Characterization of an R-Binding Site Mediating the R-Induced Activation of the Epstein-Barr Virus BMLF1 Promoter

HENRI GRUFFAT,¹ NATHALIE DURAN,¹ MONIQUE BUISSON,¹ FABIAN WILD,^{2†}
ROBIN BUCKLAND,^{2†} AND ALAIN SERGEANT^{1*}

Laboratoire de Virologie Moléculaire, Ecole Normale Supérieure de Lyon, UMR 49 CNRS-ENS, 46 Allée d'Italie, 69364 Lyon Cedex 07,¹ and Immunovirologie Moléculaire et Cellulaire, CNRS-UMR30, Faculté de Médecine Alexis Carrel, Lyon Cedex 8,² France

Received 14 February 1991/Accepted 18 September 1991

In cells latently infected with Epstein-Barr virus, the switch from latency to productive infection is linked to the expression of two Epstein-Barr virus transcription factors called EB1 and R. R is an enhancer factor, and an R-responsive element (RRE) has been identified in the BMLF1 promoter. In this study, we have used bidirectional deletion mutagenesis to delineate the BMLF1 RRE (RRE-M) to a 44-bp sequence. We also show that R expressed from a recombinant vaccinia virus protects RRE-M against digestion by DNase I. Using mobility shift assays and dimethyl sulfate interferences, we have characterized the contact points between in vitro-translated R and the DNA. R binds in vitro to one site by simultaneously contacting two sequences within the site, which are separated by 8 bp: 5'-catGTCCCtctatcatGGCGCagac-3'. Site-directed mutagenesis of this sequence completely impaired the binding of R in vitro and rendered the BMLF1 promoter nonresponsive to R. The results suggest that the R-inducible BMLF1 enhancer is composed of a single R-binding site, called RRE-M.

The human herpesvirus Epstein-Barr virus (EBV) infects and immortalizes peripheral B lymphocytes, resulting in the establishment of a latent infection. In such infected B cells, the entire EBV genome is maintained predominantly as a plasmid, and its expression is reduced to a few genes (for a review, see reference 24). The latent EBV genome is spontaneously activated in particular cell lines, in which 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*-tetradecanoylphorbol-13-acetate (29). In both cases, the activation seems to be linked to the expression of two EBV-encoded transactivators of early gene promoters, EB1 (also called ZEBRA, Z, BZLF1, or Zta) and R (also called Rta) (4–9, 12, 17, 19, 25, 27, 28). EB1 is encoded by the BZLF1 open reading frame and is expressed as a 1-kb mRNA (22) (Fig. 1). It is related to the AP-1/ATF/CREB/GCN4 family of transcription factors and interacts as an homodimer with many different DNA sequences (3, 11, 21). R is encoded by the BRLF1 open reading frame and is expressed from 3- and 4-kb mRNAs generated by alternative splicing which express both EB1 and R (17, 22) (Fig. 1).

R seems to be a factor that can act at distances over thousands of base pairs, and several R-responsive elements (RRE) have been identified (4, 9, 19). One (RRE-DR) is part of the DR/DL promoter (4, 9) and overlaps with the enhancer of the EBV origins of replication active only during the lytic cycle and called ORi1yt (16). R binds independently in vitro to two sites within RRE-DR (14) (RRE-DR1 and RRE-DR2). Each binding site covers about 18 bp, where R is thought to simultaneously contact two core sequences separated by 5 or 7 bp (14). A second RRE (RRE-M), localized in the BMLF1 promoter PM, controls the expression of two mRNAs coding for a factor acting at a posttranscriptional level (2, 19, 20) (Fig. 1).

In this report, we show that in vitro R binds to, and activates transcription from, a unique site covering 19 bp in the BMLF1 promoter. In this site, called RRE-M (2, 19, 20), R interacts with the DNA by simultaneously contacting two core sequences separated by 8 bp. RRE-M has some homologies with RRE-DR1 and RRE-DR2, but these three R-binding sites are imperfectly conserved. Site-directed mutagenesis in this R-binding sequence completely impaired induction by R of the BMLF1 promoter.

MATERIALS AND METHODS

Cloned DNA templates. The R expression vector has been described elsewhere (22). Plasmid pM13 and mutants with deletions extending in the 5'-to-3' direction from the 5' side of the BMLF1 promoter were constructed as previously described (2). Mutants with deletions extending in the 3'-to-5' direction were made as follows. Plasmid pM13 was opened at position –299 with *Pvu*II (Fig. 2A) and digested for various times with *Bal* 31 exonuclease. Selected DNAs were treated with T4 DNA polymerase and redigested with *Eco*RI. These DNA fragments were then ligated to plasmid pM13 cut by *Pvu*II and *Eco*RI. Plasmid pSV2 β (15) expresses a chimeric simian virus 40 (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 *Bam*HI. This plasmid was included in transfections to keep the amount of SV40 early promoter sequences constant, since R is expressed under the control of the SV40 early promoter.

Mutant M13m was made by site-directed mutagenesis using the MUTA-GENE M13 in vitro mutagenesis kit from Bio-Rad. All of the mutants produced were sequenced before use.

Construction of vaccinia virus recombinants expressing the R gene. A 1.8-kb *Bgl*III/*R*I fragment containing the coding region of R was cut from M13mp18R*5' (21a) and inserted into the vaccinia virus recombination vector pgpt Δ 6 (a gift

* Corresponding author.

† Present address: Institut Pasteur de Lyon, 69365 Lyon Cedex 07, France.

FIG. 1. Schematic structure of EBV transactivator genes and their targets in the BMLF1 promoter. Two open reading frames, BZLF1 and BRLF1, have been shown to code for the transcription factors EB1 and R, respectively. The BMLF1 promoter (PM) contains one EB1-responsive element (ZRE), overlapping with an AP-1 binding site, and one RRE. This promoter controls the expression of two mRNAs coding for a factor acting at the posttranscriptional level (3). Coding sequences in RNAs are represented by thick lines, and intronic sequences are represented by thin lines.

from H. Stunnenberg) cut with *Bam*HI/R1 to give pgptΔ6R. In this vector, the early/intermediate I3 promoter drives the *Escherichia coli* xanthine-guanine phosphoribosyltransferase gene and the inserted gene is driven by the 11K late promoter.

HeLa (2×10^6) cells were infected with vaccinia virus (Copenhagen strain; 0.1 PFU per cell) and 2 h later transfected with 10 μ g of CsCl-purified pgptΔ6R plasmid DNA, using the lipofectin protocol (Bethesda Research Laboratories); 24 h later, the cells were recovered and freeze-thawed twice, and recombinant viruses were selected as described elsewhere (10). The virus was plaque purified three times before use. For large-scale production of R protein, HeLa cells were infected with the recombinant at 0.1 PFU per cell, and the proteins were isolated from the cells 48 h later.

Preparation of HeLa WCEs. Whole cell extracts (WCEs) were prepared from HeLa cells or from HeLa cells infected by the recombinant vaccinia virus as previously described (26).

Heparin-agarose chromatography. WCEs were loaded onto a heparin-agarose column (heparin Ultrogel A4R [IBF]; 1 mg of protein per ml of gel) equilibrated in buffer D (20% glycerol, 20 mM Tris-HCl [pH 7.9], 20 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The column was washed with 2 volumes of buffer D, and proteins bound to the heparin were stepwise eluted with buffer D containing 240 mM, 400 mM, 600 mM, and 1 M KCl (Fig. 3A).

Production of R and mutant proteins in vitro. The BRLF1 ORF was cloned in plasmid pSPT19 (Boehringer Mannheim). Cloned inserts within the polylinker region were transcribed from the SP6 promoters. The RNA obtained was used to program protein synthesis in messenger-dependent rabbit reticulocyte lysates (Promega), using L-[^{14}C]leucine.

DNase I footprints. Footprint experiments were done by using EBV BMLF1 enhancer probes derived from plasmid pM13, 5' labeled with ^{32}P either at position -299 on the coding strand (probe pGM13C) or at position -494 on the noncoding strand (probe pGM13NC) (Fig. 4A). The experimental procedure was as previously described (14).

Electrophoretic mobility shift assay (EMSA). Two microliter of in vitro translation extract was incubated with 2×10^4 cpm of ^{32}P -labeled RREs (Fig. 3A). Incubations were carried

out in 0.5 mM $MgCl_2$ -10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9)-0.5 mM dithiothreitol-0.5 mM phenylmethylsulfonyl fluoride-150 mM KCl-10% glycerol at 25°C for 30 min. The mixture was loaded onto a 4.5% polyacrylamide gel (29:1 crosslinked)-0.2× Tris-borate-EDTA. The R-RRE complexes were separated from the noncomplexed DNA by migration at 10 V/cm and visualized by autoradiography.

DMS interference. A total of 5×10^5 cpm of DNA probe was methylated with 1 μ l of dimethyl sulfate (DMS) during 3 min at 18°C. The methylated probe was then incubated with 2 μ l of in vitro translation extract. After EMSA, the retarded (bound) DNA probe and the nonretarded (free) DNA probe were electroeluted and incubated in 100 μ l of 1 M piperidine for 30 min at 90°C. Equal amounts of the radioactive bound and free DNA probes were analyzed on 8% polyacrylamide sequencing gels and visualized by autoradiography.

Cell culture and transfections. HeLa cells were grown in Dulbecco modified Eagle medium (GIBCO) supplemented with 10% (vol/vol) 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 (13). Cells were mixed with the appropriate DNA(s), and the DNAs were in the same topological state as assayed by agarose gel electrophoresis. Usually 15 μ g of DNA was used per 100-mm dish as follows: 0.5 μ g of R-expressing vector, 5 μ g of reporter promoters, pSV0 when required, 0.5 μ g of pSV2 β as an internal control, and pUC19 up to 15 μ g.

RNA extraction and S1 nuclease mapping. Transfected cells were lysed with Nonidet P-40 as described elsewhere (18). Nuclei were pelleted, and RNA was phenol extracted from the cytoplasmic fraction. Then 10 to 40 μ g of total cytoplasmic RNA was hybridized overnight at 30°C in 50% formamide-0.3 M NaCl-0.01 M Tris-HCl (pH 7.4) to 5'- ^{32}P -labeled synthetic single-stranded DNA probes (Fig. 2A). The hybrids were digested for 2 h at 20°C with 5 U of S1 nuclease per 10 μ g of RNA. The size of the S1-protected DNA fragment was analyzed on 8% (wt/vol) 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 pSV2 β and (ii) according to the activities of the different constructions in the absence of R.

RESULTS

The BMLF1 RRE is contained within 44 bp. The BMLF1 promoter is strongly activated by R (2, 19, 20). We have previously shown, using deletion mutants extending in the 5'-to-3' direction from the 5' side of the BMLF1 promoter (Fig. 2A, mutants M13 to M10), that R-induced transcriptional activation is impaired by deletion of sequences located between positions -400 and -389 before the major initiation site (2). This is seen at both low and high R concentrations, indicating that there is no residual R-inducible activity after deletion of sequences from -400 to -389 (Fig. 2B). The increase in R-induced activity of the BMLF1 promoter followed by transfection of increasing amounts of R-expressing vector is likely to be due to increasing amounts of the R protein. In effect, as evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting,

FIG. 4. Evidence that R protects RRE-M against DNase I digestion. (A) Schematic representation of the sequences protected against DNase I digestion by the protein extracts used in the footprint experiment. On the coding strand (pGM13C), the positions of deletion mutants are indicated (M13, M16, M15, M14, and M10). Horizontal closed bars indicate footprints induced by the heparin Ultrogel fraction containing R produced in HeLa cells by a recombinant vaccinia virus. Vertical arrows and either letters or numbers indicate DNase I-hypersensitive sites. (B and C) DNase I footprints with decreasing amounts of DNase I on both strands of the DNA probe located between positions -299 and -494. The protein extracts and the probes used are indicated at the top: /, no proteins; HHep, HeLa WCE heparin fraction; HvVRHep, HeLa vaccinia virus producing R WCE heparin fraction. Vertical bars and numbers indicate footprints; horizontal arrows and numbers or letters indicate DNase I-hypersensitive sites; closed triangles indicate decreasing amounts of DNase I. G+A is the Maxam-Gilbert sequence of the probe.

source of proteins for the footprints, since the R protein was eluted at this KCl concentration from the heparin-agarose column (Fig. 3B).

One probe was 5' end labeled close to position -299 and called pGM13NC, and the other probe was 5' end labeled on the opposite strand close to position -494 and called pGM13C (Fig. 4A). No clear footprint was detected on probe pGM13C with the HeLa WCE heparin fraction (Fig. 4B, lanes 3 and 4) compared with the DNase I digestion pattern obtained with the probe alone (Fig. 4B, lane 2). However, in the presence of the HeLaVVR heparin fraction (Fig. 4B, lanes 5 to 7), a change in the digestion pattern occurred, with the appearance of several DNase-hypersensitive sites, des-

ignated a through g, and of a DNase I-protected area called region I (-365 to -393), located between DNase I-hypersensitive sites b and c. These observations suggest that R binds directly to this region.

DNase footprints were also performed on probe pGM13NC (Fig. 4C). Again, the DNase digestion pattern in the presence of the HeLa WCE heparin fraction (Fig. 4C, lanes 3 and 4) was not notably altered compared with the digestion pattern observed with the probe alone (Fig. 4C, lane 2). However, R expressed from the recombinant vaccinia virus modified the digestion pattern, with the induction of seven DNase I-hypersensitive sites and the appearance of a DNase I-protected area (region II, -349 to -414), located between the hypersensitive sites 1 and 5 (Fig. 4C, lanes 5 to 7). These results suggest that R interacts directly with RRE-M (-400 to -356), mediating the R activation in a transient expression assay, as defined by deletion mutagenesis (Fig. 1B).

R binds directly to the BMLF1 RRE in vitro. To determine further whether R directly binds to the BMLF1 RRE, we performed EMSA using the in vitro-translated R protein in conjunction with three double-stranded DNA fragments overlapping region I and region II, called M16e, M15e, and M14e (Fig. 5A). RNAs transcribed from the SP6 promoter located upstream from the R gene were used to program protein synthesis in messenger-dependent rabbit reticulocyte lysates. In these lysates, a 90-kDa [14 C]leucine-labeled polypeptide was detected and was immunoprecipitated by anti-R antibody (not shown). We also made use, as competitors, of the double-stranded oligonucleotide called B0 (Fig. 5A), which corresponds to one of the two R-binding sites in the OR1yt/DR promoter (RRE-DR1 [14]), and of a mutated version called B0* which does not bind in vitro-translated R (14).

As judged by EMSA and competitions, specific binding of R was observed to 32 P-radiolabeled probes M16e and M15e, but no complexes formed detectably on probe M14e (Fig. 5B). The in vitro-translated R bound with a high affinity to M16e (Fig. 5B, lane 2) and to M15e (Fig. 5B, lane 6) but poorly to M14e (Fig. 5B, lane 10). Specific binding was abolished by a 400-fold molar excess of unlabeled B0 (Fig. 5B, lanes 3, 7, and 11), but unlabeled B0* did not detectably compete for the binding of R (Fig. 5B, lanes 4, 8, and 12). These results, together with those of the deletion mutagenesis studies and the DNase I footprinting experiments, suggest that there is an R-binding site overlapping probes M15e and M14e.

R binds in vitro to a single site in the BMLF1 RRE. Since in vitro-translated R bound specifically to probe M16e (Fig. 5B, lanes 1 to 4), the contact points of R with a DNA fragment containing these sequences (probe M16E; Fig. 6A) were probed chemically by modification of the DNA with DMS (methylation interference analysis). After partial methylation at purine residues, the modified probe was used in the gel retardation assay. Free and bound probe DNA was recovered and subjected to chemical cleavage at methylation sites, and the products were resolved by gel electrophoresis under denaturing conditions. On both the coding and the noncoding strands of probe M16E, strong interferences were detected on guanine residues in the sequences 5'-GGCGC-3' and 5'-GTCCC-3' (Fig. 6B). Such strong interferences suggest that R simultaneously contacts both sequences rather than binding to them separately. The results of the methylation interferences (summarized in Fig. 6A) suggest that R binds to a site consisting of two regions separated by 8 bp, 5'-GTCCCTCTATCATGGCGC-3', called RRE-M.

FIG. 5. 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. Arrows and letters followed by a number indicate the positions of deletion mutants depicted in Fig. 2A. Closed rectangles over and under the sequence of probe B0 show the contact points between R and this DNA probe as determined by DMS interferences (14). Accordingly, some of these contact points have been mutated and are marked by asterisks in probe B0*. (B) In vitro-translated R was incubated with probes M16e (lanes 1 to 4), M15e (lanes 5 to 8), and M14e (lanes 9 to 12) and with DNA competitors as indicated at the top: /, no competitor added; L, lysate translated with brome mosaic virus RNA. DNA competitors were added at a mass ratio of 400. Free (F) and bound (B) DNA probes are indicated at the left. NS, nonspecific complexes.

The R-binding site called RRE-M mediates the R-induced activity of the BMLF1 promoter. To further evaluate the contribution of the R-binding site in the R-induced activity of the BMLF1 promoter, we mutated RRE-M (Fig. 7A, mutant M13m). This mutation impaired totally the binding of R to the isolated RRE-M in vitro (not shown). The mutated promoter (M13m) and the wild-type promoter (Fig. 7A, M13) were transfected into HeLa cells either alone or with 0.5 μ g of R expression vector, and their transcriptional activity was evaluated by quantitative S1 analysis. Representative results are shown in Fig. 7B. As expected, the activity of the wild-type BMLF1 promoter in mutant M13 (Fig. 7B, lane 1)

FIG. 6. Fine mapping of R-binding sites by methylation interferences. (A) Sequence of the double-stranded DNA probe M16E used for formation of protein-DNA complexes. The R-binding site detected after DNase footprinting is indicated by an open bar under the DNA sequence. The G residues involved in the formation of specific nucleoprotein complexes on the probes are shown by black squares over and under the sequence. Arrows and letters followed by numbers over and under the sequence of probe M16E located the endpoints and the orientations of the deletion mutants (Fig. 2A). (B) DNA binding assays. The assays were performed under standard conditions except that probe M16E, 5' end labeled on either the coding (M16E c) or the noncoding (M16E nc) strand, was treated with DMS before incubation with the in vitro-translated protein R. Lanes: G+A, Maxam-Gilbert sequence ladder; F, nonretarded probe; B, retarded complex. Asterisks indicate the G residues whose methylation strongly prevents the formation of complexes. (C) Evidence that three RREs are partially homologous. RRE-DR1 and -DR2 are presented as they appear on the coding strand, whereas RRE-M is presented as it appears on the noncoding strand.

FIG. 7. Evidence that a single R-binding site mediates the BMLF1 promoter R inducibility. (A) Sequences of the wild-type (M13) and the mutated (M13m) BMLF1 promoter. The contact points between R and RRE-M are shown by black squares over and under the RRE-M DNA sequence. The mutated version of RRE-M is also shown, and the modified bases are marked by asterisks. ZRE-M is the EB1-responsive element. (B) Transcription assays. HeLa cells were transfected with the different constructs and in the conditions indicated at the top panel. Transcriptional activation was determined by quantitative S1 analysis of total cellular RNA isolated from transfected cells. Plasmid pSV2 β expressing an SV40- β -globin hybrid RNA under the control of the SV40 early promoter-enhancer was cotransfected as an internal control. The specific start sites of β -globin and the early start sites of SV40 are indicated by β and SV, respectively.

was activated by R (Fig. 7B, lane 2), but when six base pairs were changed in RRE-M, the R-induced activation was strongly impaired (Fig. 7B, lanes 3 and 4). Plasmid pSV2 β , 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 (Fig. 7B, lanes 5 to 8, labeled SV). An equal amount of specifically initiated RNA was found in every transfection, indicating that the results described above could be compared.

These results suggest that the R-induced expression of the BMLF1 promoter is mediated by a single R-binding site, called RRE-M, which is located between positions -391 and -374.

DISCUSSION

In this report, we provide evidence that the BMLF1 RRE contains a single R-binding site, called RRE-M, which mediates transcriptional activation by R.

Using DNase I footprints, we have shown that R produced in HeLa cells from a recombinant vaccinia virus binds and protects against DNase I digestion a region defined by deletion mutagenesis as mediating transcriptional activation by R in transient expression assays. In addition, we show that R can interact directly in vitro with a specific sequence, RRE-M, composed of two regions and separated by 8 bp: 5'-CATGTCCTCTATCATGGCGCAGAC-3'. This site is partially homologous to the two R-binding sites characterized in the ORlyt enhancer (14), RRE-DR1 (5'-CCTGTGC

CTTGTCCTCCGTTGGACAATGTC-3') and RRE-DR2 (5'-CAA TGTCCTCCAGCGTGGTGGCTG-3').

R seems to contact directly and simultaneously the regions delineated by bold letters, and this proposal is made on the following observations. First, mutagenesis in one or the other of these two regions impairs the binding of R in vitro and impairs transcriptional activation by R mediated by these sites in transient expression assays. Second, methylation of the purine residues in the binding site induced a 100% binding interference, demonstrating that the two regions are not individual binding sites. This conclusion was confirmed by mixing an extract containing the R protein with an extract containing a truncated form of R and then adding to this mixture a radioactive probe containing an R-binding site and analyzing the protein-DNA complexes formed by EMSA. In this situation, besides the complexes of low and high mobility corresponding to the wild-type and truncated protein-DNA complexes, respectively, no complex of intermediate mobility corresponding to the binding of one R protein and one truncated mutant appeared (23).

The three sites characterized can be aligned and share some sequence homologies (Fig. 6C), but these homologies do not as yet allow us to propose a consensus. There are, however, several interesting structural features in the three RREs. First, the sequences which are thought to be directly and simultaneously contacted by R (letters in bold) are separated by 5 to 8 bp and are not therefore on the same side of the DNA double helix. Second, the 5' side of the RREs, GTG/CCC, is rather conserved, although the 3' side is not. Third, the three R-binding sites are imperfectly symmetric but R interacts in vitro as an homodimer with the three RREs, and the dimerization region and the DNA-interaction region overlap a region of 280 amino acids (23). None of these regions have obvious homologies with already described dimerization and DNA-binding domains, suggesting that the interaction of R with DNA is mediated by novel protein structures.

Finally, mutation in the BMLF1 promoter of the R-binding site completely abolished the activity of the R-inducible enhancer. This finding also suggests that the R-responsive enhancer in the BMLF1 promoter contains a single R-binding site. This conclusion was also confirmed by the deletion mutagenesis, by the DNase footprints, and by the DMS interferences studies.

However, there could be two R-binding sites, as in the DR promoter (14), but R would in this case bind cooperatively to the second site and only when the stronger adjacent site (RRE-M) is occupied. To test this possibility, we made shift-up probes containing the BMLF1 promoter sequences from positions -299 to -494 and tested the binding of increasing amounts of R either translated in vitro or produced by the recombinant vaccinia virus. In both cases, only one type of complex formed on this probe, at low and high protein concentrations, indicating that there is no cooperative binding to a second R-binding site in the BMLF1 promoter on this probe (not shown).

In conclusion, the BMLF1 R-responsive enhancer seems to contain a single R-binding site, only partially homologous to the other binding sites identified in the DR promoter (14).

ACKNOWLEDGMENTS

We thank H. Wolf for the anti-R rabbit antibody.

This work was financially supported by INSERM (contract 871015) and by the Association pour la Recherche sur le Cancer (contract 6810). H.G. and N.D. are recipients of an MRT fellowship.

REFERENCES

1. Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Séguin, P. S. Tuffnell, and B. G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature (London)* **310**:207-211.
2. Buisson, M., E. Manet, M. C. Biemont, H. Gruffat, B. Durand, and A. Sergeant. 1989. The Epstein-Barr virus (EBV) early protein EB2 is a post-transcriptional activator expressed under the control of EBV transcription factors EB1 and R. *J. Virol.* **63**:5276-5284.
3. Chang, Y. N., D. L. Y. Dong, G. S. Hayward, and S. D. Hayward. 1990. The Epstein-Barr virus Zta transactivator: a member of the bZIP family with a unique DNA-binding specificity and a dimerization domain that lacks the characteristic heptad leucine zipper motif. *J. Virol.* **64**:3358-3369.
4. Chevallier-Greco, A., H. Gruffat, E. Manet, A. Calender, and A. Sergeant. 1989. The EBV DR enhancer contains two functionally different domains: domain A is constitutive and cell specific, domain B is transactivated by the EBV early protein R. *J. Virol.* **63**:615-623.
5. Chevallier-Greco, A., E. Manet, P. Chavrier, C. Mosnier, J. Daillie, and A. Sergeant. 1986. Both Epstein-Barr virus (EBV) encoded trans-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. *EMBO J.* **5**:3243-3249.
6. Chevallier-Greco, A., E. Manet, P. Chavrier, G. Urier, M. Buisson, J. Daillie, and A. Sergeant. 1987. An EBV early promoter is activated by two viral encoded trans-acting factors, p. 157-161. *In* D. L. Ablashi (ed.), *Epstein-Barr virus and human diseases*. The Humana Press, Clifton, N.J.
7. Countryman, J. K., H. Jenson, E. Grogan, and G. Miller. 1986. A 2.7-kb rearranged DNA fragment from Epstein-Barr virus capable of disruption of latency. *Cancer Cells* **4**:517-523.
8. Countryman, J., and G. Miller. 1985. Activation of expression of latent Epstein-Barr herpesvirus after transfer with a small cloned subfragment of heterogeneous viral DNA. *Proc. Natl. Acad. Sci. USA* **82**:4085-4089.
9. Cox, M. A., J. Leahy, and M. Hardwick. 1990. An enhancer within the divergent promoter of Epstein-Barr virus responds synergically to the R and Z transactivators. *J. Virol.* **64**:313-321.
10. Falkner, F. G., and B. Moss. 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. *J. Virol.* **62**:1849-1854.
11. Farrell, P. J., D. T. Rowe, M. C. Rooney, and T. Kouzarides. 1989. Epstein-Barr virus BZLF1 transactivator specifically binds to a consensus AP-1 site and is related to c-fos. *EMBO J.* **8**:127-132.
12. Flemington, E., and S. H. Speck. 1990. Autoregulation of Epstein-Barr virus putative switch gene BZLF1. *J. Virol.* **64**:1227-1232.
13. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of adenovirus 5. *Virology* **52**:456-467.
14. Gruffat, H., E. Manet, A. Rigolet, and A. Sergeant. 1990. The enhancer factor R of Epstein-Barr virus (EBV) is a sequence specific DNA binding protein. *Nucleic Acids Res.* **18**:6835-6843.
15. Gruffat, H., N. Moreno, and A. Sergeant. 1990. The Epstein-Barr virus (EBV) ORlyt enhancer is not B cell specific and does not respond synergically to the EBV transcription factors Z and R. *J. Virol.* **64**:2810-2818.
16. Hammerschmidt, W., and B. Sugden. 1988. Identification and characterization of ORlyt, a lytic origin of replication of Epstein-Barr virus. *Cell* **55**:427-433.
17. Hardwick, J. M., P. M. Lieberman, and S. D. Hayward. 1988. A new Epstein-Barr transactivator, R, induces expression of a cytoplasmic early antigen. *J. Virol.* **62**:2274-2284.
18. Jalinet, P., and C. Kedinger. 1986. Negative regulatory sequences in the Ela-inducible enhancer of the adenovirus-2 early Ella promoter. *Nucleic Acids Res.* **14**:2651-2669.
19. Kenney, S., E. Holley-Guthrie, E. C. Mar, and M. Smith. 1989. The Epstein-Barr virus BMLF1 promoter contains an enhancer element that is responsive to the BZLF1 and BRLF1 transactivators. *J. Virol.* **63**:3878-3883.
20. Kenney, S., J. Kamine, E. Holley-Guthrie, E. C. Mar, J. C. Lin, D. Markovitz, and J. Pagano. 1989. The Epstein-Barr virus immediate-early gene product, BMLF1, acts in *trans* by a posttranscriptional mechanism which is reporter gene dependent. *J. Virol.* **63**:3870-3877.
21. Lieberman, P. M., and A. J. Berk. 1990. In vitro transcriptional activation, dimerization, and DNA-binding specificity of the Epstein-Barr Zta protein. *J. Virol.* **64**:2560-2568.
- 21a. Manet, E. Unpublished data.
22. Manet, E., H. Gruffat, M.-C. Trescol-Biemont, N. Moreno, P. Chambard, J. F. Giot, and A. Sergeant. 1989. Epstein-Barr virus bicistronic mRNAs generated by facultative splicing code for two transcriptional trans-activators. *EMBO J.* **8**:1819-1826.
23. Manet, E., A. Rigolet, H. Gruffat, J. F. Giot, and A. Sergeant. 1991. Domains of the Epstein-Barr virus (EBV) transcription factor R required for dimerization, DNA binding and activation. *Nucleic Acids Res.* **19**:2661-2667.
24. Miller, G. 1985. Epstein-Barr virus, p. 563-590. *In* B. Fields (ed.), *Virology*. Raven Press, New York.
25. Miller, G., M. Rabson, and L. Heston. 1984. Epstein-Barr virus with heterogeneous DNA disrupts latency. *J. Virol.* **50**:174-182.
26. Sergeant, A., D. Bohmann, H. W. Zentgraf, H. Weiher, and W. Keller. 1984. A transcription enhancer acts in vitro over distances of hundreds of base pairs on both circular and linear templates but not on chromatin-reconstituted DNA. *J. Mol. Biol.* **180**:577-600.
27. Takada, K., N. Shimizu, S. Sakuma, and Y. Ono. 1986. Trans activation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV DNA fragment. *J. Virol.* **57**:1016-1022.
28. Urier, G., M. Buisson, P. Chambard, and A. Sergeant. 1989. The Epstein-Barr virus early protein EB1 activates transcription from different responsive elements including AP-1 binding sites. *EMBO J.* **8**:1447-1453.
29. zur Hausen, H., E. J. O'Neil, U. K. Freese, and E. Hecher. 1978. Persisting oncogenic herpes-virus induced by tumor promoter TPA. *Nature (London)* **272**:373-375.