

# Expression of a nuclear and a cytoplasmic Epstein–Barr virus early antigen after DNA transfer: Cooperation of two distant parts of the genome for expression of the cytoplasmic antigen

(cosmid clones/transfection/transient gene expression)

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**ABSTRACT** Expression of Epstein–Barr virus (EBV) antigens was studied after transfection of cloned EBV DNA fragments into baby hamster kidney (BHK) cells. A set of seven widely overlapping clones covering the whole genome of the non-defective Epstein–Barr virus strain M-ABA was used for transfection. Transfer of the cosmid clones into BHK cells resulted in expression of two distinct antigens, as revealed by indirect immunofluorescence using human anti-EBV sera. Staining with human sera of different reactivity against EBV-associated antigens revealed that both types of antigens were related to the early antigen complex. The first type of antigen was detected only in the nuclei of BHK cells that had received DNA of a clone containing *HindIII*-G, -H, -E, -I2, -O, -I1, and -P. The second type of antigen was found in the cytoplasm of cells cotransfected with clones containing *Sal*-A and *HindIII*-I2, -O, -I1, -P, and -C, whereas transfection of both individual clones failed to induce the antigen. Further analysis with subclones identified *HindIII*-G (5 kilobases) and *HindIII*-I2 (3 kilobases) plus the rightmost 3 kilobases of *Sal*-A as the sequences responsible for expression of the nuclear and the cytoplasmic antigen, respectively. The fact that two distant regions of the viral genome are required for expression of a viral antigen provides evidence for intergenomic regulation that can be studied *in vitro*.

Infection of human B lymphocytes by Epstein–Barr virus (EBV) leads to unlimited growth of the cells *in vitro*. Cells transformed or immortalized by EBV have acquired the viral genome usually in many copies and express the viral nuclear antigen EBNA (for review, see ref. 1). In EBV genome-carrying lymphocytes the lytic cycle of the virus is usually repressed in most of the cells and only a small percentage of cells is spontaneously induced to produce virus particles. Treatment of the cells with the tumor promoter phorbol 12-myristate 13-acetate increases substantially the number of cells that produce early or late antigens associated with the lytic cycle of the virus (2, 3). Because cells in which the virus is induced to synthesis of early antigens are bound to die, repression of the lytic cycle is obviously an important function to maintain the transformed state of the cell.

Recently, the natural barrier of the restricted host range of EBV was overcome by several investigators who used different techniques of gene transfer to introduce intact EBV DNA or EBV DNA fragments into recipient cells (4–9). Implantation of the EBV receptor into cells of different origin has also been successful to mediate the uptake of EBV in non-natural target cells (10). These experiments allow the synthesis of early and late viral antigens in different monolayer cells and even the production of biologically active virus has been reported after transfection of human placental

cells with intact EBV DNA (5). Even though transfer of EBV DNA into recipient cells has allowed the expression of viral antigens, only the nuclear antigen EBNA has so far unequivocally been assigned to a specific fragment of the viral genome by DNA transfer (8). The existence of additional EBNA proteins, one being encoded by *Bam*HI-M, has recently been reported by two groups (11, 12). For assignment of viral antigens to specific regions of the genome by transfection it is essential that the gene encoding the antigen is not inactivated by the restriction endonuclease that was used for cloning of the respective fragment. To minimize this possibility we have constructed a set of overlapping cosmid clones of M-ABA EBV DNA. This virus strain, originally derived from a nasopharyngeal carcinoma (13), has transforming capacity and was shown to have the genomic organization of an EBV prototype (14) in contrast to the widely used B95-8 strain, which is known to be an unusual deletion derivative (14, 15). The set of cosmid clones contains the complete viral genome with the exception of a few hundred base pairs, which are not stably replicated in *Escherichia coli* and overlap by 3–22 kilobase pairs to each other (16).

Here we report the expression of two distinct EBV early antigens after transfer of the cosmid clones into baby hamster kidney (BHK) cells. Expression of one of the two antigens requires the simultaneous presence of two distant regions of the genome providing evidence for intergenomic regulation of antigen expression.

## MATERIALS AND METHODS

**Cells.** BHK cells were maintained in Eagle's minimal essential medium (ME medium) supplemented with 5% fetal calf serum, penicillin (100 international units/ml), and streptomycin (100 µg/ml). Cells were passaged twice weekly by diluting them 1:8 in fresh medium.

**Transfection.** The calcium phosphate precipitation technique was used for transfection (17), with the modification described by Wigler *et al.* (18). Twenty-four hours prior to transfection,  $4 \times 10^4$  BHK cells were seeded into Costar six-well tissue culture plates containing a  $24 \times 24$  mm coverslip in each well, resulting in 60–70% confluency at the time of transfection. Four hours before transfection the medium was replaced by fresh medium containing 10% fetal calf serum. A calcium phosphate precipitate was formed by mixing 0.25 ml of 250 mM  $\text{CaCl}_2$  containing 10 µg of salmon sperm DNA and an appropriate amount of the cloned viral DNA fragment with 0.25 ml of  $2 \times$  concentrated HEPES-buffered saline (pH 7.08). After 20–30 min at room temperature, the mixture was added directly to the well containing the coverslip with the recipient cells. After 4 hr at 37°C, the cells were washed once with Eagle's ME medium and then treated with 20% dimethyl sulfoxide in medium for 90 sec as described by Stow

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Abbreviations: EBV, Epstein–Barr virus; kb, kilobase(s); BHK, baby hamster kidney.

and Wilkie (19). After washing the cells in medium they were cultured in 5% fetal calf serum/Eagle's ME medium for 48 hr.

**Immunofluorescence.** Forty-eight hours after transfection the coverslip was removed from the medium, washed once with phosphate-buffered saline (P<sub>i</sub>/NaCl), and fixed with acetone at -20°C for 5 min. By the use of nail varnish the coverslip was divided into four areas of 7 × 7 mm each for convenience in counting the number of fluorescence-positive cells. Cells expressing EBV antigens were stained by indirect immunofluorescence by the standard procedure described by Henle and Henle (20) using human antisera with different reactivities against EBV-specific antigens. The results of the transfection were scored by counting the number of fluorescent cells per 7-mm square. The number of cells varied between 5 × 10<sup>4</sup> and 10 × 10<sup>4</sup> cells per 7-mm square.

**EBV DNA Clones.** A detailed description of the complete set of M-ABA (EBV) DNA clones is given elsewhere (16). A restriction map of *Bam*HI, *Eco*RI, *Hind*III, and *Sal* I sites of M-ABA (EBV) with a description of the clones used in this study is given in Fig. 1.

**RESULTS**

**Expression of a Nuclear Antigen in BHK Cells by Transfection of Overlapping Clones.** To study viral antigen expression by transfection we have used seven of the EBV DNA clones shown in Fig. 1, which span over the entire viral genome and which overlap by at least 3 kilobases (kb). Ten micrograms of each DNA was precipitated with calcium phosphate and applied to BHK cells as recipient cells. Forty-eight hours after transfection the cells were fixed and stained with an antiserum containing anti-VCA, anti-EA, and anti-EBNA antibodies by indirect immunofluorescence. The clones cM B14 and cM 302-23 induced the synthesis of an EBV-specific nuclear antigen. Both clones were generated by partial digestion of M-ABA virus DNA by *Hind*III and subsequent cloning of the partial digestion products into the *Hind*III site of pHC79. cM B14 contains the *Hind*III fragments B, M, N, J, and G, and cM 302-23 contains the fragments G, H, E, I<sub>2</sub>, O, I<sub>1</sub>, and P, both clones thus sharing the *Hind*III-G fragment. Both clones showed the same pattern of fluorescence with the staining confined to the nucleus of the recipient cells (Fig. 2). The dose-response between the amount of cM 302-23 DNA used for transfection and the number of fluorescence-positive cells was linear up to a DNA concentration of about 20 μg applied per well (data not shown). Under optimal conditions, about 0.5-1% of the total number of cells expressed the antigen.

Staining of the nuclear antigen was apparently associated

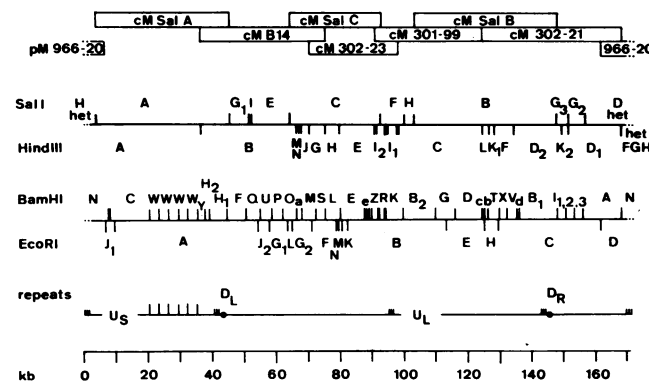


FIG. 1. Restriction map of *Sal* I, *Hind*III, *Bam*HI, and *Eco*RI in M-ABA (EBV) DNA. Clones used for transfection are represented above the map. D<sub>L</sub> and D<sub>R</sub>, left and right duplication; U<sub>S</sub> and U<sub>L</sub>, short and long unique region. pM 760-22 contains the *Hind*III-*Sal* I fragment overlapping between *Sal*-A and *Hind*III-B.

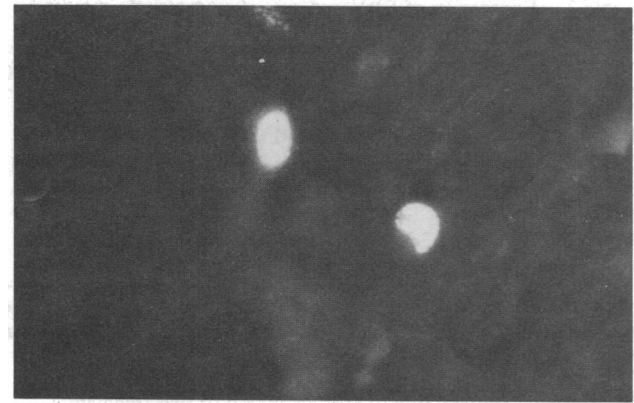


FIG. 2. Nuclear antigen expressed in BHK cells transfected with DNA of cM 302-23. (×250.)

with the presence of anti-EA antibodies and not with the presence of anti-VCA or anti-EBNA antibodies in the different sera. Only sera containing anti-EA antibodies stained positive, whereas sera containing relatively high anti-EBNA and anti-VCA titers, but no anti-EA antibodies, did not react with the antigen (Table 1). This indicates that the antigen induced by transfection of cM B14 and cM 302-23 DNA belongs to the early antigen complex. None of the other five cloned DNAs (cM *Sal*-A, cM *Sal*-B, cM 301-99, cM 302-21, and pM 966-20) induced an antigen that reacted by indirect immunofluorescence with any of the sera tested.

***Hind*III-G Is Coding for the Nuclear Antigen of the Early Antigen Complex.** The observation that the cosmid clones that induce the nuclear antigen share the *Hind*III-G fragment suggested that the antigen might be coded for by this fragment. This could, indeed, be demonstrated by using the subcloned *Hind*III fragments of cM 302-23 for transfection. Only the subclone containing the 5-kb *Hind*III-G fragment induced the nuclear antigen, whereas clones containing *Hind*III-H, -E, -I<sub>2</sub>, and -I<sub>1</sub> failed to induce this antigen (data not shown).

Table 1. Reactivity of human sera to EBV antigens expressed in BHK cells after transfection of cM 302-23

Reactivity	Serum no.*	Titer			Positive cells per 7-mm square†
		Anti-VCA	Anti-EA	Anti-EBNA	
VCA +, EA +	9923	2048	1024	32	714
	2625	1024	256	64	648
	8280	2048	1024	64	613
	8213	2048	512	64	195
	8181	1024	256	16	155
VCA +, EA -	7526	512	256	64	399
	9922	512	<8	16	0
	9680	512	<8	8	0
	9009	512	<8	256	0
	8994	256	<8	32	0
VCA -, EA -	8080	128	<8	128	0
	7413	512	<8	64	0
	10097	<8	<8	<8	0
	10085	<8	<8	<8	0
	9932	<8	<8	<8	0
	9596	<8	<8	<8	0

Ten micrograms of cloned DNA was cotransfected with 10 μg of salmon sperm DNA.

\*Sera were used for immunofluorescence at a dilution of 1:30.

†Mean values of fluorescent cells per four 7-mm squares. Similar results were obtained with cM B14 and cM *Sal*-C. Transfections with cM *Sal*-A, cM 301-99, cM *Sal*-B, cM 302-21, and pM 966-20 were negative.

Table 2. Expression of EBV antigens after cotransfection of DNA of two clones

	<i>Sal-A</i>	B14	302-23	301-99	<i>Sal-B</i>	302-21	966-20
<i>Sal-A</i>	0	593*	1448*†	424†	0	0	0
B14		142*	1103*†	591*	279*	139*	136*
302-23			417*	428*	214*	142*	347*
301-99				0	0	0	0
<i>Sal-B</i>					0	0	0
302-21						0	0
966-20							0

Ten micrograms of each DNA was used for transfection. The numbers represent mean values of fluorescent cells per four 7-mm squares.

\*Nuclear antigen due to the presence of *HindIII-G*.

†Cytoplasmic antigen due to cotransfection of two fragments.

**Expression of a Cytoplasmic Antigen After Cotransfection of Two Different EBV Cosmid Clones.** Transfection of intact EBV DNA was reported to induce a cytoplasmic antigen in addition to a nuclear antigen in human placental cells (6). Similarly, after cotransfection of DNA of seven overlapping clones into BHK cells, we observed cells in which the cytoplasm as well as the nuclei were stained by indirect immunofluorescence with anti-EBV specific antibodies. Since a cytoplasmic antigen could not be detected after transfection of the seven individual cosmid clones, we attempted to induce the cytoplasmic antigen by cotransfection of all possible combinations of two cosmid clones.

All combinations of clones containing either cM B14 or cM 302-23, which both contain *HindIII-G*, induced the synthesis of a nuclear antigen in accordance with the results described above. However, a significantly higher number of cells expressed virus-specific antigens after cotransfection of cM 302-23 plus cM *Sal-A* or cM 302-23 plus cM B14 (Table 2). Additionally, after transfection of both combinations of clones a different pattern of fluorescence staining was observed with a cytoplasmic fluorescence in addition to the nuclear staining. Similar results were obtained, when cM B14 and cM 301-99 DNA were transfected into BHK cells together. Moreover, cotransfection of cM *Sal-A* and cM 301-99 generated the synthesis of a cytoplasmic antigen (Fig. 3), even though both clones individually failed to induce any antigen.

**Identification of the Sequences Responsible for the Synthesis of the Cytoplasmic Antigen.** The cotransfection assay always revealed expression of the cytoplasmic antigen if combinations of cM 302-23 or cM 301-99 and cM *Sal-A* or cM B14 were applied to the recipient cells. Both pairs of clones overlap significantly. cM 302-23 and cM 301-99 have *HindIII-I2*, -O, -I1, and -P in common, and cM *Sal-A* and cM B14 share the rightmost and leftmost 8 kilobase pairs, respectively, which carry the D<sub>L</sub> region (see Fig. 1) (21, 22). To narrow down the regions required for the expression of the cytoplasmic antigen, the subcloned *HindIII* fragments of cM 302-23 were cotransfected with cM *Sal-A*. The cytoplasmic antigen was only induced when *HindIII-I2* was cotransfected with

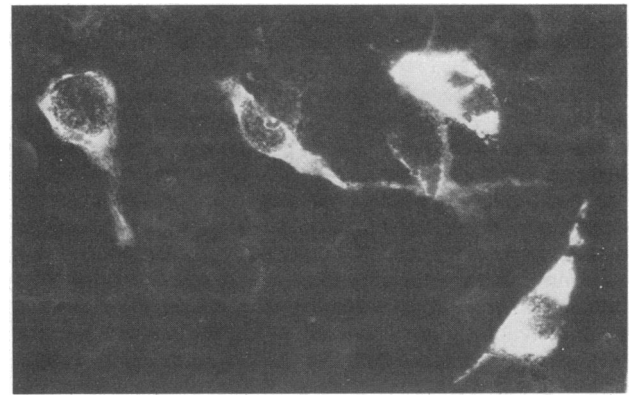


FIG. 3. Cytoplasmic antigen expressed in BHK cells cotransfected with DNA of pM *HindIII-I2* and pM 760-22. ( $\times 250$ .)

cM *Sal-A* (Table 3, left). Cotransfection of *HindIII-I2* with cM B14 resulted in expression of antigens in the cytoplasm as well as in the nucleus. Analysis of the cytoplasmic antigen with sera with different reactivities against EBV-EA and -VCA, similar to that described above for the nuclear antigen, revealed that the cytoplasmic antigen induced by cotransfection also belongs to the early antigen complex (data not shown).

The cooperation between *HindIII-I2* and sequences in cM *Sal-A* was studied further by cotransfection of *HindIII-I2* with subcloned fragments of *Sal-A*. The *Bam*HI fragments F, H2, Y, and W and the *Bgl* II fragments C and U as well as the part of *Sal-A* to the left-hand side of the large internal repeat (pM 765-10) failed to induce the antigen upon cotransfection with *HindIII-I2*. *Bam*HI-H1 cotransfected with *HindIII-I2* induced a weak diffuse cytoplasmic fluorescence pattern, quite distinct from the brilliant fluorescence observed after cotransfection of cM *Sal-A* and pM *HindIII-I2*. Only the clone containing the right-hand side of *Sal-A* (pM 760-22), which overlaps completely to cM B14, was capable of inducing the brilliant staining pattern in the cytoplasm in cooperation with *HindIII-I2* (Table 3, center), thus localizing the sequences important for the antigen induction to the 8-kb *HindIII-Sal I* fragment shared by cM *Sal-A* and cM B14. Cotransfection experiments, in which one of the two components was varied and the second kept constant, revealed identical dose-response curves for both fragments (Fig. 4).

**Inactivation of the Antigen-Inducing Activity in pM 760-22 by Restriction Nuclease Digestion.** *HindIII-I2* and the insert of pM 760-22 are 3 and 8 kb long, respectively. To narrow down the respective region in pM 760-22, the DNA was digested with various restriction endonucleases and controlled on a gel before being used for transfection together with *HindIII-I2*. Digestion with *Bgl* II and *Sma* I inactivated completely the capacity of pM 760-22 DNA to induce the cytoplasmic antigen in collaboration with *HindIII-I2*, whereas *Kpn* I, *Not* I, *Sac* I, *Sac* II, and *Sph* I did not destroy the antigen-inducing activity of this fragment. After cotransfection of *HindIII-*

Table 3. Expression of a cytoplasmic antigen after cotransfection of DNA of subclones of cM 302-23 and cM *Sal-A*

Clone	cM <i>Sal-A</i>		Clone	pM <i>HindIII-I2</i>		Clone	pM <i>HindIII-I2</i>	
	Nucleus	Cytoplasm		Nucleus	Cytoplasm		Nucleus	Cytoplasm
pM <i>HindIII-G</i>	671	0	pM 760-22	0	227	pM <i>Bgl</i> II-C	0	0
pM <i>HindIII-H</i>	0	0	pM <i>Bam-F</i>	0	0	pM <i>Bgl</i> II-U	0	0
pM <i>HindIII-E</i>	0	0	pM <i>Bam-H1</i>	0	0*	pM 765-10	0	0
pM <i>HindIII-I2</i>	0	262	pM <i>Bam-H2</i>	0	0	cM <i>Sal-A</i>	0	185
pM <i>HindIII-I1</i>	0	0	pM <i>Bam-Y</i>	0	0			

Five micrograms of DNA of each clone and 10  $\mu$ g of salmon sperm DNA were cotransfected onto BHK cells. The numbers represent mean values of fluorescent cells per four 7-mm squares.

\*Cells with faint fluorescence different from the staining pattern of the positive controls were present.

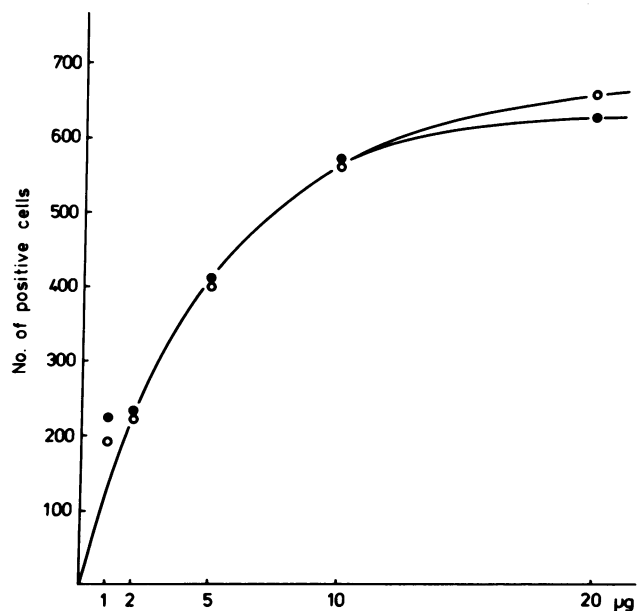


FIG. 4. Number of cells expressing the cytoplasmic antigen in relation to the amount of DNA used for transfection. BHK cells were cotransfected with a constant amount of pM *HindIII-I2* DNA (5 μg) and variable amounts of pM 760-22 DNA (●) or with a constant amount of pM 760-22 DNA (5 μg) and variable amounts of pM *HindIII-I2* DNA (○). The cells were fixed after 48 hr for immunofluorescence staining. The number of positive cells represents a mean value of four 7-mm squares.

I2 with *Bam*HI-digested pM 760-22 DNA, the cytoplasmic fluorescence pattern was faint, indicating that the antigen-inducing activity is partially abolished by *Bam*HI digestion. *Bam*HI, *Bgl* II, and *Sma* I have restriction sites on the right-hand side of pM 760-22 outside the D<sub>L</sub> region (Fig. 5). This indicates that sequences of 3 kb at the right-hand side of pM 760-22 close to the *Sal* I site are required for induction of the cytoplasmic antigen.

**DISCUSSION**

We have described two distinct EBV-related antigens that were detected in BHK cells transfected with DNA of overlapping clones of the M-ABA (EBV) strain. This strain was originally derived from a nasopharyngeal carcinoma and has the biological properties and the genome organization of an EBV prototype. One of the induced antigens is located in the nucleus, whereas the second antigen is confined to the cyto-

plasm of the recipient cell. Both antigens belong to the early antigen complex.

Expression of viral antigens after transfer of cloned DNA fragments has been reported by Grogan *et al.* (6), Summers *et al.* (8), and Glaser and co-workers (9, 23). The first antigen that has clearly been assigned to a specific region of the genome is EBNA, which is encoded by *Bam*HI-K (8). Glaser and co-workers have reported that B95-8 *Bam*HI-H induced a cytoplasmic antigen upon transfection into D98/P3HR-1 hybrid cells (9) and upon microinjection into EBV-negative epithelial cells, which is related to the R component of the early antigen complex (23, 24). Additionally, they reported that another cytoplasmic antigen (EA-D) is synthesized upon injection of DNA of a clone encompassing part of the sequences of B95-8 virus *Bam*HI-K and -B (clone EB 61-72; ref. 25). We have been unable to induce cytoplasmic antigens by transfection of BHK cells with cM *Sal*-A or cM 301-99 alone, which span completely over the B95-8 clones used by Glaser *et al.* However, since different techniques of DNA transfer, different target cells, and different cloned fragments were used in these experiments, it is difficult to determine the reason for the discrepancies.

Transfection with subcloned fragments has located the gene encoding the nuclear antigen on the *Hind*III-G fragment, which is 5 kb long. This antigen is obviously different from EBNA. It shows a homogenous brilliant staining in contrast to the characteristic dotted fluorescence pattern of EBNA and is stained only by anti-EA-positive and not by anti-EA-negative sera, irrespective of the anti-VCA and anti-EBNA titers. Therefore, this antigen appears to be different from that encoded by *Bam*HI-M (12). *Bam*HI-M overlaps significantly to *Hind*III-G. To further clarify the nature of these proteins it will be important to map the coding regions in more detail and to characterize the respective polypeptides themselves.

We have also presented evidence that a cytoplasmic antigen of the early antigen complex is expressed when two fragments distantly located on the viral genome are simultaneously transferred into BHK cells. Individually, neither of the fragments exerted a biological effect upon transfection. The biologically active sequences are located at the right-hand end of *Sal*-A (pM 760-22) and in *Hind*III-I2, ≈50 kb apart. The clone pM 760-22 is 8 kb in size and contains the complete D<sub>L</sub> region including the *Not* I repeats and about 1.5 kb to the left and 3 kb to the right of the D<sub>L</sub> region. Digestion with *Bgl* II and *Sma* I completely abolished the antigen-inducing activity in cooperation with *Hind*III-I2, whereas digestion with *Kpn* I, *Not* I, and *Sph* I had no effect. *Bam*HI treatment partially inactivated the antigen-inducing activity

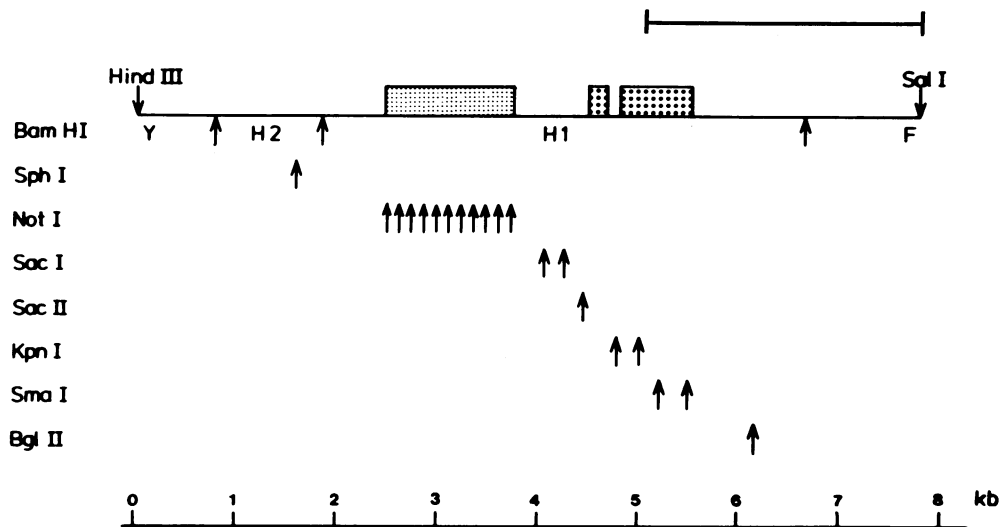


FIG. 5. Inactivation of the antigen-inducing activity of pM 760-22 by cleavage with various restriction enzymes. The *Not* I repeats are marked by fine dots, and the region of homology to D<sub>R</sub> (see Fig. 1) is marked by large dots. The upper line represents the region required for induction of the cytoplasmic antigen in the cotransfection assay with the *Hind*III-I2 fragment.

of *Sal-A* and pM 760-22. This pattern of inactivation allowed us to narrow down the region involved in antigen induction to 3 kilobase pairs to the right of the duplicated region ( $D_L$ ).

Several possibilities exist to explain the cooperative effect of two distant regions in antigen induction. First, cotransfection of two separate fragments has been shown to result in ligation of fragments to each other allowing an interaction of both fragments in "*cis*" (26). An action in *cis* can be ruled out, however, by experiments in which both fragments were separately introduced into BHK cells. The first fragment was cotransfected with a gene for a dominant marker, giving rise to a cell line containing the respective EBV DNA fragment. Introduction of the second fragment into this cell line revealed that expression of the cytoplasmic antigen is readily accomplished under these conditions (unpublished data), thus excluding a cooperation of both fragments in *cis*. Both regions could cooperate in "*trans*" in such a way that one of the two codes for the antigen itself and the second for a protein, whose prior synthesis is required for the production of the antigen. Alternatively, both regions could possibly code for two different polypeptide chains, which are then assembled into the same protein with the known antigenic properties. At present we cannot differentiate between both possibilities.

The viral early antigens, which are induced by phorbol 12-myristate 13-acetate in Raji or NC37 cells, have been shown to consist of three major components of 85, 58, and 35 kilodaltons (27). Several other components have also been identified in producer cell lines treated with phosphonoacetic acid, which inhibits viral DNA replication and synthesis of late viral proteins. Eleven different early polypeptides have been assigned to the B95-8 virus genome by hybrid selection of RNA with cloned DNA fragments and *in vitro* translation of the eluted RNA species (28). Three polypeptides of 36, 44, and 47 kilodaltons have been obtained by translation of RNAs selected by *Bam*HI-M. This fragment overlaps with *Hind*III-G. Whether the antigen expressed by transfection corresponds to one of these polypeptides remains to be answered. Additionally, whether these antigens are recognized by monoclonal antibodies raised against distinct components of the early antigen complex remains to be studied. The monoclonal antibodies, recently described by Pearson *et al.* (29), have not yet been available to us during the course of our experiments.

It would be desirable to establish cell lines carrying the corresponding fragments that express the respective antigens constitutively. Such cell lines would easily allow the generation of specific antibodies and the biochemical characterization of the viral proteins. They might also be of diagnostic value to dissect the antibody response in patients with EBV-associated diseases. Such cell lines have not yet been established by cotransfection with a plasmid conferring a dominant selectable marker. Expression of early antigen components, however, might be cytopathic and thus incompatible with survival of the cells.

Expression of early antigens occurs in association with the lytic cycle of the virus, whereas EA expression is repressed in EBV-transformed lymphocytes. This repression of the lytic cycle is at least one of the basic functions required for immortalization, since induction of the lytic cycle leads to cell death and thus terminates the transformed state. Whether the repression is mediated by a viral or cellular function is unknown. By cotransfection with two different EBV DNA clones into BHK cells, we have so far been unable to identify a fragment that represses the expression of an antigen. However, it is conceivable that the natural target cells for immortalization by EBV, the human B lymphocytes, provide a

function involved in repression of the lytic cycle. Therefore, it will be important to study expression of EBV antigens in lymphocytes transfected by EBV DNA fragments and to establish systems in which the regulation of antigen expression can be studied *in vitro*.

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