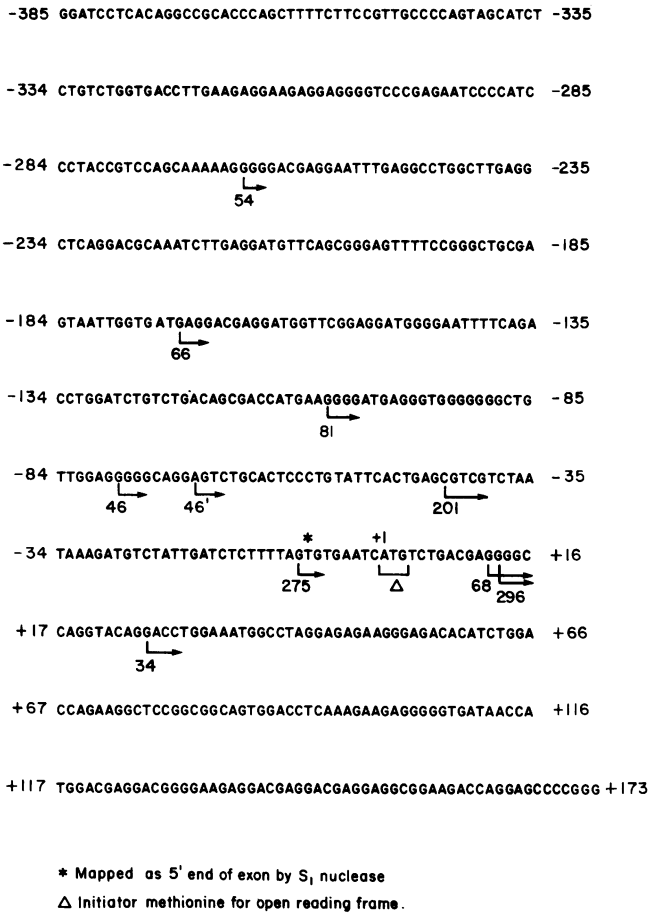

FIG. 6. Sequence of the *Bam*HI/*Sma*I subfragment of IIf in EBV strain FF41. Symbols: +1, the A of the initiator methionine; Δ, initiator methionine; *, the start of the exon as mapped by S1 nuclease analysis (29); →, the locations of the endpoints of eight deletion mutations that map in this subfragment. Each number refers to an individual mutant (Table 1).

The sixth mutant in this group, clone 275, begins at −9, 1 bp upstream from the start of the exon. Mutants 296 and 68 (68 at +12 and 296 at +13), and mutant 34 (at +26) invade the open reading frame. The two mutants with large deletions are not shown in Fig. 6; their endpoints lie in IR3, well into the open reading frame, about +454 (clone 111) and +733 (clone 5).

DISCUSSION

The mutants we have isolated provide a biologic description of the influence of sequences immediately 5′ to the exon

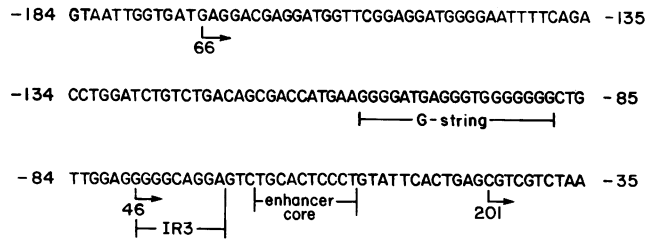

FIG. 7. Sequences 5′ to the EBNA exon that influence expression of the polypeptide.

encoding the EBNA 1 polypeptide on expression of this partial gene when it is cloned on a simian virus 40 vector and transferred into COS-1 cells. The mutants can be divided into four categories: (i) those that do not alter EBNA expression, (ii) those that decrease the level of EBNA polypeptide synthesized, (iii) those that markedly affect the amount of protein made, and (iv) those that eliminate detectable expression of EBNA. The endpoints of those 20 deletion mutants that fail to affect EBNA expression (Table 1) are all located at least 116 bp upstream of the start of the open reading frame. The three mutants that do not express antigen all extensively invade the open reading frame. The two most interesting classes of mutants are those that invade the reading frame, but nonetheless reproducibly induce a low level of antigen detectable by immunofluorescence, and those that fail to invade the open reading frame, but show a decreased level of EBNA polypeptide expression.

Mutants that lack part of the open reading frame. There are two possible explanations for antigen which is expressed by the five mutants that invade the open reading frame: (i) it may not be EBNA, but a replicative antigen or (ii) it may represent a truncated EBNA polypeptide. As to the first possibility, there is a replicative gene whose promoter and structural gene lie partially in Ilf (29). Therefore, we considered the possibility that the low level of antigen seen by immunofluorescence was caused by such a replicative product. However, a human serum that has high titers of antibodies to replicative antigens, but lacks antibody to the *Bam*HI-K EBNA, does not react with antigen induced by any of the mutants (data not shown). Therefore, it seems more likely that the antigen we detect by immunofluorescence is contained on a polypeptide or group of polypeptides composed of segments of the entire EBNA product. We have not yet been able to identify such altered polypeptides on immunoblots because the deletion mutants also remove signals in the flanking 5′ upstream region that are required for the efficient expression of the plasmids in COS-1 cells. To identify the putative altered EBNA polypeptide(s), it will be necessary to replace the regulatory signals. If such truncated EBNA polypeptides are made, they might use alternative splice acceptor sites and a different initiator methionine. The first internal methionine in Ilf is located far downstream; if it were utilized, the resulting product would have a molecular weight of about 11,000.

Mutant 275 behaves similarly to the five mutants that invade the open reading frame; i.e., it makes a small amount of antigen that cannot be detected on an immunoblot. Its deletion removes the A residue from the sequence AG found at the end of the putative intron. Therefore, this mutant probably also uses an alternate splicing signal and an internal initiator methionine.

Mutants with decreased levels of expression of a full-sized EBNA polypeptide. There were three mutants that have been sequenced, clones 46, 46′, and 201, which make reduced amounts of EBNA polypeptide. By using densitometry tracings of the immunoblots, we found that the level of EBNA expression by clone 46, whose endpoint is −78 bp upstream of the open reading frame, is about 33% of wild type (data not shown). Clone 46′, which removes only 8 bp more than clone 46, exhibits EBNA expression at only 13% of wild type, and clone 20 (−44 bp) induces only 5% of the wild-type level of EBNA (data not shown).

Thus, a region lying between −45 and −106 bp from the start of the open reading frame regulates the expression of EBNA 1 in COS-1 cells. Since mutants in this region each differ in level of EBNA expression, there may be more than one regulatory signal or a continuum of regulatory sequences.

DNA sequences in the regulatory region. There are several possible candidates for these regulatory signals. Beginning at −106 bp, there is a run of 19 residues, 15 of which are G. It has been observed that the molecular structure of poly(dG)-poly(dC) is the A form of DNA and rarely if ever assumes the B form (1). DNA in the A form fails to form chromatin-like structures when mixed in vitro with histones, although it might interact with other proteins (23, 30). Recently, an S1 nuclease-hypersensitive site has been mapped to such a "G string" in the 5′ flanking sequence of chicken β-globin gene (16). A similar site may be important in the expression of EBNA in the gene transfer system under study because clone 46, which eliminates the G string, makes reduced levels of EBNA (Fig. 4 and 5).

Beginning at −78 bp, there is one copy of the sequence 5′-GGGGCAGGA-3′. This element is a major figure in IR3 where it is repeated 52 times and gives rise to the glycine-alanine copolymer that is part of EBNA. The sequence is also found in three other locations in the EBV genome; once in *Bam*HI-S and twice in *Bam*HI-D. Statistically, any 9-mer would occur at random once per 240,000 bp. Thus, the presence of this sequence in the 5′ flanking region of a gene where it encodes a polymer seems remarkable and is likely to be biologically important. The related sequence 5′-GGCCAGGA XG-3′ has recently been found to be common to hypervariable minisatellite regions in human DNA (14).

Significance of findings. The experiments presented represent early steps in defining EBV DNA sequences that participate in the efficient synthesis of the *Bam*HI-K EBNA. While we have identified DNA sequences that are important in regulating EBNA expression after gene transfer into monkey cells, it is clear that we are unable to extrapolate these findings to the behavior of EBNA in the usual target cells and in the background of intact virus.

These initial studies define the phenotype of the mutants and regulatory DNA sequences that would be located in an intron in the intact genome. The studies do not yet provide a mechanism for the observed variations in expression. Clearly, a detailed study of transcription by these mutants is required next. Quite likely, the sequences influence splicing. Recent studies using comparable techniques of deletional mutagenesis in globin genes and adenovirus genes have shown that a minimal amount of DNA, upstream of a splice acceptor site, is needed for accurate and efficient splicing (3, 27).

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