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The 5' flanking region of the gene for the Epstein–Barr virus-encoded nuclear antigen 2 contains a cell type specific *cis*-acting regulatory element that activates transcription in transfected B-cells

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ABSTRACT

We have recently identified the promoter that positions the initiation (cap) site for RNA encoding the Epstein-Barr virus (EBV) determined nuclear antigen 2 (EBNA2) in transfected COS-1 cells. The cells were transfected with recombinant vectors that contained the *Bam*HI WYH region of the EBV genome. In order to delineate regulatory DNA sequences required for the expression of EBNA2 the 5' flanking region of the gene was linked to reporter genes in expression vectors and transfected into EBV genome-negative lymphoid DG75 cells. We demonstrate that several *cis*-acting elements contribute to a transciptional enhancer activity found in the region between nucleotides -553 and -86 relative to the cap site. The enhancer was active in lymphoid DG75 cells but not in HeLa cells and stimulated transcription also from the heterologous thymidine kinase (TK) and β-globin promoters. Nuclear extracts of lymphoid cells contained protein factors that bound to the enhancer. The *in vitro* introduction of a mutation in the enhancer sequence that substantially reduced the transcription stimulatory activity concurrently blocked the binding of one of the factors.

INTRODUCTION

Epstein-Barr virus (EBV) is the aetiological agent of infectious mononucleosis and is involved in the development of Burkitt's lymphoma (BL) and anaplastic nasopharyngeal carcinoma (NPC) by so far undefined mechanisms. EBV also infects human B-lymphocytes *in vitro* and transforms them into continuously dividing, immortalized lymphoblastoid cell lines (LCLs). Only a very small fraction of the cells enters the lytic cell cycle and releases infectious virions. The large majority of the growth transformed lymphoblastoid cells remains in a state of "latent infection", expressing only a limited number of viral genes: a set of nuclear proteins designated EBNA -1, -2, -3, -4, -5, and -6, respectively (1 - 15), a membrane protein (LMP; 16), and two small RNA molecules (17 - 19). The entry of the virus particle into the lymphocyte is mediated by the C3d receptor molecule on the cell surface acting as the EBV receptor (20). Immortalization by EBV infection *in vitro* of other cell types has so far not been achieved. A possible reason might be the absence of virus-specific receptors on the cells. However, recent observations show that a

subset of epithelial cells in primary culture carries the C3d receptor and can be infected by EBV *in vitro* (21, 22). EBNA was detected in infected epithelial cells in the early stages of maturation whereas antigens associated with lytic infection were found in cells approaching senescence. These results suggest that the expression of EBV genes and the outcome of the EBV infection at the cellular level is determined not only by the virus but by intracellular host factors specific for the cell type or differentiation stage.

The observation that EBV-induced growth transformation of lymphoid cells and expression of the EBNA complex are concomitant events, is the main basis for the assumption, that some or all of the proteins constituting the EBNA complex are required for the initiation or maintenance of the immortalized state. A role for EBNA2 in the early phase of B-cell transformation is supported by several additional observations. The P3HR-1 substrain of EBV, which lacks the EBNA2 gene, does not stimulate DNA synthesis in resting B lymphocytes and fails to transform them into immortal cell lines (23, 24). Furthermore, transfected Rat-1 cells, that express EBNA2, grow at lower serum concentrations than the untransfected parental cells (25), and LCLs transformed by strains of EBV, that contain different alleles of the EBNA2 gene, have different growth characteristics (26).

A possible mechanism for the function of EBNA2 is suggested by the finding that transfection of an EBV-negative BL cell line with the EBNA2 gene led to changes in the expression of several cell surface antigens, notably an increase in the expression of the cellular activation antigen CD23 (27). Proteolytic fragments of CD23 reportedly function as an autocrine growth factor for normal and transformed B-cells (28) and might conceivably mediate some of the effects of EBNA2 on cellular growth. Being a nuclear protein EBNA2 presumably has its primary action at the level of transcription and regulates the expression of an assortment of cellular genes. Moreover, EBNA2 might also, directly or indirectly, affect the expression of EBV genes. This is suggested by the observation that EBV-negative BL cell lines converted to EBV genome positivity by *in vitro* infection with the P3HR-1 EBV strain (lacking the EBNA2-encoding exon) in contrast to the corresponding B95-8 EBV-converted lines did not express detectable levels of LMP (29).

The DNA sequence coding for EBNA2 is contained *in toto* within a continuous rightward long open reading frame (BYRF1) in the *Bam*HI Y and H fragments of B95-8 EBV DNA (**Fig. 1**; 25, 30). Transcription of EBNA2 mRNA in COS-1 cells transfected with a subfragment of the *Bam*HI WYH region was initiated at a site 25 bp 3' to a promoter consensus sequence in the *Bam*HI W fragment. The promoter was essential as shown by the fact that EBV-specific RNA synthesis and EBNA2 expression were abolished if the promoter part of the gene was deleted (Ricksten *et*

al., 1987). Furthermore, in several cDNA clones that contained the BYRF1 reading frame the EBV DNA sequence began at or close to the cap site used in the transfected COS-1 cells (12, 31). It thus seems likely that the RNA polymerase II promoter in the *Bam*HI W region is utilized for the synthesis of EBNA2 mRNA in EBV-infected cells at least at some stage of the transformation process.

In this paper we have examined whether the *Bam*HI W promoter region contains enhancer elements that might regulate the expression of the adjacent EBNA2 gene. We demonstrate that several *cis*-acting elements contribute to the transcriptional enhancer activity found in the region between nucleotides -553 and -86 relative to the transcription initiation site. We also show that nuclear extracts of lymphoid cells contain protein factors that bind to the EBV enhancer, and that the introduction of a mutation in the enhancer region, which substantially reduces the transcriptional stimulatory activity, also prevents the binding of one of the factors.

MATERIALS AND METHODS

Cell culture, DNA transfections, and CAT assays

DG75 is an EBV genome-negative lymphoid B-cell line derived from an Israeli Burkitt lymphoma-like case (32). The cells were maintained as suspension cultures in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, penicillin and streptomycin in 5% CO_2 at 37°C. Medium was changed every 3 to 4 days.

Monolayer cultures of HeLa cells were cultured in Iscove's modification of Dulbecco's medium (Gibco) containing 10% fetal calf serum and antibiotics.

The lymphoid cells were transfected using the DEAE-dextran technique of McCutchan and Pagano (33). The cells were subcultured at a density of 0.4×10^6 cells per ml 24 h prior to transfection. The cells were harvested by centrifugation, washed, and portions of 5×10^6 cells were suspended in 1 ml of a transfection solution (30) containing DEAE-dextran (0.5 mg per ml; mol. wt. 500 000; Pharmacia Fine Chemicals), 10 μg of the vector DNA to be tested, and 2.5 μg of the internal control pCH110 DNA that carries the gene for β-galactosidase. The cells were incubated for 1 h at 37°C, washed, exposed to 20% (v/v) dimethylsulphoxide for 2 min at 20°C, washed, suspended in fresh cell culture medium and incubated at 37°C. Forty h after transfection the cells were harvested, suspended in 0.25 ml of 0.25 M Tris-HCl (pH 8.0), and lysed by sonication on ice. Aliquotes of the cell lysates were assayed for chloramphenicol acetyl transferase (CAT) activity by the method of Gorman *et al.* (34) using 80 nmoles acetylcoenzyme A (Sigma) and 0.5 μCi [^{14}C]chloramphenicol (54 mCi per mmol; New England Nuclear) in a total volume of 0.10 ml. The reaction mixtures were incubated for 120 min at 37°C. The amounts of cell extract added were adjusted to give a less than 50% conversion of the ^{14}C-

labelled substrate to product. The acetylated ^{14}C-labelled products were quantitated by liquid scintillation counting after separation by thin layer chromatography and visualisation by autoradiography. The β-galactosidase activity of the cell lysates was assayed by the method of Herbomel *et al.* (35) and used to normalize the CAT activity of the extracts for differences in transfection efficiency.

DNA transfection of HeLa cells was performed by the calcium phosphate coprecipitation technique (36). The cells were seeded at 0.6×10^6 cells per 60 mm culture dish 24 h before transfection. Each dish recieved 5 ml of a suspension containing the calcium phosphate-precipitated plasmid DNAs (7.5 μg of the vector to be tested and 2.5 μg of the pCH110 internal control plasmid) and was then incubated for 4 h at 37°C. The cells were treated for 2 min with 15% glycerol, washed, and incubated at 37°C in fresh cell culture medium. The cells were harvested 40 h later and lysed by sonication on ice. The cell extracts were centrifuged for 5 min at 7000 x g and the supernatants were assayed for CAT and β-galactosidase activities as described above.

Plasmid construction

All manipulations involved in the plasmid constructions were carried out by standard procedures (37) or under the conditions recommended by the enzyme supplier. A library of cloned restriction endonuclease fragments of B95-8 EBV DNA was established earlier (38). The plasmid pSVECAT, which carries the reporter CAT gene under the control of the simian virus 40 (SV40) early promoter, was obtained from M. Yaniv (Institute Pasteur, Paris, France). The plasmid pCH110, which contains the *E. coli* β-galactosidase gene linked to the SV40 early promoter, was purchased from Pharmacia Fine Chemicals.

The recombinant vector pBΔWCAT-1, which carries the 5' flanking region of the EBNA2 gene including the transcription initiation site linked to the CAT gene (**Fig. 1**), was constructed in the following manner. An *Ava*I subfragment of the *Bam*HI W fragment of B95-8 EBV DNA (nucleotides 44547 to 45180) was isolated by agarose gel electrophoresis, and the staggered ends were filled in using the large fragment of DNA polymerase I. The *Ava*I fragment was ligated to *Hind*III linkers and cloned into *Hind*III cleaved pSVECAT DNA replacing the SV40 early control region in this vector.

A series of derivatives of the pBΔWCAT-1 vector in which 5' parts of the EBNA2 control region are deleted (**Fig. 1**) was constructed by cleaving the purified *Ava*I subfragment with *Bgl*II, *Sac*I, *Nar*I, *Apa*I, or *Sna*BI, isolating the fragments corresponding to the 3' end of the *Ava*I fragment, and cloning them into the *Hind*III site of the deleted SVECAT plasmid using *Hind*III linkers as described above. The

resulting recombinant vectors were designated pBΔWCAT-2, -3, -4, -5, and -6, respectively.

The pTE1 plasmid (39) was kindly provided by T. Edlund (University of Umeå, Sweden). In this plasmid transcription of the CAT gene is regulated by the herpes simplex virus (HSV) thymidine kinase (TK) promoter. A polylinker has been inserted about 600 bp upstream of the TK promoter, and restriction endonuclease sites allow easy removal of the intervening pBR322 sequences (**Fig. 3A**). To generate the pTKΔW series of derivatives of pTE1 the EBNA2 promoter-containing *Ava*I fragment and the larger *Sna*BI subfragment of the *Ava*I fragment (nucleotides 44547 to 45014), which does not contain the TATAAA sequence or the cap site (**Fig. 1**), were inserted in both directions into the *Hind*III site of pTE1 using *Hind*III linkers. These constructs were designated pTKΔW-1, -1(*i*), -2, and -2(*i*), respectively. The pBR322 sequences separating the EBNA2 transcriptional control region and the TK promoter in these vectors were removed by cleavage with *Xba*I and *Nru*I and recircularization, generating the plasmids pTKΔW-3, -3(*i*), -4, and -4(*i*), respectively.

The plasmid pβCAT-3 (**Fig. 3A**), kindly provided by C. Svensson (Karolinska Institutet, Stockholm, Sweden), contains the mouse major β-globin promoter linked to the CAT gene and an SV40 DNA fragment carrying the early polyadenylation signal (40). The *Ava*I and the *Ava*I-*Sna*BI subfragments of the *Bam*HI W fragment of EBV DNA defined above were inserted in both directions in front of the β-globin promoter in the vector using *Sal*I linkers. The cloned recombinants were denoted pβΔW-1, -1(*i*), -2, and -2(*i*), respectively.

In the pSVCAT-β plasmid constructed by C. Svensson (Karolinska Institutet, Stockholm, Sweden) the SV40-derived polyadenylation signal of pSVECAT has been replaced by the rabbit β-globin polyadenylation signal. The *Ava*I fragment of EBV DNA defined above and the small EBNA2 promoter-carrying *Sna*BI subfragment of the *Ava*I fragment (**Fig. 1**) were cloned in the pSVCAT-β vector replacing the SV40 early control region. The plasmids were denoted pBΔWCAT-1β and pBΔWCAT-6β, respectively.

Plasmids pBΔWglobin-1 and pBΔWglobin-6 were constructed in the following manner. The *Ava*I fragment and the small *Sna*BI subfragment of the *Ava*I fragment defined above (**Fig. 1**) were cloned into the *Hind*III site of the pTE1 plasmid using *Hind*III linkers. The segment of these constructs that contains the pBR322 sequences between the polylinker and the CAT gene, the CAT gene, and the SV40-derived polyadenylation signal, was removed by *Bam*HI cleavage and replaced by a *Pvu*II-*Bgl*II fragment of psx-β+ DNA (41) using *Bam*HI linkers. This fragment carries the rabbit β-globin coding exons and polyadenylation signal but not the promoter

sequence (nucleotides -9 to + 1200 relative to the transcription initiation site). It was linked immediately 3' to the EBV transcription regulatory sequences in the plasmids.

RNA analysis

Cytoplasmic RNA was prepared by a modification of the method of Favaloro *et al.* (42) using 10 mM vanadyl-ribonucleoside complexes as RNAase inhibitor in the RNA extraction buffer. For RNAase mapping analysis a 426-bp fragment of pBΔWCAT-1 DNA containing the EBNA2 transcription initiation site and the 5' end of the CAT gene (**Fig. 2**) was cloned into the pSP65 vector (Promega Biotec) in the antisense orientation relative to the SP6 promoter. Synthesis of ^{32}P-labelled RNA of high specific activity was carried out under the conditions indicated by Promega Biotec using SP6 RNA polymerase and [^{32}P]UTP (3000 mCi per mmol; New England Nuclear). Full length transcripts were isolated by electrophoresis in denaturing 4% polyacrylamide gels. Before hybridization traces of DNA contaminating the cytoplasmic RNA preparations were removed by incubating 40 µg aliquotes of RNA with DNAase I (RNAase-free; Promega Biotec) for 30 min at 37°C in the presence of RNAase inhibitor (Boehringer), followed by phenol extraction and ethanol precipitation. Hybridization was performed for 16 h at 46°C under conditions described earlier (30) using approximately 10^6 cpm of ^{32}P-labelled RNA probe per reaction. Single stranded material was hydrolyzed by incubation with 5 µg of RNAase A and 10 units of RNAase T1 per ml for 1 h at 37°C . The digests were treated with proteinase K, phenol extracted, ethanol precipitated, and electrophoresed through a 4% polyacrylamide, 7 M urea sequencing gel.

S1 endonuclease analysis of cytoplasmic RNA was performed as described previously (30). Plasmid psx-β + DNA, which contains the rabbit β-globin gene (41), was cleaved with *Bam*HI, end-labelled using T4 polynucleotide kinase and [^{32}P]ATP, denatured, and annealed to 80 µg of RNA. After hybridization the mixture was treated with S1 endonuclease, precipitated with isopropanol, and electrophoresed in a denaturing 4% polyacrylamid gel. The end-labelled fragment of the probe protected by globin RNA is 200 nucleotides long.

Mobility shift binding assays

Nuclear extracts were prepared as described by Dignam *et al.* (43) and stored frozen in aliquotes at -70°C.

DNA fragments were end labelled with ^{32}P by linearization of plasmid DNA with the appropriate restriction endonuclease, and repair of the single stranded ends with [α-^{32}P]dNTP (3 000 Ci/mmol, New England Nuclear) and the Klenow fragment of DNA polymerase I (Boehringer Mannheim). The DNA was cleaved with a second restriction endonuclease, and the fragments separated by electrophoresis on a 4% polyacrylamide gel (acrylamide: bisacrylamide 30:1) in 100 mM Tris, 100 mM boric

acid, 2 mM EDTA (pH 8.3). The wet gel was autoradiographed, and the DNA fragment was excised, electroeluted by isotachophoresis (44), and precipitated with ethanol.

Binding reactions (25 µl) contained 10 mM TrisHCl (pH 7.5), 50 mM NaCl, 1 mM dithiotreitol, 1 mM EDTA, 5% glycerol, 8 µg poly(dI-dC), ^{32}P-labelled DNA (approximately 1 ng, 40000 cpm), and 5 µg of nuclear extract (always added last). For the competition experiments various quantities (0-100 ng) of unlabelled competitor fragments were added to the reaction mixtures before the ^{32}P-labelled DNA. After incubation at room temperature for 25 min the mixture was electrophoresed at 12V/cm for 45 min on a 4% polyacrylamide gel (acrylamide:bisacrylamide 30:1) in 3.7 mM TrisHCl (pH 7.5), 3.3 mM sodium acetate, 1 mM EDTA. The gel was soaked 15 min in 5% glycerol, dried and autoradiographed.

DNAase I footprinting

Binding reactions were as described above except that the reaction volume was 40 µl and the amounts of poly(dI-dC), ^{32}P-labelled fragment, and nuclear extract were increased approximately 5-fold. After incubation at room temperature for 25 min $MgCl_2$ and DNAase I were added to a concentration of 2 mM and 1.25 µg/ml, respectively, and the incubation was continued for 1 min. The DNAase reaction was stopped by the addition of EDTA to 3.5 mM, and the mixture was directly loaded on to a 4% polyacrylamide gel and electrophoresed as described above. The wet gel was autoradiographed for 2 h at 4°C to visualize the various complexes and the bands were excised. The DNA was electroeluted using isotachophoresis (44), precipitated with ethanol, denatured, and electrophoresed on an 8% polyacrylamide gel (19:1) in 7 M urea.

RESULTS

5' flanking sequences of the EBNA2 gene enhance transcription in DG75 cells

We have recently identified the promoter and the transcription initiation site used for the synthesis of EBNA2-specific mRNA in COS-1 cells transfected with recombinant vectors that carry a segment of the *Bam*HI WYH region of the EBV genome (30). In order to delineate regulatory DNA sequences required for the transcription of the EBNA2 gene we have constructed a series of recombinants, in which the 5' flanking region of the gene (nucleotides 44547 to 45180 of B95-8 EBV DNA) or fragments of this region with deletions progressively removing the 5' end were linked to the reporter CAT gene (see **Fig. 1** and Methods). The constructs were transfected into the EBV-negative lymphoid DG75 cell line, and the CAT activity was determined in cell extracts after 48 h as a measure of promoter activity. The plasmid

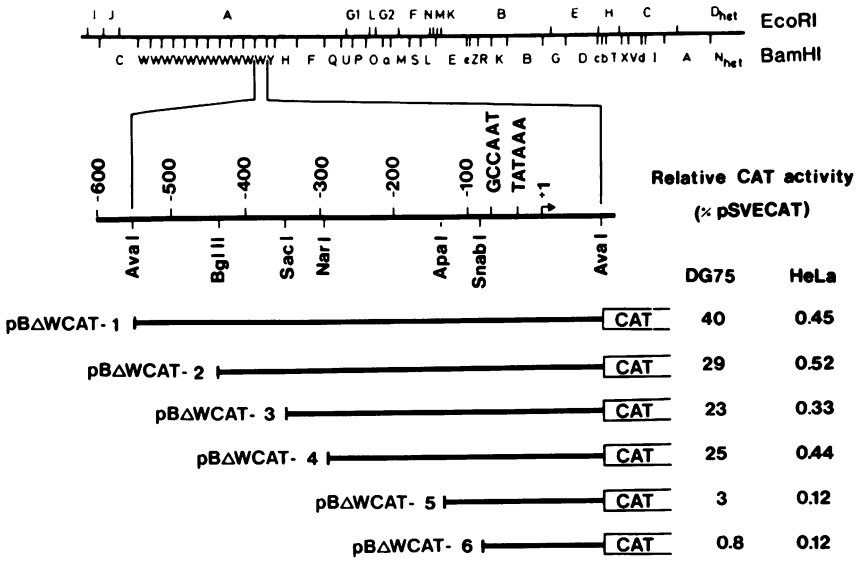

Figure 1. Schematic representation of the B95-8 EBV genome and the pBΔWCAT series of recombinant vectors. The pBΔWCAT-1 plasmid contains an *Ava*I subfragment of the *Bam*HI W fragment of B95-8 EBV DNA corresponding to nucleotides 44547 to 45180. The EBNA2 transcription initiation site at position 45100 is denoted by an arrow and numbered as + 1. CAT gene expression in DG75 and HeLa cells transfected by the vectors is expressed as the percentage of the CAT activity obtained with pSVECAT DNA.

pCH110, which contains the SV40 early promoter and the *E. coli* β-galactosidase gene, was included in all transfections, and the β-galactosidase activity of the cell extracts was used to correct for the variability in transfection efficiency. The results of the transfection experiments are shown in **Figure 1**. The pBΔWCAT-1 vector induced the expression of CAT at about 40% of the level obtained with a construct (pSVECAT) in which the SV40 early promoter controls the expression of the CAT gene. Deletion of the 5' part of the *Bam*HI W promoter region from the *Ava*I to the *Bgl*II site (position -436 relative to the cap site) reduced CAT expression to about 30%. Deletion to the *Nar*I site (position -297) had a minor additional effect, but further deletion to the *Apa*I site (position -136) led to a substantial decrease of CAT activity to 3%. Finally, deletion to the *Sna*BI site (position -86) resulted in an additional 4-fold reduction of the activity (0.8%).

The transcriptional enhancement of the 5' flanking region of the EBNA2 gene was also analyzed directly at the RNA level. The pBΔWCAT-1, pBΔWCAT-6, and pSVE-CAT plasmids, respectively, were transfected into DG75 cells together with psxβ+ DNA. This plasmid contains the rabbit β-globin gene under the control of the SV40

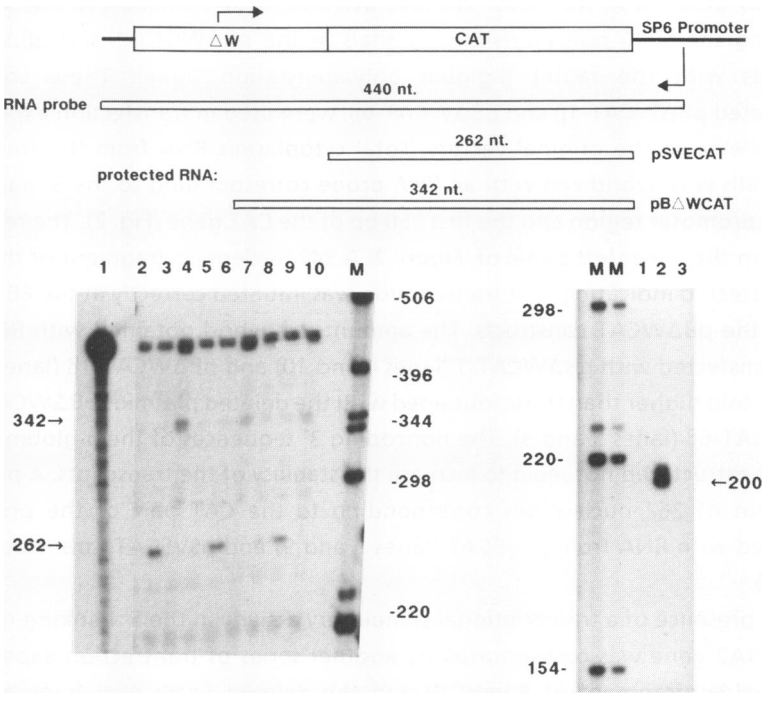

Figure 2. Hybridization analysis of RNA from DG75 cells transfected with recombinant vectors. ^{32}P-labelled antisense RNA was synthesized *in vitro* using SP6 RNA polymerase and linearized plasmid DNA as a template. The plasmid contains the 3' end of the CAT gene and an EBNA2 cap site-carrying segment of the *Bam*HI W fragment as detailed in Methods. The RNA probe and the fragments protected from RNAase hydrolysis by RNA from cells transfected with pBΔWCAT-derived vectors and pSVE-CAT, respectively, are indicated. The lower left panel shows the results of RNAase protection experiments with RNA from cells transfected with: no DNA (lane 2), pSVECAT DNA (lanes 3 and 9), pBΔWCAT-1 (lanes 4 and 10), pBΔWCAT-6 (lane 5), pSVCAT-β DNA (lane 6), pBΔWCAT-1β (lane 7, pBΔWCAT-6β (lane 8). Lane 1 contains the probe annealed without cell RNA, and lanes marked M contain molecular size markers. The lower right panel shows the results of S1 nuclease protection analysis of RNA from cells transfected with: no DNA (lane 1), pBΔW-globin1 (lane 2); pBΔW-globin6 DNA (lane 3). The probe was psxβ+ DNA labelled as described in Methods. A 200 nucleotide fragment of the probe should be protected in the hybrid.

early promoter. Total cytoplasmic RNA was analyzed by S1 mapping using appropriate end-labelled DNA probes. Although substantial amounts of a protected fragment were obtained with the β-globin probe, no fragment of correct size could be identified with the pBΔWCAT probe (data not shown). In spite of the seemingly large amounts of CAT in the cells the steady state concentration of CAT mRNA was below the detection level presumably due to a short half-life of CAT RNA in the transfected cells. To increase the sensitivity of the detection method, defined RNA probes labelled to high specific activity were employed for the hybridization

assays as detailed in Methods. We also attempted to stabilize the transcripts by replacing the SV40 polyadenylation signals in the pBΔWCAT-1 and pBΔWCAT-6 plasmids with the rabbit β-globin polyadenylation signal. These constructs, designated pBΔWCAT-1β and pBΔWCAT-6β, were used in transfection experiments in parallel with the original vectors. Total cytoplasmic RNA from the transfected DG75 cells was hybridized with an RNA probe corresponding to the 3' part of the EBNA2 promoter region and the first 250 bp of the CAT gene (**Fig. 2**). The results are shown in the lower left panel of **Figure 2**. A 342 nucleotide fragment of the probe was protected indicating that transcription was initiated correctly at the EBNA2 cap site of the pBΔWCAT constructs. The amounts of hybrid obtained with RNA from cells transfected with pBΔWCAT-1 (lanes 4 and 10) and pBΔWCAT-1β (lane 7) were several-fold higher than those obtained with the deleted plasmids pBΔWCAT-6 and pBΔWCAT-6β (lanes 5 and 8). The noncoding 3' sequences of the β-globin gene in the β constructs did not seem to increase the stability of the transcripts. A protected fragment of 262 nucleotides corresponding to the CAT part of the probe was obtained with RNA from pSVECAT (lanes 3 and 9) and pSVECATβ transfected cells (lane 6).

The presence of a transcriptional stimulatory activity in the 5' flanking region of the EBNA2 gene was corroborated by another series of transfection experiments. The *Ava*I subfragment of *Bam*HI W and the deleted *Sna*BI-*Ava*I fragment were linked to the β-globin gene without its promoter in the constructs pBΔW1-globin and pBΔW6-globin. Total cytoplasmic RNA from DG75 cells transfected with these plasmids was analyzed by S1 mapping using an endlabelled fragment of psxβ+ DNA as a probe. A fragment of 200 bp from the second exon of the β-globin gene should be protected in the DNA-RNA hybrid. A high level of specific transcripts was found in cells transfected with the pBΔW1-globin construct (**Fig. 2**, right panel lane 2), whereas no vector-induced transcription could be detected in cells transfected with pBΔW6-globin DNA (lane 3).

Taken together these data suggested that the 5' flanking region of the EBNA2 gene contained *cis*-acting regulatory elements that activate transcription in the lymphoid cell line DG75, and that the enhancer region was composed of several positive regulatory domains.

The Bam*HI W promoter region is not active in HeLa cells*

The pBΔWCAT-1 vector induced CAT expression in HeLa cells at only 0.45% of the level obtained with pSVECAT (**Fig. 1**). This corresponds to an almost 100-fold reduction of the transcription stimulatory activity of the *Bam*HI W enhancer in HeLa cells as compared with DG75 cells. Deletion of sequences from the 5' end of the promoter-containing fragment to position -297 had no effect on CAT expression,

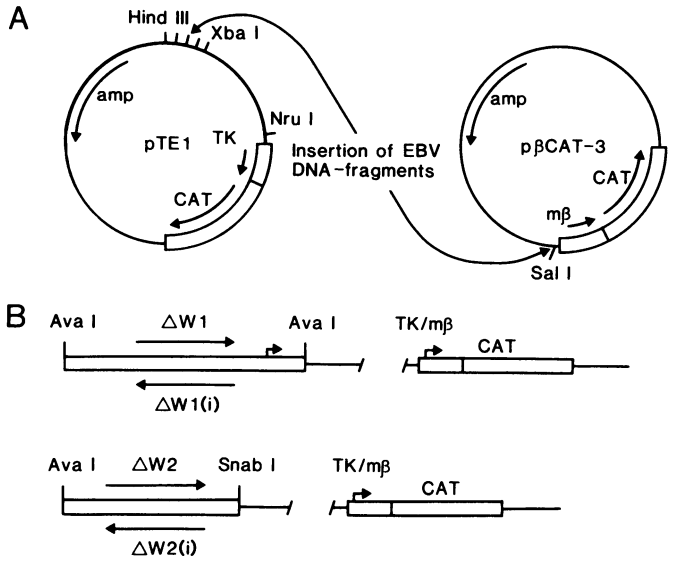

Figure 3. (A) Schematic structure of the pTE1 and pβCAT-3 plasmids. Single lines are pBR322 sequences and the open boxes represent thymidine kinase (TK), β-globin (mβ), and CAT gene sequences as indicated. (B) Schematic representation of the EBV DNA-containing derivatives of the plasmids. The 5' flanking region of the EBNA2 gene isolated as an *Ava*I fragment was cloned into the vectors in both orientations as detailed in Methods generating the BΔW1 series of recombinants. The *Sna*BI subfragment lacking the cap site was similarly cloned into the vectors generating the BΔW2 series of recombinants.

but further deletion to position -86 caused an approximately 4-fold reduction of the CAT inducing activity. The results showed that the EBNA2 enhancer activated its homologous promoter only to a very low extent in HeLa cells and thus was cell type specific.

The Bam*HI W enhancer activates heterologous promoters*

Common characteristics of viral enhancers are their ability to activate *cis*-linked heterologous promoters and to function over considerable distances in an orientation-independent manner. To investigate the *Bam*HI W enhancer with regard to these properties derivatives of the pTE1 and pβCAT-3 vectors, which contain the herpes virus TK and mouse β-globin promoters linked to the CAT gene, respectively, were employed (**Fig. 3**).

The enhancer-promoter containing *Ava*I fragment of *Bam*HI W and the 5' end *Sna*BI subfragment of the *Ava*I fragment that lacks the TATA box, were inserted in both orientations 598 bp upstream of the TK promoter in the pTE1 plasmid generating the constructs pTKΔW1, pTKΔW1(*i*), pTKΔW2, and pTKΔW2(*i*) (**Fig. 3**). A

Table 1. Effect of the *Bam*HI W enhancer on heterologous promoters in DG75 cells.

Plasmid	*Bam*HI W enhancer		Promoter	Relative CAT expression
	orientation	distance		
pTE1			TK	1
pTKΔW1	original	distal	TK	6
pTKΔW1(*i*)	inverse	distal	TK	3
pTKΔW3	original	proximal	TK	39
pTKΔW3(*i*)	inverse	proximal	TK	16
pTKΔW2	original	distal	TK	4
pTKΔW2(*i*)	inverse	distal	TK	2
pTKΔW4	original	proximal	TK	39
pTKΔW4(*i*)	inverse	proximal	TK	12
pβCAT-3			β-globin	1
pβΔW1	original	proximal	β-globin	8
pβΔW1(*i*)	inverse	proximal	β-globin	5
pβΔW2	original	proximal	β-globin	19
pβΔW2(*i*)	inverse	proximal	β-globin	8

The recombinant plasmids were cotransfected with the internal control plasmid pCH110 into DG75 cells and the transient expression of CAT and β-galactosidase was determined as described in Methods. CAT expression is given as the ratio between the normalized CAT activity obtained with the enhancer-containing vector and that obtained with the original vector (pTE1 and pβCAT-3, respectively).

related series of recombinants was also constructed in which the intervening DNA sequence between the *Bam*HI W enhancer and the TK promoter had been deleted (pTKΔW3, pTKΔW3(*i*), pTKΔW4, and pTKΔW4(*i*)). The recombinant vectors were transfected into DG75 cells and the transient expression of CAT determined (**Table 1**). The results showed that the *Bam*HI W enhancer activated the TK promoter in both orientations, but its efficiency was considerably lower in the position more distant to the promoter. The pTKΔW series was also transfected into HeLa cells. The *Bam*HI W enhancer did not provide a significant enhancement of the TK promoter activity in these cells (data not shown).

In a corresponding series of constructs the *Ava*I fragment and its 5' end *Sna*BI subfragment were cloned in both orientations into a *Sal*I site immediately 5' to the β-globin promoter in the pβCAT-3 plasmid (vectors pβΔW1, pβΔW1(*i*), pβΔW2, and

pβΔW2(*i*); **Fig. 3**). All four constructs induced a much higher expression of CAT in DG75 cells than the enhancerless original vector (**Table 1**) showing that the β-globin promoter was activated by the *Bam*HI W enhancer. No enhancement was obtained in HeLa cells.

Nuclear factors interact in trans *with the* Bam*HI W enhancer*

When the *Ava*I-*Apa*I or *Ava*I-*Sna*BI fragments (**Fig. 1**) of the pBΔWCAT-1 vector that contain the *Bam*HI W enhancer, were excised and reinserted in the opposite direction using *Xho*I linkers (8-mer) the ability of the vectors to induce transient expression of CAT in DG75 cells was reduced more than 10-fold. To answer the question if the manipulations of the restriction endonuclease cleavage sites *per se* were responsible for the inactivation of the enhancer a linker scanning experiment was performed. Insertion of an *Xho*I linker (8-mer) in the *Bgl*II, *Sac*I, or *Nar*I site of the vector (**Fig.1**) did not affect the expression of the CAT gene. However, linker insertion into the *Apa*I or *Sna*BI site drastically reduced CAT expression (data not shown). In a subsequent experiment pBΔWCAT-1 DNA was digested with *Apa*I or *Sna*BI, the 3' single stranded ends of the *Apa*I cleaved DNA were removed with T4 DNA polymerase (*Sna*BI produces blunt ends), and *Xho*I linker oligonucleotides of three different lengths (8-mers, 10-mers, and 12-mers) were ligated into the restriction sites. Upon transfection into DG75 cells the three derivatives of pBΔWCAT-1 with linkers in the *Sna*BI site all expressed the CAT gene at levels corresponding to about 10% of that of the unmodified vector. Plasmid DNAs with linkers inserted into the *Apa*I site expressed the CAT gene at 18% (8-mer), 34% (10-mer), and 85% (12-mer), respectively, of the level obtained with unmodified pBΔWCAT-1 DNA (average of 3 determinations). Nucleotide sequencing across the modified *Sna*BI sites showed that the insertions had resulted in the net addition of 3, 5, and 8 nucleotides, respectively, to the BΔW fragment.

The results suggested that the inserted oligonucleotides interfered directly and/or indirectly with protein-binding sites in the promoter-proximal part of the EBNA2 regulatory region. In order to demonstrate directly the binding of nuclear proteins to this region nuclear extracts of DG75 cells were incubated with ^{32}P-labelled promoter-carrying *Apa*I-*Ava*I subfragment (nucleotides -136 to +80, see **Fig. 1**) under binding conditions, and the DNA-protein complexes formed were separated from unbound DNA by polyacrylamide gel electrophoresis. Two distinct bands, C_D1 and C_D2, with retarded mobility appeared on the gel (**Fig. 4A**, lane 2). The radioactivity in both bands could be diluted out by an excess of unlabelled fragment in the reaction mixture (**Fig. 4A**, lanes 3-6). Competition with a modified *Apa*I-*Ava*I fragment, in which an *Xho*I linker had been inserted in the *Sna*BI site, resulted in the disappearance of the C_D1 complex band on the gel but left the C_D2

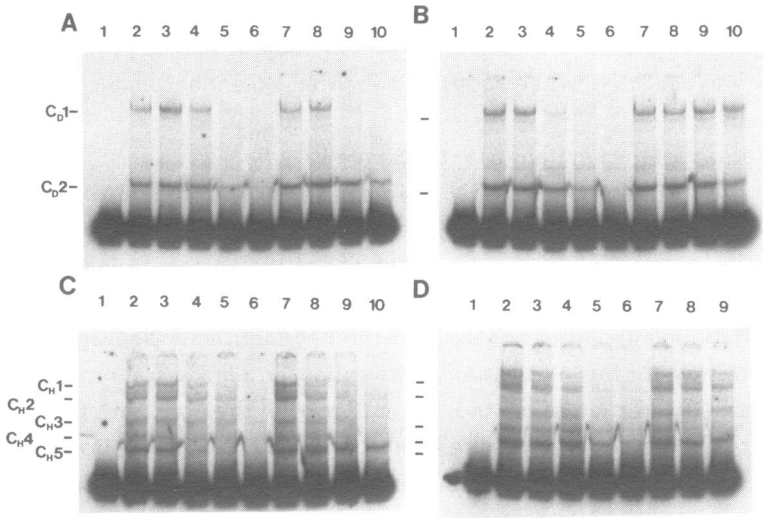

Figure 4. Nuclear factors bind to the promoter-proximal region of the EBV *Bam*HI W enhancer. Mobility shift assays of nuclear extracts from DG75 (**A** and **B**) and HeLa (**C** and **D**) cells with the ^{32}P-labelled 197 bp *Apa*I-*Ava*I fragment containing the *Bam*HI W promoter. (**A**). Lane 1, binding reaction without nuclear extract; lane 2, binding reaction with a nuclear extract from DG75 cells; lanes 3-6, competition analysis with 1, 5, 15, and 45 ng of the unlabelled *Apa*I-*Ava*I fragment, respectively; lanes 7-10, competition analysis with similar amounts of a modified *Apa*I-*Ava*I fragment in which an *Xho*I linker has been inserted in the *Sna*BI site. Retarded complexes are indicated by the designations C_D1 and C_D2. (**B**). Lane 1, binding reaction without nuclear extract; lane 2, binding reaction with a nuclear extract from DG75 cells; lanes 3-6, competition analysis with 1, 5, 15, and 45 ng, respectively, of the *Ava*I-*Sna*BI subfragment of the *Apa*I-*Ava*I fragment; lanes 7-10, competition analysis with similar amounts of the *Sna*BI-*Ava*I subfragment. (**C**). Lanes 1-10, binding reactions and competition analyses were similar to those in (**A**) except that a nuclear extract from HeLa cells was used. Retarded complexes are indicated by the designations C_H1 to C_H5 (**D**). Lanes 1-9, binding reactions and competition analyses were similar to those in (**B**) except that a nuclear extract from HeLa cells was used and the incubation containing 15 ng competitor DNA was omitted in the last series of competition analyses.

band unchanged (**Fig. 4A**, lanes 7-10). This suggested that nuclear factors interacted with DNA sequences in or close to the *Sna*BI cleavage site. The slow-moving C_D1 band was also competed out by the *Apa*I-*Sna*BI part (-136 to -80) of the *Apa*I-*Ava*I fragment (**Fig. 4B**, lanes 3-6). This subfragment also seemed to have some competitive effect on the C_D2 band. The *Sna*BI-*Ava*I fragment (-86 to +80) had no effect on either of the bands (**Fig. 4B**, lanes 7-10). Thus, the results indicated that DG75 cells contained nuclear proteins that bound to two different sites in the *Apa*I-*Sna*BI part of the regulatory region. This was confirmed using DNAase I footprinting. The factor(s) forming the C_D1 complex protected a sequence (5'-TCGCGTTGCTAGGCCACCTT-3') in the *Apa*I-*Sna*BI fragment corresponding to

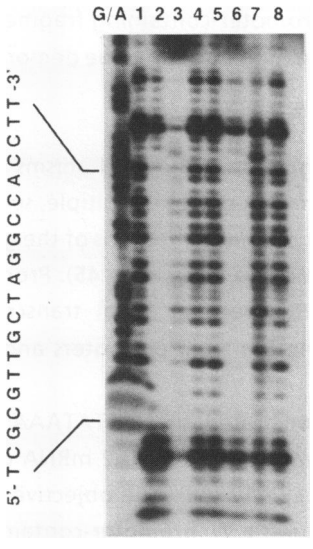

Figure 5. DNAase I protection analysis of the interaction of nuclear factors in DG75 cells with a segment of the *Bam*HI W promoter region. The *Apa*I-*Sna*BI fragment, labelled in the *Apa*I end, was incubated with nuclear extracts from DG75 cells and DNAase treated as described in Methods. The DNA-protein complexes were purified by electrophoretic separation in a 4% polyacrylamide gel and the DNA component was analyzed on an 8% sequencing gel. The protected sequence is indicated. Lane G/A, the fragment subjected to a chemical A + G sequencing reaction; lanes 1 and 5, fragment DNA which has not been incubated with nuclear extract; lanes 2 and 6, DNA correspondig to the C_D1 complex in the mobility shift assay; lanes 3, 4, 7, and 8, DNA from the unbound fraction after incubation with nuclear extract.

nucleotides 44977 to 44997 of B95-8 EBV DNA (**Fig. 5**). We were not able to identify protected DNA sequences in the C_D2 complex with this technique.

Similar experiments were also performed using nuclear extracts of HeLa cells. A more complex fragment retardation pattern consisting of at least 5 distinct bands, C_H1 to C_H5, was obtained (**Fig. 4C**, lane 2). All bands could be diluted out by an excess of the unlabelled *Apa*I-*Ava*I fragment (**Fig. 4C**, lanes 3-5). As with DG75 nuclear extracts all bands except the most fast-moving one (C_H5) were competed out by a modified *Apa*I-*Ava*I fragment having a linker oligonucleotide inserted in the *Sna*BI site (**Fig. 4C**, lanes 7-10). The C_H1, C_H2, and C_H3 bands were competed out by the *Apa*I-*Sna*BI subfragment (**Fig. 4D**, lanes 3-6). Competition with the *Sna*BI-*Ava*I subfragment clearly reduced the amount of the C_H4 complex without affecting the others (**Fig. 4D**, lanes 7-9). Thus, the fragment retardation assays demonstrated the presence of nuclear factors in HeLa cells binding to the promoter-proximal enhancer region. At least one of these (C_H5) had binding properties similar to factors found in DG75 cells. In addition the HeLa cells contained a factor (C_H4)

binding to the *Sna*BI-*Ava*I promoter-containing fragment, which was not present in DG75 cells in a concentration high enough to be demonstrable in the system.

DISCUSSION

Increasing evidence suggests that the mechanisms responsible for transcription regulation conform to a common pattern. Multiple, sequence-specific DNA-protein interactions occur in distinct regulatory regions of the gene determining the degree of transcriptional activation (for a review see 45). Promoters position the start site of transcription and are required for basal transcriptional activity. Enhancers increase the rate of transcription from promoters and are often inducible or cell-type specific.

We have recently demonstrated that a TATAAA sequence in the *Bam*HI W fragment of EBV DNA determines the EBNA2 mRNA cap site in transfected COS-1 cells transiently expressing EBNA2 (30). The objective of the present investigation was to characterize the *Bam*HI W promoter-containing region with regard to sequence elements required for the regulation of genes transcribed by RNA polymerase II. DNA fragments of the *Bam*HI W region were linked to the CAT gene and the transient expression of the gene was determined in lymphoid DG75 cells and in HeLa cells. Our experiments indicate that the 5' flanking region of the EBNA2 gene contain *cis*-acting regulatory elements that activate transcription in a cell-type specific manner.

The *Bam*HI W regulatory element posesses several of the properties commonly ascribed to viral transcriptional enhancers. The putative EBNA2 enhancer activated promoters in *cis*-configuration in both orientations, even if the enhancement was more pronounced in the original orientation. The enhancer functioned in conjunction with at least two heterologous promoters, the herpes simplex virus TK and the rabbit β-globin promoters. The enhancer also retained part of its stimulatory activity when moved to a position about 600 nucleotides further upstream from the cap site in a construct containing the TK promoter. Deletion analysis suggested that the EBNA2 enhancer is composed of several positive regulatory domains, the main ones being located between nucleotides -553 to -436, -297 to -136, and -136 to -86 relative to the EBNA2 cap site.

Unlike viral enhancers in general the EBNA2 regulatory element seemed to be active only in certain cell types and furthermore was not dependent on viral gene products for its activity. The data presented here showed that the putative EBNA2 enhancer is 100-fold more active in the EBV negative DG75 B-cell line than in HeLa cells. This is in contrast to our previous findings that the *Bam*HI W promoter was active and indispensible for the transient expression of the EBNA2 gene in

transfected monkey kidney COS-1 cells (30). The impression that the *Bam*HI W promoter is highly active in monkey cells was presumably caused by the fact that the recombinant vectors used in this study contained the SV40 origin allowing the plasmids to replicate to high copy numbers in COS-1 cells thereby amplifying EBNA2 expression. We have now analyzed the enhancer activity of the EBNA2 regulatory region in CV-1 cells using the pSVECAT and pBΔWCAT-1 constructs described above. This cell line is the parental line of COS-1 and does not contain SV40 DNA and T antigen. The expression of CAT in these cells after transfection with pBΔWCAT-1 was only 0.7% of the expression obtained with pSVECAT (unpublished data). These results support our conclusion that the putative EBNA2 enhancer is cell type specific. Obviously, it is not possible to extrapolate from the results obtained with DG75 cells to other B-cell lines or normal B lymphocytes without further information. DG75 is a tumor cell and possibly only representative of B-cells at a certain stage of the lymphocytic differentiation process. The same reservation should be made for HeLa cells. These cells have been maintained in culture since they were established from a tumor almost 40 years ago, and should not be considered as representing normal epithelial cells.

B-cell specific enhancer elements have been localized within the introns of the immunoglobulin (Ig) μ heavy-chain and κ light-chain genes (46 - 51). Comparison of the sequence of the Ig enhancer elements with that of the *Bam*HI W regulatory region did not reveal any obvious structural homologies. Specifically, the sequence motif 5'-ATGCAAAG-3', designated the Ig octamer, was not present in the putative EBNA2 enhancer.

The results of our linker scanning experiments emphasize the importance of a correct helical orientation of the DNA sequence elements of the *Bam*HI W regulatory region. Insertion of nucleotides in position -136 between the promoter and the enhancer domains influenced the transcriptional activity of the promoter in a manner depending on the length of the insert. A net insertion of 3 bp reduced the activity by 80%. However, an insertion of 8 bp, or almost a full turn of the DNA helix, largely restored the promoter activity. This observation, in conjunction with studies of repressor and activator interactions in other systems (52 - 55), suggested that one or several proteins bound to DNA sequence elements in the enhancer region interact with a protein(s) bound to the promoter, and that this interaction requires stereospecific alignment of the proteins on the DNA helix.

The electrophoretic mobility shift and DNAase footprinting experiments provide direct evidence for the binding of protein factors to the promoter-proximal part of the *Bam*HI W enhancer element. The DNA sequence protected from DNAase I cleavage by proteins in DG75 nuclear extracts did not show significant homology

with known transcription signals or with sequences further upstream in the *Bam*HI W regulatory region. The insertion of a linker oligonucleotide at position -86 (the *Sna*BI site) prevented the binding of a nuclear protein to this region as shown by the binding competition experiments. The same linker insertion mutation also drastically reduced the transcriptional activity of the *Bam*HI W regulatory region in DG75 cells. Thus, the results demonstrate a clear correlation between the specific binding of a protein and transcriptional activity.

It has recently been shown that a component of the *ori*P region in the EBV genome can function as a transcriptional enhancer with some heterologous promoters and that the enhancer activity is dependent on EBNA1 acting as a *trans*-activator (56). It was suggested that the synthesis of the highly spliced family of EBNA mRNAs is initiated at a promoter consensus sequence at position 11303 in the *Bam*HI C region and that *ori*P might regulate transcription from this promoter. This idea has been substantiated by work showing that the transcription initiation site for several EBNA RNAs, including EBNA2-encoding mRNA, is located about 30 nucleotides 3' of the *Bam*HI C promoter sequence in B95-8 cells (57). Apparently the promoter was active in most but not all of the human EBV-transformed cell lines investigated. On the other hand the cDNA sequencing data of Sample *et al.* (12) and Speck *et al.* (31) and the results of our transfection experiments in COS-1 cells (30) and DG75 cells (this paper) clearly suggest that the transcription initiation site of EBNA2 mRNA is determined by a promoter in the internal repeat region of the EBV genome. These seemingly contradictory results could be reconciled assuming that the two promoters are active under different circumstances. The constitutively active *Bam*HI W promoter, which does not need viral gene products for its activity, could function during the initial phase of EBV infection, whereas the *Bam*HI C promoter might dominate at later stages, when EBNA1 has accumulated in the cells. It is also conceivable that the *Bam*HI W enhancer, which depends on cellular factors for its activity and is cell type specific, does not stimulate transcription in lymphoid cells at certain differentiation stages.

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