The enhancer factor R of Epstein-Barr virus (EBV) is a sequence-specific DNA binding protein

Henri Gruffat, Evelyne Manet, Agnès Rigolet and Alain Sergeant*
Laboratoire de Virologie Moléculaire, Ecole Normale Supérieure de Lyon, UMR 49 CNRS-ENS, 46 Allée d'Italie, 69364 Lyon Cedex 07, France

Received September 20, 1990; Revised and Accepted October 26, 1990

ABSTRACT

In cells latently infected with EBV, the switch from latency to productive infection is linked to the expression of two EBV transcription factors called EB1 (or Z) and R. EB1 is an upstream element factor which has partial homology to the AP1/ATF family, whereas R is an enhancer factor. In the R-responsive enhancer of the replication origin only active during the EBV lytic cycle (ORIlyt), R-responsive elements are located in a region of about 70 bp (RRE-DR). Here we show that R, produced either by in vitro translation, or present in nuclear extracts from HeLa cells constitutively producing R, binds directly to and protects against DNAase I digestion, two regions in RRE-DR. Using mobility shift assay and DMS interference, we have characterized the contact-points between R and the DNA. Two binding sites, RRE-DR1 and RRE-DR2, were characterized and are contiguous in RRE-DR. R binds to these two sites probably by simultaneously contacting two sequences within the sites, which are separated by 7 bp in RRE-DR1, cctGTGCCttgtcccGTGGACaatgtccc, and by 6bp in RRE-DR2, caatGTCCCtccagcGTGGTGgctg. Direct interaction of R with its cognate sequences is conferred by its N-terminal 355 amino-acids. Directed mutagenesis in RRE-DR, of either R-binding site, impaired binding of R in vitro and, as assayed by transient expression in HeLa cells, impaired R-activation by a factor of two. This suggests that RRE-DR1 and RRE-DR2 do not respond cooperatively to R.

INTRODUCTION

The human herpes virus EBV (Epstein-Barr virus) infects and immortalizes peripheral B lymphocytes, resulting in the establishment of a latent infection. In such latently infected B cells, the entire EBV genome is maintained mainly as a plasmid, and its expression reduced to a few genes: those encoding two small RNAs (EBERS) (1), the six Epstein-Barr Nuclear Antigens (EBNA-1, -2, -3A, -3B, 3C and LP) (For a review see ref 2), the BHRF1 encoded protein (3; 4), the latent membrane protein (LMP) (5) and the terminal membrane proteins (TP1 and TP2), whose coding sequences are created by joining the ends of the linear virus (6; 7).

The latent EBV genome is spontaneously activated in particular cell lines, where between 0.5% and 5% of the cells produce viruses. It can also be activated by various chemical agents including the tumor promoter 12-O-tetradecanoyl-phorbol 13-acetate (TPA) (8). In both cases, the activation seems to be linked to the expression of two EBV-encoded transactivators of early gene promoters, EB1 (also called Z) and R (9; 10,11; 12; 13; 14; 15). EB1 is encoded by the open reading frame (ORF) BZLF1 and is expressed from two promoters, PZ and PR, either as a 1kb monocistronic mRNA or as 3 and 4 kb mRNAs generated by alternative splicing and expressing both EB1 and R, the BRLF1 ORF encoded factor (Figure 1A) (16).

EB1 seems to have a key role in the induction of the lytic cycle (10; 11; 12). It is a DNA binding protein (17; 18; 19) that positively autoregulates its own promoters but also activates transcription from quite different responsive elements including AP-1 binding sites (20; 21). EB1 does not seem to be a factor that can act at distances more than 100 to 200 bp from the TATA box (20). R, however, seems to be a factor that can act at distances over thousands of base pairs and several R targets have been identified (22; 23a; 23b; 24; 25). One is part of the duplicated promoter DR/DL (26; 22), and overlaps with the enhancer of the EBV origins of replication active only during the lytic cycle and called ORIlyt (27).

ORIlyt activity is dependent on the EBV-encoded DNA polymerase, on the BZLF1 encoded transcription factor EB1, and on the presence of the enhancer located upstream from the DR/DL TATA boxes (27) (Figure 1A). This enhancer has two functionally distinct regions, A and B. Region A is constitutively active in all cell lines tested so far except lymphoid B cells, whereas region B was transactivated by R in all cell lines tested (22). One R target in the DR enhancer B region has been reduced to 28 bp and contains the double palindromic sequence B0, CCTGTGCC*TTGTCC*CGT*GGACAA*TGTCC (22). When this 28bp DNA fragment was placed upstream from the rabbit β-globin promoter, it mediated an 8 fold R-induction (28). However, the double palindrome *TTGTCC*CGT*GGACAA*TGTCC either alone or duplicated did not confer R-responsiveness to the β-globin

* To whom correspondence should be addressed

promoter (28). The contiguous and partially overlapping sequence GTCCCTCCAGCGTGGTGGCTGCC, called B1, mediated a 3 fold R-activation when placed upstream of the β-globin promoter (28). However, when linked to each other, B0 and B1 (RRE-DR), mediated a 25 fold R-activation (28).

We show in this report that R binds in the B enhancer region, to sequences which fall within the RRE-DR. Moreover, we show by mobility shift assay and DMS interference, that R binds independently in vitro to two sites within the RRE-DR (the RRE-DR1 and the RRE-DR2). Each binding site covers about 18 bp, where R probably simultaneously contacts two core sequences separated by 6 or 7 bp. In the RRE-DR, mutations that impaired binding of R to one site or the other, only reduced the R-transactivation by a factor of 2, suggesting that the RRE-DR1 and the RRE-DR2 do not respond cooperatively to R.

MATERIALS AND METHODS

Cloned DNA templates

The EB1 and R expression vectors have been described extensively elsewhere (16). Briefly, they are pUC18 derivatives containing the ORFs BZLF1 and BRLF1 that code respectively for the EBV transactivating factors EB1 and R, placed under the control of the SV40 early promoter-enhancer. Plasmid pG2 contains the rabbit β-globin gene with the M13mp12 polylinker cloned 5′ to the β-globin promoter (29). Plasmids pG2.899/741, pG2.899/752, pG2.899/775, pG2.899/812, pG2.737/805, were made by ligating subregions of the B region of the DR enhancer 425 bp 5′ to the β-globin promoter (see figures 2B and 3B). All clones were verified by sequencing. Numbers after pG2 describe the position and the orientation of the border of the inserted enhancer subregions and they refer to the map coordinates described in Figures 1A and 2A. Plasmid pSV2β (28) expresses a chimeric SV40-β-globin RNA and was cotransfected as an internal control for transient expression experiments. Plasmid pSV0 contains the SV40 Hpa II (map position 346) to Hind III (map position 5171) fragment cloned in pUC19 digested with Hind III and BamH I. This plasmid was included in transfections to keep the amount of SV40 early promoter sequences constant, since EB1 and R are expressed under the control of the SV40 early promoter.

Production of HeLaN and HeLaR cells

HeLa cells were transfected with plasmids p36/7polyA or p36/7polyAR (Figure 2A), by the calcium precipitate method (30). 20 hrs after transfection, the cells were exposed to G418 at a concentration of 800mg/L. Every three days, the cells were washed and placed in fresh medium plus G418. Several G418 resistant clones were isolated, amplified and frozen. Two of them were selected and called HeLaR (for cells transfected with p36/7polyA R) and HeLaN (for cells transfected with p36/7polyA).

HeLa cells nuclear extracts

Nuclear extracts were prepared from HeLaN and HeLaR cells, by the method of Dignam (31), with the modifications introduced by Wildeman et al. (32).

Production of R and mutant proteins in vitro

The BRLF1 ORF was cloned in plasmids pSPT18 or pSPT19 (Boehringer Mannheim). Cloned inserts within the polylinker region were transcribed from either the SP6 or the T7 promoters. The RNA obtained were used to program protein synthesis in messenger dependent rabbit reticulocyte lysates (Promega) using ^{14}C-L-Leucine.

DNAase I footprints

Footprints experiments were made using an EBV ORIlyt enhancer probe derived from plasmid pG2−899/741, 5′-labelled by 32P at either position 741 (probe pG2EC) or at position 899 (plasmid pG2ENC) (Figure 3). The experimental procedure is described in ref 33.

Electrophretic Mobility Shift Assay (EMSA)

2μl of in vitro translation extract, were incubated with 2×10^4 cpm of ^{32}P-labeled-RREs (figure 1A). Incubations were carried out in 0,5 mM $MgCl_2$, 10 mM HEPES-KOH (pH 7.9), 0.5 mM DTT, 0.5 mM PMSF, 150 mM KCl, 10% glycerol, at 25°C for 30 minutes. The mixture was loaded onto a 4.5% polyacrylamide gel (29 to 1 crosslinked), 0.2×TBE. The R-RRE complexes (B) were separated from the non-complexed DNA (F) by migration at 10 V/cm and visualized by autoradiography.

DMS interferences

5×10^5 cpm of the DNA probes was methylated using 1 μl of DMS during 3 mn. at 18°C. The methylated probe was then incubated with 2μl of in vitro translation extract. After EMSA assay, the retarded DNA probe (B) and the non-retarded DNA probe (F) were electroeluted and incubated in 100 μl of 1M piperidine for 30 mn. at 90°C. An equal amount of the radioactive B and F probes was analysed on 8% polyacrylamide sequencing gels and visualized by autoradiography.

Cell culture and transfections

HeLa cells were grown in DMEM (Gibco) supplemented with 10% (v/v) fetal calf serum. The plasmids used for transfection were prepared by the alkaline lysis method and purified through two CsCl gradients. HeLa cells were seeded at 10^6 cells per 100 mm Petri dish 8 h prior to transfection. Transfections were performed by the calcium precipitate method (30). Cells were mixed with the appropriate DNA(s), and the DNAs were in the same topological state as assayed by agarose gel electrophoresis. Usually 15μg of DNA were used per 100 mm dish including: 0,5μg of R-expressing vector, 5μg of reporter promoters, pSV0 when required, 0,5μg of pSV2β as internal control and pUC19 up to 15μg.

RNA extraction and S1 nuclease mapping

The cells transfected were lysed by NP40 as described elsewhere (29). Nuclei were pelleted and RNA phenol extracted from the cytoplasmic fraction. 10 to 40 μg of total cytoplasmic RNA was hybridized overnight at 30°C in 50% formamide, 0.3M NaCl, 0.01M Tris-HCl pH 7.4 to 5′- 32P-labelled synthetic single-stranded DNA probes (Figure 1B). The hybrids were digested 2 hours at 20°C with 5U of S1 nuclease per 10 μg of RNA. The size of the S1 protected DNA fragment was analysed on 8% (w/v) acrylamide/8.3 M urea gels. Quantification was made by cutting the specific S1-protected bands out of the gel and counting the radioactivity. The results were corrected as follows: (i) according to the efficiency of transfection as evaluated by counting the radioactivity present in S1-protected probes corresponding to specific SV40 early RNA expressed from plasmid pSV2β and (ii) according to the activities of the different constructions in the absence of R.

RESULTS

The ORIlyt RRE contains two regions responding to R

We have previously shown that the ORIlyt enhancer sequences located between positions −741 to −899 (region B) upstream from the IR4 gene CAP site (Figure 1A), responded to R in a non cell-specific manner when linked to an heterologous promoter (22; 28). We show here, that 69bp within the R-responsive enhancer contains the R-responsive element. Enhancer deletion mutants placed 425 bp upstream of the rabbit β-globin promoter in plasmid pG2 (Figure 1B), were transfected in HeLa cells, and their response to increasing amounts of R-expressing vector was evaluated by quantitative S1 analysis. We do not show that increasing amounts of R-expressing vector result in increasing amounts of R protein. However, since specific R-induced transcription increased in response to increasing amounts of R-expressing vector (see figure 1B), we assume that this is rather due to increasing amounts of R-protein. The enhancer fragments inserted increased 2 fold the basal activity of the β-globin promoter in plasmid pG2 (Figure1B, compare lane 1 with lanes 2 and 3), suggesting that cellular factors participate in the function of this enhancer. R highly stimulated the expression of specific β-globin transcripts when region B (plasmid pG2−899/741), was inserted 5′ to the β-globin promoter (Fig. 1B, lanes 7, 11 and 15). Deletion of sequences between bp −741 and −752 (plasmid pG2-899/752), decreased the R-activation effect by a factor 2, and this was seen at low R concentrations (Figure 1B, lanes 8 and 12), but not at high R concentrations (Figure 1B, lane 16). Therefore, the effect of the deletion can be compensated by the amount of R protein expressed. Deletion of sequences between bp −752 and −775 (plasmid pG2-899/775), impaired the induction of β-globin transcripts by R, and this effect could not be compensated by the amount of R expressed (Figure1B, lanes 9, 13 and 17). However, the remaining sequences retained a low but detectable R-inducibility. In conclusion, it seems that there are at least two R-responsive elements within or overlapping with the sequences located between positions −742 and −775. This is likely to be the case, since a DNA fragment overlapping this region and placed upstream of the β-globin gene (plasmid pG2-737/805), rendered this promoter responsive to R (Figure 1B, lanes 6, 10 and 14), and the level of R-activation was comparable with that obtained with region B at all R concentrations tested (compare lanes 7, 11 and 15 with lanes 6, 10 and 14). However, there could be only one R-responsive element, and the stimulatory effect might be due to a stabilizing effect of flanking sequences (−741 to −752). Plasmid pSV2β, expressing a chimeric SV40-β-globin RNA under the control of the SV40 early promoter-enhancer sequences, was cotransfected as a control for transfection efficiency (see figure 1B, pSV2β internal control). An equal amount of specifically initiated RNA was found in every transfection, indicating that the results described above could be compared.

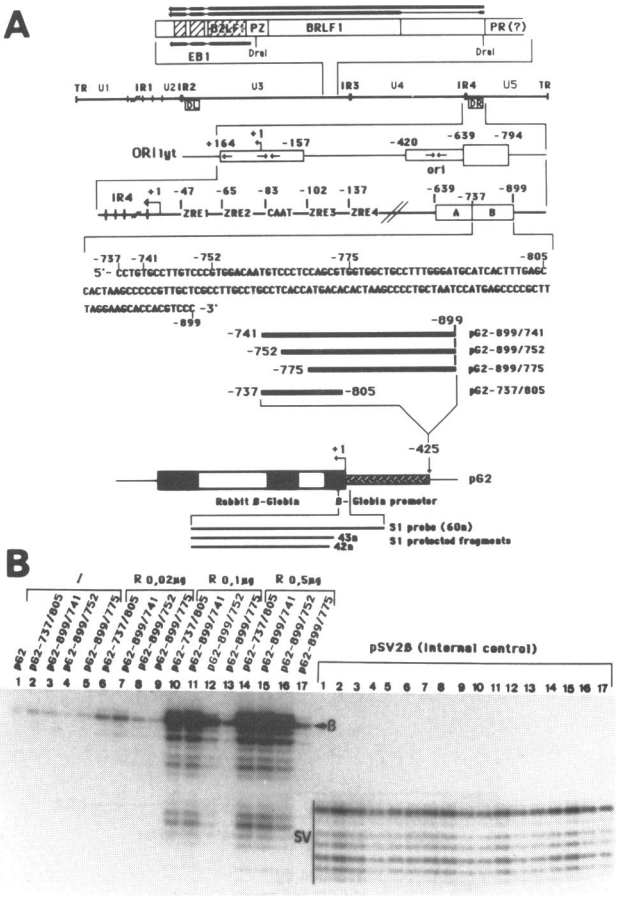

Figure 1. An R-responsive enhancer overlaps with ORIlyt. **(A)** Two EBV ORFs have been shown to code for transcription factors: BZLF1 codes for EB1 and BRLF1 codes for R. ORIlyt overlaps with the DR promoter where four EB1 binding sites have been mapped (ZRE1 to ZRE4). ORIlyt is composed of three regions, indicated by open rectangles (27). Numbers over the rectangles indicate the end-points of the regions with respect to position 52787 as +1 on the EBV B95-8 sequence (34). Horizontal arrows indicate palindromic sequences. ori indicates the origin of replication. Orilyt is overlapping with a promoter controlling the expression of a short repeted sequence called IR4. Proximal to the CAP site, there are four EB1 binding sites called ZRE1 to 4. Distal to the CAP site, region −639 to −794 is the ORIlyt enhancer. The sequence of the R-responsive region in the ORIlyt enhancer and denoted domain B is shown, and has been mutated by progressive deletion. The structures of the mutants are shown (thick lines). Numbers at both ends of the thick lines indicate the end points of the deletions in domain B, with respect to position 52787 as +1 on the EBV B95-8 sequence. These mutants have been linked to the β-globin promoter (plasmid pG2). The schematic structure of S1 nuclease DNA probes and the size of the S1 protected DNA fragments are also presented. **(B)** HeLa cells were transfected with the different constructs in the conditions indicated in the top panel. Transcriptional activation was determined by quantitative S1 analysis of total cellular RNA isolated from transfected cells . Plasmid pSV2β expresses an SV40-β-globin hybrid RNA under the control of the SV40 early promoter-enhancer was cotransfected as an internal control (28). The specific start sites of β-globin and the early-early start sites of SV40 are indicated by β and SV respectively.

HeLaR cells constitutively produce a functional R protein

Having shown that at least two R-responsive elements are located between positions −737 and −805, we wanted to determine if R binds directly to this region and where. First, we made a HeLa cell line (HeLaR) constitutively producing the R protein. HeLaR cells contain an integrated plasmid (p36/7polyAR, Figure 2A) carrying the neomycin gene as a selection marker, and the R gene (Figure 2A, ORF BRLF1), fused to the metal-inducible human metallothionein IIA promoter. These HeLaR cells produce a polypeptide recognized by a rabbit anti-R polyclonal antibody (Figure 2B, lane 2), not detected in HeLaN cells containing only the plasmid p36/7polyA (Figure 2B, lane 1). This polypeptide migrated with the same apparent molecular weight (90 Kda), as the R protein expressed in Raji cells treated by TPA (Figure 2B, compare lanes 2 and 4). We transfected the rabbit β-globin gene into HeLaR cells, either enhancerless (plasmid pG2), or linked to the R-responsive enhancer (plasmid pG2-737/805) (Figure 2C). Treatment of the HeLaR cells by $ZnCl_2$, did not lead to

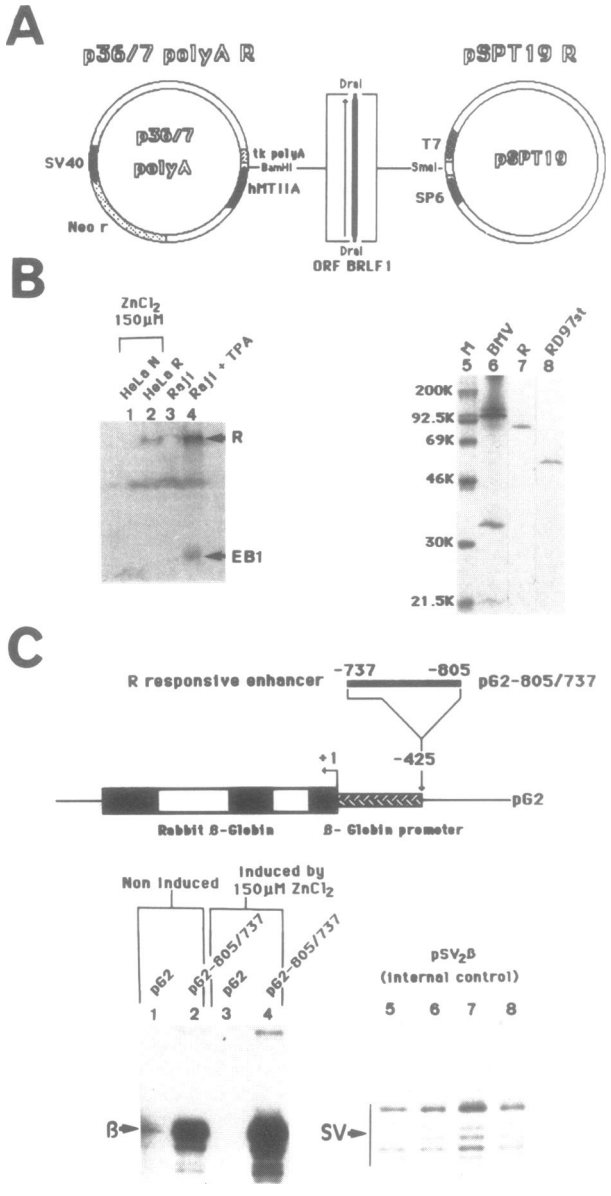

Figure 2. HeLaR cells produce a functional R polypeptide **(A)** Schematic representation of the plasmids used to generate HeLaR and HeLaN cells, and to produce R by in vitro translation. **(B)** HeLaR cells but not HeLaN cells induced by $ZnCl_2$, produce a 90 Kda polypeptide immuno-related to R produced in Raji cells where the EBV early gene products EB1 and R have been induced by TPA (lanes 1 to 4). The proteins were revealed by western blot using two rabbits antisera, one anti R (35) and one anti EBI, mixed together. R and a truncated version of R (RD97st) were also produced by in vitro translation and separated on PAGE (lanes 5 to 8). **(C)** To determine if the 90 Kda polypeptide produced by HeLaR cells was transactivating the RRE-DR linked to the β-globin promoter, HeLaR cells were transfected with plasmid pG2-805/737 as indicated on the top panel. The level of correctly initiated β-globin RNA (β) was determined by S1 nuclease analysis (lanes 1 to 4). The amount of RNA expressed from pSV2β (SV) cotransfected as an internal control, is presented in lanes 5 to 8, and corresponds to the RNA samples presented in lanes 1 to 4.

an increased expression of specifically initiated β-globin mRNA from plasmid pG2 (Figure 2C, lanes 1 and 3). However, in HeLaR cells, β-globin RNA levels were increased when the R-responsive enhancer was inserted in front of the β-globin promoter (Figure 2C, compare lanes 1 and 2). Moreover, this R-enhanced β-globin RNA transcription was further increased by treatment of the cells by ZnCl2 (Figure 2C, compare lanes 2 and 4). An equal amount of RNA was expressed from plasmid pSV2β cotransfected as an internal control, suggesting that the results described above could be compared (Figure 2C, lanes 5 to 8). In conclusion, HeLaR cells produce a functional R polypeptide, and the amount of R produced can be increased by $ZnCl_2$. Wild type R protein and a C-terminal deleted mutant of R (Manet et al, unpublished data) were also produced by in vitro translation. RNAs transcribed from the SP6 promoter located upstream from the R gene cloned in plasmid pSPT19 (Figure 2A, plasmid pSPT19R) or of the deleted mutant gene cloned in the same plasmid, were used to programm protein synthesis in messenger-dependent rabbit reticulocyte lysates. In these lysates, a 90Kda 14C-Leucine-labelled polypeptide corresponding to the wild type R was detected after SDS-PAGE and autoradiogaphy (Figure 2B, lane 7) whereas a 50 Kda 14C-Leucine-labelled polypeptide was detected for the C-terminal deleted mutant.

R binds to the RRE-DR in vitro

Four sources of proteins, HeLaR and HeLaN cells nuclear extracts, and reticulocyte lysates containing R or BMV (Bromomosaic virus) proteins translated in vitro were used in conjunction with 5′-end-labeled double stranded DNA probes covering the RRE-DR to determine the sequence-specific DNA properties of R by DNAase I footprinting. One probe was 5′-end-labeled close to position −741 and called pG2ENC (Figure 3A), and the other probe was 5′-end-labeled on the opposite strand close to position −899 and called pG2EC (Figure 3C). Several regions of probe pG2ENC, called I, II, III, IV and V, were protected against DNAase I digestion by a HeLaN nuclear extract (Figure 3B, lanes 5 and 6), as compared to the DNAase I digestion pattern of the probe in absence of protein extract (Figure 3B, lanes 2 to 4). Moreover, two DNAase I hypersensitive sites called b and c, were also induced. However, the HeLaR nuclear extract strongly increased the protection of regions I (−741 to −760) and II (−760 to −800) (Figure 3D, lanes 7 and 8), and induced the DNAase I hypersensitive site a. These results suggest that R is binding directly to regions I and II. They are also compatible with the idea that the HeLaR extracts contain cellular factors induced by R that bind to region I and II. The same factor might be present in HeLaN cells at lower concentrations. To determine further if R directly binds to these regions, DNAase I footprints were performed with extracts containing in vitro translated R. In this case, only region I and a shorter segment of region II were protected against DNAase I digestion (Figure 3B, lanes 16 and 17). As such a footprint is not observed with extracts containing proteins translated in vitro from BMV RNA in the same conditions (Figure 3B, lanes 13 to 15), the DNAase I footprint obtained with R in vitro translated can be considered as specific. As shown in Figure 3A, regions I and II overlap the sequences located between positions −737 and −775, which are those that mediate R-activation of the ORIlyt enhancer (see Figure 1).

DNAase I footprints were also performed on probe pG2EC (Figure 3C). No clear footprint was detected on this strand with either the HeLaN or the HeLaR nuclear extract (Figure 3D, lanes 1 to 9). However, a strong DNAase I hypersensitive site called a, was detected with the HeLaR nuclear extract (Figure 3D, lanes 7 to 9). This strong hypersensitive site was also induced by the in vitro translated R protein, but this time there was a clear footprint overlapping the sequences located between positions −737 and −775 (Figure 3D, lanes 14 and 15). As previously, such a footprint is not seen with extracts containing proteins in vitro translated from BMV RNA (Figure 3D, lanes 12 and 13).

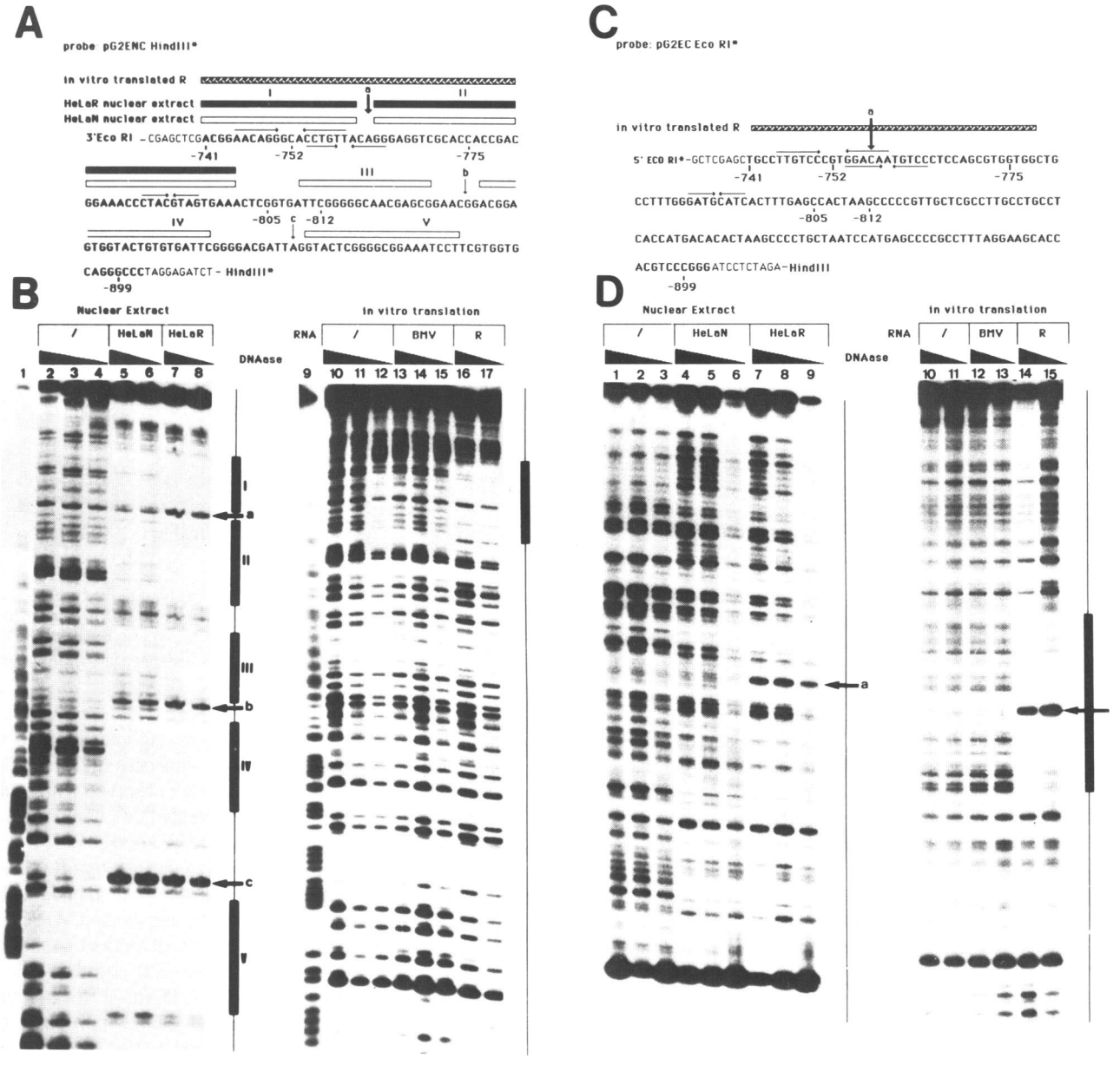

Figure 3. R binds to the RRE-DR. **(A)** and **(C)** Schematic representation of the sequences protected against DNAase I digestion by the different protein extracts used in the footprint experiment. Horizontal braids indicate footprints induced by in vitro translated R. Horizontal closed bars indicate footprints induced by HeLaR extracts. Open bars indicate footprints induced by HeLaN extracts. Vertical arrows and letters indicate DNAase I hypersensitive sites. **(B)** and **(D)** DNAase I footprints with decreasing amounts of DNAase I, on both strands of a DNA probe located between positions −741 to −899. The protein extracts used are indicated in the top panel. Vertical bars and numbers indicate footprints. Horizontal arrows and letters indicate DNAase I hypersensitive sites. Closed triangles indicate decreasing amounts of DNAase I.

Thus, in conclusion, R seems to bind directly to the sequences (RRE-DR) mediating the R-activation in a transient expression assay, as defined by deletion mutagenesis (Figure 1B).

The R DNA-binding domain is located in the N-terminal 355 amino-acids

As discussed above, R seems to bind in vitro to a region overlapping positions −741 and −780 in the RRE-DR, and possibly to two sites within region I and II. Interestingly, deletion mutagenesis in this region also showed a two-step decrease in the R-responsiveness, corresponding approximately to regions I and II. To localize further where R bound in the RRE-DR, we made mobility shift assays using the in vitro translated R protein in conjunction with three synthetic double-stranded oligonucleotides overlapping regions I and II, and called B0, B1 and BR (Figure 4A). We also made use of a fourth double stranded oligonucleotide called B2 (Figure 4A), which corresponds to a region protected by HeLaR extracts but not by in vitro translated R (−787 to −807). Probe B2 should not therefore bind in vitro translated R. As judged by mobility shift assays and competitions, specific binding of R was observed to probes B0, B1 and BR, but not to probe B2. The in vitro translated R bound with a higher affinity to B0 (Figure 4B, lane 2) and to BR (Figure 4B, lane 14), than to B1 (Figure 4B, lane 8). This was also seen when a 400 fold molar excess of unlabeled B0, B1 and BR probes were used as competitors. Probe B0

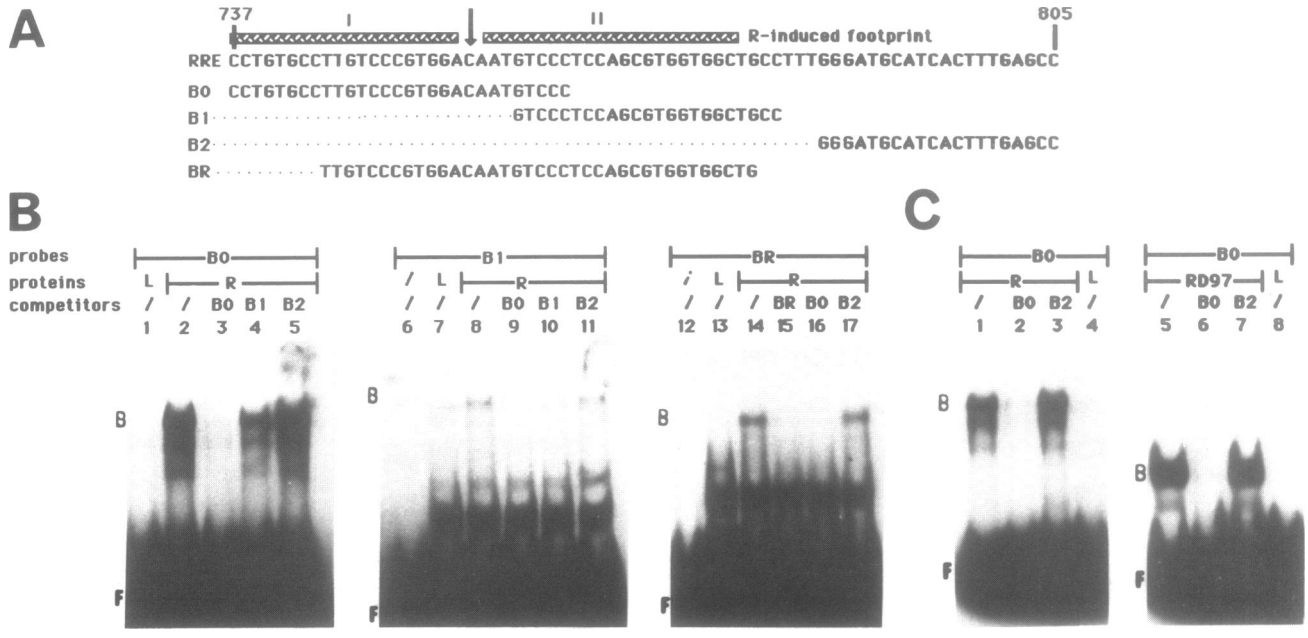

Figure 4. Mapping of R binding sites by gel retardation assay and DNA competition **(A)** Schematic representation of DNA probes used in the binding assays and competitions. **(B)** Probes B0, B1 and BR were incubated with protein extracts and with DNA competitors as indicated in the top panel. **(C)** In vitro translated R and RD97st proteins were incubated with probe B0 and DNA competitors as indicated in the top panel. In **(B)** and **(C)**, /, indicates no protein added; L, indicates lysate translated without exogenous RNA; DNA competitors were added at a mass ratio of 100; Free (F) and bound (B) DNA probe are indicated on the left of the autoradiographies.

competed efficiently for the binding of R on probes B0 (Figure 4B, lane 3), B1 (Figue 4B, lane 9) and BR (Figure 4B, lane 12). However, probe B1 competed less efficiently than probe B0 for the binding of R on probe B0 (Figure 4B, lane 4). As expected no R binding to the non-specific probe B2 could be detected (not shown), and unlabeled B2 probe did not detectably compete for the binding of R on labeled probes B0 (Figure 4B, lane 5), B1 (Figure 4B, lane 11), and BR (Figure 4B, lane 17). The truncated R protein (RD97st) generated by in vitro-transcription/translation of the C-terminal deleted BRLF1 mutant construct (Figure 2B, lane 8), still bound to probe B0. RD97st contains the N-terminal 355 amino-acids of R, and has a higher affinity for probe B0 than the wild type R protein (Figure 4C, compare lanes 1 and 5). Again, the interaction was specific since binding of RD97st to probe B0 was competed out by an excess of unlabelled probe B0 (Figure 4C, lane 6), but not by an excess of probe B2 (Figure 4C, lane 7). Similar results were obtained with probes B1 and BR (not shown). These results suggest that there are at least two R binding sites in the RRE-DR. Furthermore, specific binding to these sites is still observed when the R protein is reduced to the N-terminal 355 amino-acids.

R binds to two contiguous sites in the RRE-DR

Since in vitro translated R bound to probes B0 and BR, the contact points of R with these double-stranded oligonucleotides were probed chemically by modification of the DNA with dimethyl sulfate (methylation interference analysis). After partial methylation at purine residues, the modified probes were used in the gel retardation assay. Free (F) and bound (B) probe DNA was recovered, subjected to chemical cleavage at methylation sites, and the products were resolved by gel electrophoresis under denaturing conditions. On both the coding (C) and the non-coding (NC) strands of probe B0, strong interferences were detected on guanine residues in the sequences 5′-GTGCC-3′ and 5′-GTGGAC-3′ (Figure 5B). Such strong interferences suggest that R contacts simultaneously both sequences rather than binding to them separately. Similar results were observed with probe BR, where strong interferences were detected on guanine residues in the sequences 5′-GTCCC-3′ and 5′-CGTGGGTG-3′ (Figure 5C). Again, such strong interferences suggest that R contacts the two sequences simultaneously. This was strengthened by the fact that there was no interference on the sequence 5′-GTGGAC-3′ present alone in probe BR, whereas interferences on this sequence were observed in probe B0 (see Figure 5B). Similarly, there was no interference on the sequence 5′-GTCCC-3′ located between positions −760 and −765 and present alone in probe B0 (Figure 5B), whereas interferences were found on this sequence in probe BR (Figure 5C). Moreover, on both the coding and the noncoding strands of a double stranded oligonucleotide covering the entire RRE-DR, interferences were observed on the four sequences described above but with a much weaker intensity (not shown). The results of the methylation interferences are summarized in Figure 5A, and suggest that R binds to two contiguous sites in the RRE-DR. Each site probably consists of two regions directly contacted by R and separated by 6 or 7 bp, 5′-**GTGCC**TTGT-CCC**GTGGAC**-3′ and 5′-**GTCCC**TCCAGC**GTGGTG**-3′, called RRE-DR1 and RRE-DR2 respectively. Similar results have been obtained with the R deletion mutant RD97st (not shown).

The RRE-DR1 and the RRE-DR2 do not respond cooperatively to R

In order to further evaluate the contribution of each R-binding site in the R-induced activity of the RRE-DR, we mutated the RRE-DR1 (Figure 6A, plasmid pG2M2), the RRE-DR2 (Figure 6A, plasmid pG2M3) or both (Figure 6A, plasmid pG2M4). These mutations impaired totally the binding of R on the isolated RRE-DR1 or RRE-DR2 in vitro (not shown). Each mutant and the wild type RRE were placed upstream of the β-globin promoter

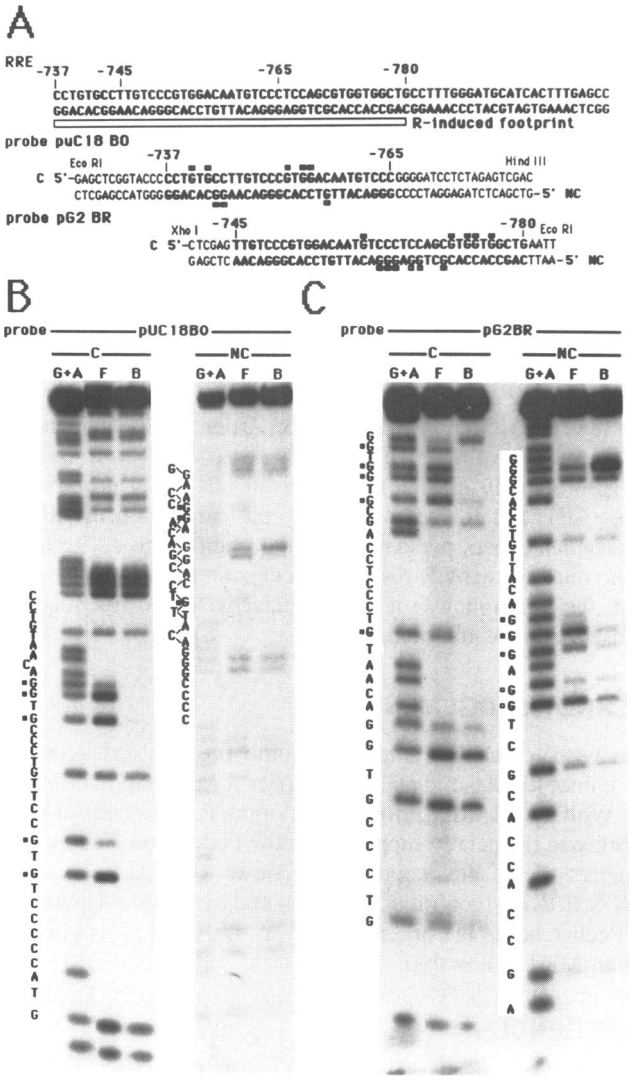

Figure 5. Fine mapping of R binding sites by methylation interference. **(A)** Sequences of the R-Responsive Enhancer (RRE) and of double stranded DNA probes used for formation of protein-DNA complexes (pUC18BO and pG2BR). The R binding site is indicated by an open bar under RRE. The G residues involved in the formation of specific nucleoprotein complexes on the probes are shown by black squares. DNA binding assays were performed under standard conditions except that probes pUC 18 B0 and pG2 BR (5'-end-labeled either on the coding (C) or the noncoding (NC) strand) were treated with dimethyl sulfate before incubation with the in vitro translated protein R. **(B)** and **(C)** G+A is a Maxam and Gilbert sequence ladder. F, is the non-retarded probe, and B, is the retarded complex. Black squares indicate the G residues whose methylation strongly prevents the formation of complexes, open squares those which are weakly involved.

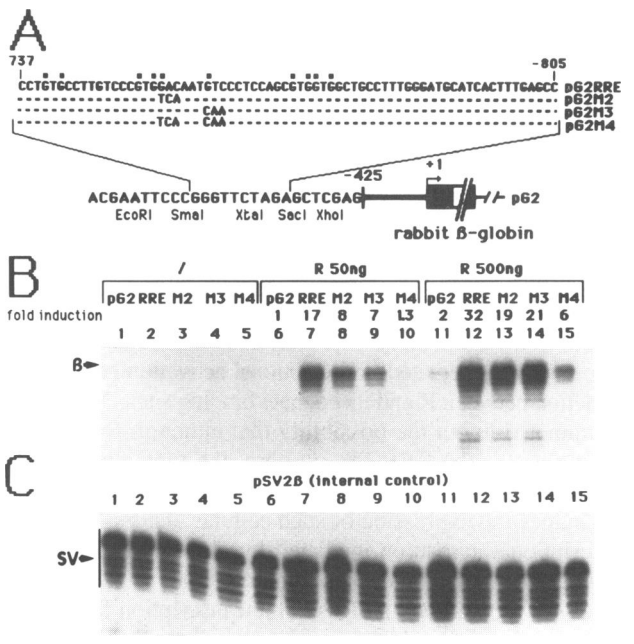

Figure 6. The paired non-homologous RRE sites in the RRE-DR do not respond synergically to R. **(A)** Schematic representation of the β-globin gene constructions in which the RRE-DR (pG2RRE), or mutants in the RRE-DR1 (pG2M2), in the RRE-DR2 (pG2M3), and in both RREs (pG2M4), have been inserted. **(B)** The various constructs were transfected in HeLa cells as indicated in the top panel, and the level of correctly initiated β-globin transcripts(β) was evaluated by quantitative S1 nuclease assay. Fold induction represents the ratio of the radioactivity counted in each S1-protected band obtained in presence of R on the radioactivity counted in the corresponding S1-protected band obtained in absence of R. This has been done for the two R concentrations. **(C)** S1 nuclease analysis of RNA initiated from plasmid pSV2β (SV) cotransfected as an internal control. RNA samples 1 to 15 are the same as in **(B)**.

in plasmid pG2 (Figure 6A). These reporter plasmids were transfected into HeLa cells either alone or along with various amounts of R-expression vector, and their transcriptional activity was evaluated by quantitative S1 analysis. Representative results are shown in Figure 6B. As expected, the RRE-DR located upstream of the β-globin promoter resulted in a strong transcriptional induction dependent on the presence of R (lane 7), and this induction reached a plateau at 500ng of R-expression vector (lane 12). The three base-pairs change in the RRE-DR1 (mutant M2, lane 8), or in the RRE-DR2 (mutant M3, lane 9), resulted in a reduction of about 50% of the R-induced activation of the β-globin promoter, and this was more obvious at non-saturating (lanes 8 and 9) than saturating amounts of R (lanes 13 and 14). Finally, when both the RRE-DR1 and the RRE-DR2 were mutated (mutant M4), the R-induced activation was strongly impaired, and this was seen at 500 ng as well as 50 ng of R-expression vector (lanes 10 and 15). These results suggest that the RRE-DR1 and the RRE-DR2 do not respond cooperatively to R. An equal amount of RNA was expressed from plasmid pSV2β (Figure 6C, lanes 1 to 15), indicating that the results presented above could be compared. However, it should be noted that the β-globin promoter, which lacks R-binding sites, is weakly stimulated by R at high concentrations of the factor, suggesting that R present at high concentrations can activate by binding non specifically to the β-globin promoter. Moreover, β-globin specific transcription from mutant M4 was still weakly activated by high concentrations of R (Figure 6B, compare lanes 2 and 6), suggesting that the mutations did not impair totally R binding in vivo.

DISCUSSION

In this report, we provide the first evidence that the N-terminal 355 amino-acids of the EBV-encoded transcription factor R, confer sequence-specific DNA binding properties to this protein in vitro.

Furthermore, using DNAase I footprints we have also shown that HeLa cell proteins bound to regions I and II in the RRE-DR, where two R-binding sites have also been identified. The

protection against DNAase I digestion of regions I and II was much more pronounced with HeLa cell extracts containing R than with HeLa cell protein extracts. At present, we do not know if this reflects the induction by R of such cellular factor, or if there is cooperativity between R and these cellular factors, or if R competes with these cellular factors for binding on the RRE-DR. Whether these cellular factors are contacting DNA at the same sites as R, and if such proteins contribute to the R-activation of the enhancer is also at present unknown. However, mutations which affect the purine residues contacted by R, impaired R-binding in vitro and also impaired the R-induced transcriptional activation in a transient expression assay (Figure 6B). This suggests that R-mediated transcriptional activation requires direct interaction between R and its cognate binding sites. Nevertheless, one cannot rule out the possibility that although R can directly bind in vitro to the RRE-DR, in vivo, the function of R would be to stabilize the binding of cellular factors to the RRE-DR, or to induce the expression of such cellular factors. In that case, transcriptional activation mediated by R in vivo would be due solely to the cellular factors stably bound to the RRE-DR. Therefore, it could be that R is not a transcription factor per se, and in addition to the domain required for specific DNA binding and/or stabilization of cellular protein interaction with DNA, it could be that R does not have a domain responsible for transcriptional activation. We have shown here that the R N-terminal 355 amino-acids are sufficient for specific DNA binding. However, this part of the R protein did not permit transcriptional activation from the RRE-DR (E. Manet, unpublished data). It remains to be established if the C-terminal part of R linked to an heterologous DNA binding domain will allow transcriptional activation from the appropriate binding sites, demonstrating that R is a transcription factor per se.

In this report we also show that the contiguous site RRE-DR1 and RRE-DR2 act additively but not synergically at low R concentrations (Figure 6B). However we have previously reported that the probe B0 containing the RRE-DR1 mediated R-activation of an heterologous promoter and that probe B1 containing the RRE-DR2 did not, whereas when linked to each other the paired RRE-DR sites responded synergically to R (28). These previous results can now be explained by the fact that R binds poorly to the RRE-DR2 in probe B1 (Figure 4B) due to the absence of flanking sequences which probably stabilize the binding. In effect, in probe BR, which contains these flanking sequences, R binds as efficiently to the RRE-DR2 as it does to the RRE-DR1 in probe B0 (Figure 4B). Therefore, in our original report (28), the apparent synergy between the paired RRE sites probably reflected stabilization of R binding to the RRE-DR2 by flanking sequences.

The additive effect described above between the RRE-DR1 and the RRE-DR2 is not seen anymore at high R concentrations. This could be due to the fact that binding of R to a single RRE, or R-induced binding of a cellular factor to a single RRE, results in maximal stimulation of transcription.

The two R-binding sites characterized in this report are partially homologous. Each seems to be composed of two regions contacted directly by the protein, and separated by 7 bp in the case of the RRE-DR1, CCTGTGCCTTGTCCCGTGGACA-ATGTCCC, and by 6bp in the case of the RRE-DR2, CAATGT-CCCTCCAGCGTGGTGGCTG. The binding sites are not palindromic and whether R interacts with the RRE sites as a monomer or as a dimer is not as yet known. We, and others, have characterized another R-responsive enhancer in the EBV early promoter PM, controlling the expression of a factor acting at the post-transcriptional level (23b; 24). Bidirectional deletion mutagenesis and DNAase I footprints indicate that there could be four R-binding sites, with sequences only partially related to the one described in this publication (H. Gruffat and M. Buisson, unpublished results). Therefore, as in the case of the EBV-encoded upstream element factor EB1 (18), the EBV enhancer factor R could bind to many sites which are imperfectly homologous and rather degenerated. This possibility is interesting if one considers the regulation of EBV early genes expression. Two transcription factors, EB1 and R, seem to be involved in the activation of EBV early genes expression. EB1 expressed in EBV latently infected B cells, activates the expression of R and of many EBV early promoters (12; 22). Many EB1 binding sites but fewer EB1 responsive elements (ZRE) have been identified. These ZRE are different but partially conserved (18). R expressed in EBV latently infected cells activates as many promoters as does EB1, but R does not induce the expression of EB1 (A. Chevallier-Greco, personal communication). This suggests, that there must be many R-responsive elements in the EBV genome, and the multiplicity of these elements calls for imperfect conservation of the binding sites.

ACKNOWLEDGEMENTS

We wish to thank Conrad B. Bluink for editorial assistance, P. Jalinot for plasmid pG2, B. Verrier for plasmid p36/7polyA, H. Wolf and H. Miller for the gift of the R and Z antisera. This work was financially supported by the Federation Nationale des Centres de Lutte contre le Cancer (F.N.C.L.C.C.), by I.N.S.E.R.M. (contrat n° 871015), and by the Association pour la Recherche sur le Cancer (A.R.C., n° 6810). H.G. is a recipient of an MRT fellowship.

REFERENCES

1. Lerner, M. , Andrews, N., Miller, G. and Steitz, J. (1981) Proc. Natl. Acad. Sci. USA, 78, 805–809.
2. Miller, G. (1985) In B. Fields (ed.), Virology. Raven Press, Publishers, New York, 563–590.
3. Bodescot, M., and Perricaudet, M. (1986) Nucleic Acids Res., 14, 7103–7114.
4. Pfitzner, A. J., Strominger, J. L. and Speck, S. H. (1978) J. Virol., 61, 2943–2946.
5. Hennessy, K. , Fennewald, S., Hummel, M., Cole, T. and Kieff., E. (1984) Proc. Natl. Acad. Sci. USA, 81, 7207–7211.
6. Laux, G. , Perricaudet, M. and Farrell, P. J. (1988) EMBO J., 7, 769–744.
7. Sample, J., Liebowitz, D. and Kieff, E. (1989) J. Virol., 63, 933–937.
8. Zur Hausen, H. , J. O'Neil, E. , Freese, U. K. and Hecher, E. (1978) Nature (London), 272, 373–375.
9. Miller, G., Rabson, M. and Heston, L. (1984) J. Virol., 50, 174–182.
10. Countryman, J. and Miller, G. (1985) Proc. Natl. Acad. Sci. U. S. A., 82, 4085–4089.
11. Countryman, J. K., Jenson, H., Grogan, E. and Miller, G. (1986) Cancer Cells, 4, 517–523.
12. Chevallier-Greco, A. , Manet, E., Chavrier, P., Mosnier, C., Daillie, J. and Sergeant, A. (1986) EMBO J., 5, 3243–3249.
13. Takada, K., Shimizu, N., Sakuma, S. and Ono, Y. (1986) J. Virol., 57, 1016–1022.
14. Chevallier-Greco, A., Manet, E., Chavrier, P., Urier, G., Buisson, M. Daillie, J. and Sergeant, A. (1987) In D. L. Ablashi (ed), Epstein-Barr virus and Human diseases. The Humana Press, Clifton, N. J., 157–161.
15. Hardwick, J. M., Lieberman, P. M. and Hayward, S. D. (1988) J. Virol., 62, 2274–2284.
16. Manet, E. , Gruffat, H., Trescol-Biemont, M-C., Moreno, N., Chambard, P., Giot, J. F. and Sergeant, A. (1989) EMBO J., 8, 1819–1826.
17. Farrell, P. J., Rowe, D. T., Rooney, M. C. and Kouzarides, T. (1989) EMBO J., 8, 127–132.

18. Lieberman, P. M. and Berk, A. J. (1990) J. Virol., 64, 2560–2568.
19. Chang, Y. N., Dong, D. L. Y., Hayward, G. S. and Hayward, S. D. (1990) J. Virol., 64, 3358–3369.
20. Urier, G. , Buisson, M., Chambard, P. and Sergeant, A. (1989) EMBO J., 8, 1447–1453.
21. Flemington, E. and Speck, S. H. (1990) J. Virol., 64, 1227–1232.
22. Chevallier-Greco, A. , Gruffat, H., Manet, E., Calender, A. and Sergeant, A. (1989) J. Virol., 63, 615–623.
23a. Kenney, S., Holley-Guthrie, E., Mar, E. C. and Smith M. (1989) J. Virol., 63, 3878–3883.
23b. Kenney, S., Kamine, J., Holley-Guthrie, E., Mar, E. C., Lin, J. C., Markovitz, D. and Pagano, J. (1989). J. Virol., 63: 3870–3877.
24. Buisson, M., Manet, E., Biemont, M. C., Gruffat, H., Durand, B. and Sergeant, A. (1989) J. Virol., 63, 5276–5284.
25. Cox, M. Leahy, J. and Hardwick, M. (1990) J. Virol., 64, 313–321.
26. Laux, G., Freese, U. K. and Bornkamm, G. (1985) J. Virol., 56, 987–995.
27. Hammerschmidt, W., and Sugden, B. (1988) Cell, 55, 427–433.
28. Gruffat, H., Moreno, N. and Sergeant, A. (1990) J. Virol., 64, 2810–2818.
29. Jalinot, P. and Kedinger, C. (1986) Nuc. Acids Res., 14, 2651–2669.
30. Graham, F. L. and Van Der Eb, A. J. (1973) Virology, 52, 456–467.
31. Dignam, J. D., Lebowitz, R. M. and Roeder, R. G. (1983) Nucl. Acids Res., 11, 1475–1489.
32. Wildeman, A. G., Sassone-Corsi, P., Grudström, T., Zenke, M. and Chambon, P. (1984) EMBO J., 3, 3129–3133.
33. Galas, D. and Schmitz, A. (1978) Nucl. Acids Res., 5, 3157–3170.
34. Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S.C., Séguin, C., Tuffnell, P. S. and Barrell, B. G. (1984) Nature , 310, 207–211.
35. Marshall, M., Leser, U., Seibl, R. and Wolf, H. (1989) J. Virol. 63, 938–942.