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A Second Nuclear Protein Is Encoded by Epstein-Barr Virus in Latent Infection

Abstract. *A region of the Epstein-Barr virus (EBV) genome that is important in inducing cell proliferation includes a single long open reading frame. Part of this open reading frame has been fused to the* lacZ *gene and expressed in* Escherichia coli. *Antisera to the fusion protein identify a protein in the nuclei of latently infected growth-transformed lymphocytes and in Burkitt tumor cells grown in vitro. This nuclear protein is encoded by a different virus gene than that which encodes the previously described EBV nuclear antigen, EBNA.*

Epstein-Barr virus (EBV) infection of human B lymphocytes results in cellular proliferation (*1*). The virus causes infectious mononucleosis and is believed to be an etiologic factor in two prevalent human tumors, Burkitt lymphoma and nasopharyngeal carcinoma (*2*). EBV does not productively replicate in most infected B lymphocytes or tumor cells but persists in a latent state (*3*). The latent virus genome is usually episomal (*4*) but may integrate into the cell genome (*5*). At least three messenger RNA's (mRNA's) are transcribed from widely separated regions of the 170-kilobase (kb) EBV genome in latently infected cells (*6–8*). One of these RNA's, encoded from the IR1-U2 region (Fig. 1A), appears to be important in initiation of growth transformation since a virus isolate (P3HR-1) with a deletion in the U2 domain cannot initiate growth transformation (*9, 10*). Recombinants between P3HR-1 and cloned U2 DNA can be obtained that are capable of transforming cells (*11*). Furthermore, the U2 domain transfected into lymphocytes induces DNA synthesis (*12*). We now demonstrate that the U2 domain of EBV encodes a recently identified nuclear antigen, EBNA2, that is characteristic of latently infected, growth-transformed cells (*13*).

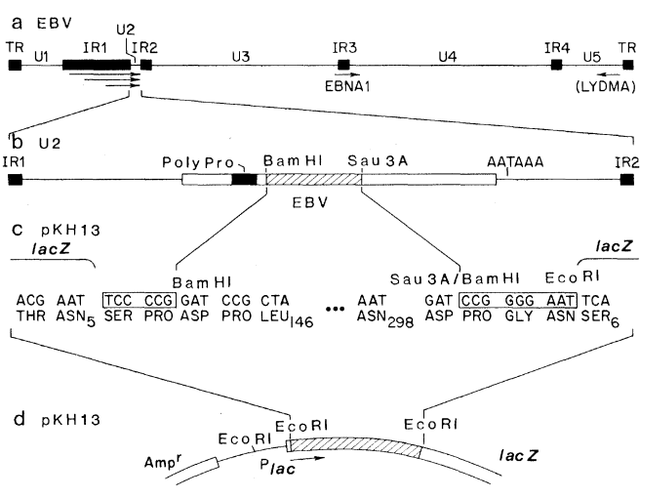

Fig. 1. EBV genome and pKH13 construction. (a) The EBV genome showing the location of major internal repeats (IR), terminal repeats (TR), and single-copy (U) domains (*3*). Transcripts and proteins characteristic of latently infected cells are indicated by arrows. Two other polyadenylated RNA's and two small nonpolyadenylated RNA's have also been detected from U1. Parentheses indicate tentative mapping of the LYDMA function to the membrane protein encoded by the U5 RNA (*8, 14*). (b) The U2 domain (*15*). The major open reading frame (open boxes) contains a polyproline-encoding triplet repeat (solid box). The location of the 465-bp Bam HI–Sau 3A fragment expressed in bacteria is shown (hatched box). (c) DNA sequence of the boundaries between the pKH13 insert and vector and the predicted amino acid sequence. Amino acids are numbered for their position in β-galactosidase (*25*) or in the EBV open reading frame (*15*). The boxed regions are characteristic of the polylinker in the pSKS104 vector (*17*). (d) Diagram of pKH13 showing the relationship of the gene fusion to the *lac* promoter.

A membrane protein and two nuclear proteins have been detected in cells latently infected with EBV (*8, 13–15*). The membrane protein is encoded by the 3′ end of the EBV genome (*8, 14*) (Fig. 1). This region probably is the source of the new cell membrane antigen, LYDMA, which is recognized by T cells from previously infected individuals. Its amino acid sequence is known from RNA mapping and DNA sequencing (*8*). One nuclear protein, EBNA, has been mapped to an EBV open reading frame which contains a polymorphic triplet repeat element (*6*). Mapping of this protein was facilitated by correlating the DNA repeat reiteration polymorphism among different EBV isolates with a polymorphism in protein size (*6*). Transfection with an EBV DNA fragment containing the open reading frame resulted in induction of a nuclear antigen detected by polyvalent, human sera (*16*). Antibody to a protein expressed in *Escherichia coli* from part of the EBNA or putative LYDMA gene identified these proteins in latently infected cells, thus confirming the earlier results (*13, 14*). A second, antigenically unrelated nuclear protein, EBNA2, was identified by EBV-immune human sera in all cells of most latently infected B lymphocyte cultures (*13*). Recent evidence has suggested that EBNA2 is encoded by the U2 domain of EBV DNA, a region which contains a polymorphic 1.5-kb open reading frame transcribed in latently infected, growth-transformed cells (*7, 15*) (Fig. 1). Five EBV isolates with similar U2 domains were shown to induce similar EBNA2 proteins; a virus with a shorter U2 open reading frame induced a smaller EBNA2 protein and a virus in which U2 was deleted lacked EBNA2 (*15*).

To prove that the U2 open reading frame encodes EBNA2, we inserted a 465-base-pair (bp) U2 Bam HI–Sau 3A fragment into the Bam HI site of the *lacZ* bacterial expression vector

pSKS104 (*17*) (Fig. 1). This gene fusion should result in expression of a U2/*lacZ* fusion protein with a mass of 135 kilodaltons (kD); galactosidase is 116 kD and the EBV U2 Bam HI–Sau 3A fragment should translate a 17-kD polypeptide. As shown in Fig. 2, recombinant clones did express a fusion protein of the expected size. One recombinant clone, pKH13, was used to purify the fusion protein. The fusion protein was relatively unstable and degraded protein was evident in all preparations (Fig. 2). Attempts to express more of the U2 open reading frame resulted in fusion proteins that were less stable (*18*). Under optimal conditions for bacterial growth and protein extraction, equal amounts of the 135-kD fusion protein and a 120-kD degradation product were obtained from *E. coli* carrying pKH13 (Fig. 2). Both the intact fusion protein and the 120-kD degradation product reacted with EBV-immune human sera that detect EBNA2 (Fig. 2). The reactivity was specific for the EBV domain of the fusion protein since prior absorption had removed all reactivity with β-galactosidase and other *E. coli* proteins (Fig. 2). One human serum reacted preferentially with the 135-kD fusion protein, while a second reacted equally with the 135- and 120-kD proteins. This indicates that there are at least two epitopes in the expressed protein segment that can be recognized by immune human sera.

Sera from rabbits immunized with the partially purified 135- and 120-kD fusion proteins reacted with EBNA2 after electrophoretic blotting and immunostaining of nuclear proteins (Fig. 3). The EBNA2 protein detected with the rabbit sera was 88 kD in IB4, II100-1, and I100-1 cells, which are independently derived lymphocyte cell lines that were infected with the B95-8 EBV isolate; 86 kD in Lamont cells, 86-kD in W91 cells, and 87 kD in Raji cells (Fig. 3). As expected, EBNA2 protein was not detected in extracts of B cell lines which had not been infected with EBV, such as the BJAB cell line. These results are identical to those obtained with EBNA2-reactive human sera (*13, 15*) (Fig. 3). After absorption with uninfected lymphoblastoid cells, the rabbit sera were used in indirect immunofluorescence tests with the latently infected IB4 and uninfected BJAB cell lines. Weak granular nuclear fluorescence was observed in all IB4 cells, similar to that found with EBNA2- and EBNA1-reactive human sera (*13*). No reactivity was found with BJAB nuclei. The EBV genome in the Daudi line of Burkitt lymphoma cells was recently shown to be deleted for part of U2 (*19*). We extended the correlation between U2 and EBNA2 by demonstrating that there is no EBNA2 protein in the nuclei of Daudi cells (Fig. 3).

These results show that EBV encodes EBNA2 and that part of EBNA2 is located in the U2 open reading frame (Fig. 1b). The polyproline-encoding triplet repeat element in this open reading frame (Fig. 1b) is also likely to encode part of EBNA2 as the AG876 isolate has a shorter polyproline repeat and a smaller EBNA2 (*15*). Differences in repeat reiteration frequency are known to result in variability in other EBV proteins (*8, 13, 14*). It is conceivable that the U2 open reading frame encodes for all of EBNA2. The sequence *ATCATG* (A, adenine; T, thymine; C, cytosine; G, guanine) near the beginning of the U2 open reading frame has the characteristics of a translational initiator (*15, 20*). The 3′ end of the exon has been mapped to a site immediately preceded by a termination codon and a polyadenylation signal (*7, 15, 21*). Although the B95-8 open reading frame would only encode a 53-kD protein, the discrepancy between this and the apparent EBNA2 size of 88 kD on denaturing gels is not unusual for a protein of such high proline content (28 percent) as is encoded by the open reading frame (*15, 22*). Precise mapping of the 5′ end of the U2 exon and the 5′ exons of this RNA will reveal whether translation is initiated within the U2 open reading frame.

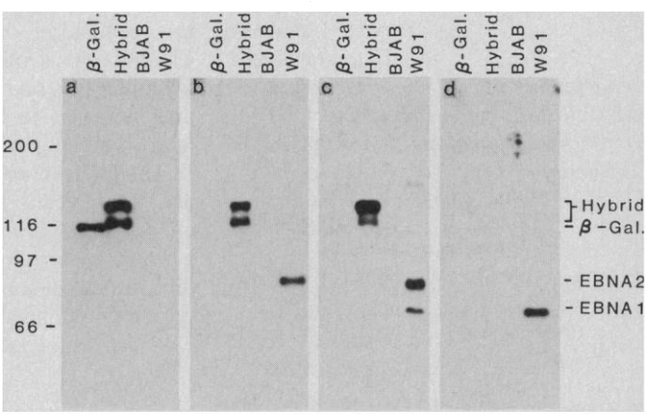

Fig. 2. Human EBNA2-reactive sera also react with the hybrid protein. Electrophoretic blots (*26*) were reacted with (a) a rabbit antiserum to β-galactosidase; (b and c) two human EBNA2 reactive sera, or (d) a human EBNA1 reactive serum (*13, 15*). β-Galactosidase, nuclear proteins from BJAB (EBV-negative) and W91 (EBV-positive) cells, and partially purified hybrid protein were separated by discontinuous 6 percent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to nitrocellulose (*13*). Hybrid protein was prepared by boiling *E. coli* cell pellets in sample buffer (*13*), followed by chromatography on Sephacryl-400 columns in SDS (0.1 percent).

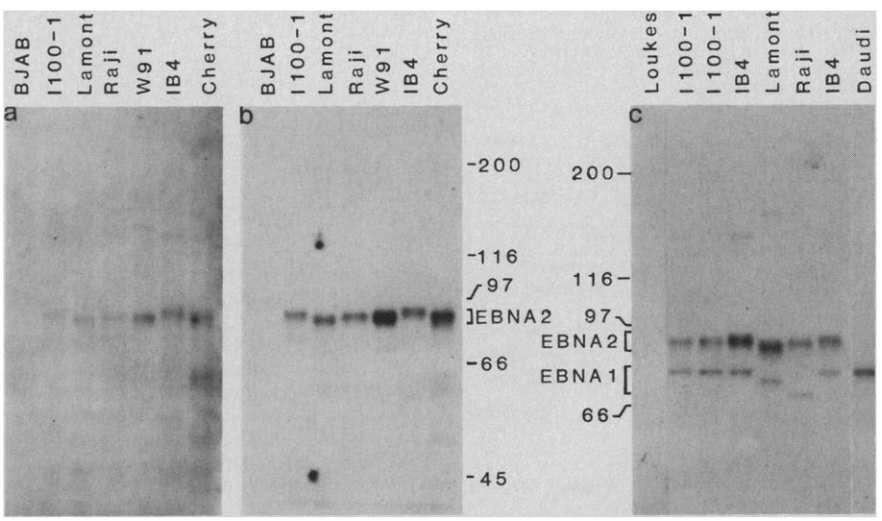

Fig. 3. EBNA2 is encoded by the EBV U2 open reading frame. Immune staining of electrophoretic blots of nuclear proteins of cell lines selected to show the variability in size of EBNA2 with (a) rabbit antiserum to pKH13 hybrid protein, (b) human serum reactive with EBNA2, and (c) a human serum reactive with both EBNA1 and EBNA2. Electrophoresis was done on either 7.5 percent (a and b) or 6 percent (c) SDS-PAGE (*13*). Rabbit antiserum was prepared by injecting rabbits with 0.1 mg of hybrid protein plus complete Freund's adjuvant followed by two booster injections of protein in incomplete Freund's adjuvant. Sera were preabsorbed with β-galactosidase and an EBV-negative B cell line, Loukes. Cell lines were previously described (*15*).

P3HR-1 and virus isolated from Daudi cells have the entire U2 domain deleted (*9, 19*) and do not express an EBNA2 protein (*15*) (Fig. 3). The absence of U2 from these growth-transformed cells is in apparent conflict with the proposed role of EBNA2 in growth transformation. However, after virus-induced growth transformation, the function of EBNA2 may have been superseded by the increased expression of c-*myc* which is known to be activated in these Burkitt tumor cell lines (*23, 24*). In support of the hypothesis that deletion of U2 occurred long after growth transformation and c-*myc* translocation is the observation that Jijoye, the Burkitt tumor cell line from which P3HR-1 is derived, has a normal-sized U2 domain (*9*).

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Rapid Switching of Plant Gene Expression Induced by Fungal Elicitor

Abstract. *The pattern of messenger RNA synthesis in suspension-cultured bean cells (*Phaseolus vulgaris *L.) was analyzed by blot hybridization and in vitro translation of newly synthesized messenger RNA. The RNA was separated from pre-existing RNA by organomercurial affinity chromatography after in vivo labeling with 4-thiouridine. The elicitor induced the synthesis of messenger RNA's encoding phenylalanine ammonia-lyase, chalcone synthase, and chalcone isomerase, three enzymes of phenylpropanoid metabolism involved in the synthesis of isoflavonoid-derived phytoalexins. This is part of a rapid and extensive change in the pattern of messenger RNA synthesis directing production of a set of proteins associated with expression of disease resistance.*

The natural process of disease resistance in plants involves inducible defense mechanisms such as the accumulation of host-synthesized phytoalexin antibiotics, deposition of lignin-like material, accumulation of hydroxyproline-rich glycoproteins and proteinase inhibitors, and increases in the activity of certain hydrolytic enzymes (*1*). Such responses can be induced not only by infection but also by glycan, glycoprotein, or lipid elicitor preparations obtained from fungal and bacterial cell walls and culture filtrates and in some cases by structurally unrelated artificial elicitors or mechanical damage (*1, 2*).

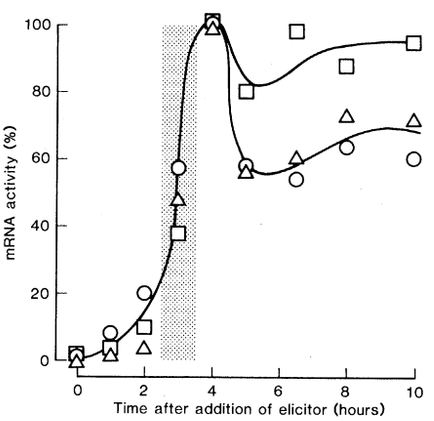

Fig. 1. Labeling of bean cells with 4-thiouridine during elicitor induction of mRNA activities encoding PAL (○), CHS (△), and CHI (□). Cells (*18*), were labeled for 1 hour (shaded area) with 4-thiouridine (1 millimolar; Sigma) and [5,6-^{3}H]uridine (0.04 micromolar, 38.4 Ci/mmol; New England Nuclear). For measurement of mRNA activities, total RNA (isolated by extraction directly into phenol) (*19*) was translated in vitro with a rabbit reticulocyte lysate translation system. Labeled enzyme subunits were visualized by fluorography following separation by immunoprecipitation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*6*). The mRNA activity was defined as the incorporation of [^{35}S]methionine into immunoprecipitable subunits as a percentage of incorporation into total protein, and was expressed relative to the activity at maximal induction (4 hours).

Biological stress causes extensive characteristic changes in the pattern of protein synthesis that are related to expression of specific defense responses (*3–6*). Induction of messenger RNA's (mRNA's) encoding phytoalexin biosynthetic enzymes and hydroxyproline-rich glycoproteins after stress has been observed by means of RNA blot hybridization with appropriate cloned DNA sequences as probes (*7, 8*). These observations suggest that specific changes in gene expression might be an early stage in plant defense. To test this hypothesis we have studied the effect of elicitor on the pattern of RNA synthesis in suspension-cultured bean cells (*Phaseolus vulgaris* L.) by means of a technique based on in vivo labeling with 4-thiouridine (*9*).

Treatment of bean cells with a fungal elicitor prepared from *Colletotrichum lindemuthianum* causes rapid coordinated increases in the rate of synthesis of three enzymes of phenylpropanoid biosynthesis, phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and chalcone isomerase (CHI), concomitant with the onset of accumulation of phaseollin and related phenylpropanoid-derived isoflavonoid phytoalexins (*5*). The increase in enzyme synthesis reflects increases in the corresponding mRNA activities which, together with modulation of the apparent stability of the biosynthetic enzymes in vivo, are responsible for the marked increases in enzyme activity that regulate expression of the defense response (*5, 6*). Using cloned CHS complementary DNA (cDNA) sequences as probes in RNA blot hybridizations, a close correspondence between induction of hybridizable mRNA and increased mRNA activity is observed (*8*).

Maximum rates of increase in the mRNA activities encoding PAL, CHS, and CHI (Fig. 1) were observed between 2.5 and 3.5 hours after treatment with an elicitor prepared from *C. lindemuthianum* (*10*), which causes anthracnose