Identification of transactivator and nuclear localization domains in the Epstein–Barr virus DNA polymerase accessory protein, BMRF1

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The Epstein–Barr virus (EBV) BMRF1 gene product is an essential component of the viral DNA polymerase and is absolutely required for lytic virus replication. In addition to its polymerase accessory protein function, we recently demonstrated that BMRF1 is a transactivator, inducing expression of the essential oriLyt promoter, BHLF1. However, the regions of BMRF1 required for transactivation of BHLF1 are unknown. Here we demonstrate that the carboxy-terminal portion of the BMRF1 protein (amino acids 378–404), although not required for DNA binding or polymerase processivity function, is required for transactivator function as well as nuclear localization. Site-directed mutagenesis of this region allowed us to separate the transactivator and nuclear localization motifs of BMRF1. The two DNA-binding domains of BMRF1 are also sufficient for efficient transactivation of the BHLF1 promoter.

Epstein–Barr virus (EBV) is a human gammaherpesvirus that infects both epithelial and lymphoid cells (Rickinson & Kieff, 1996). EBV causes the clinical syndrome infectious mononucleosis and is closely associated with several types of human malignancy, including B-cell lymphomas and nasopharyngeal carcinoma (Rickinson & Kieff, 1996). In B cells, EBV usually establishes a latent infection and is replicated as an episome by using oriP and the cellular DNA replication machinery (Kieff, 1996; Reissman et al., 1985; Rickinson & Kieff, 1996). In contrast, EBV-infected epithelial cells commonly have the lytic form of virus replication (Li et al., 1992; Sixbey et al., 1984), in which the virus is replicated by a virally encoded polymerase and a separate origin of replication, oriLyt, is used (Hammerschmidt & Sugden, 1988; Schepers et al., 1993a, b). Replication from oriLyt requires the products of six viral genes: BALF5 (the catalytic component of the viral DNA polymerase), BMRF1 (the polymerase accessory protein), BALF2 (single-stranded DNA-binding protein), BSLF1 (primase), BBLF4 (helicase) and BBLF2/3 (helicase–primase–associated protein) (Fixman et al., 1992, 1995). In addition, the immediate-early gene product BZLF1 binds directly to oriLyt and is also required for activation of the viral replicative genes (Fixman et al., 1995; Schepers et al., 1993a, b).

The BMRF1 gene product is the major early phosphoprotein induced during EBV lytic replication and is a double-stranded (ds) DNA-binding protein that is essential for processive DNA synthesis by the viral polymerase (Chen et al., 1995; Chiou et al., 1985; Cho et al., 1985; Kallin et al., 1985; Kiehl & Dorsky, 1991, 1995; Li et al., 1987; Pearson et al., 1983; Tsurumi et al., 1993). Apart from its role as the DNA polymerase accessory protein, we have recently reported that BMRF1 transactivates the oriLyt early BHLF1 promoter, while not affecting the oriLyt BHRF1 promoter (Zhang et al., 1996, 1997). BMRF1-induced activation of BHLF1 is mediated through the essential downstream component of oriLyt, a region of oriLyt that is required for oriLyt replication (Schepers et al., 1993b; Zhang et al., 1997). Furthermore, the downstream component of oriLyt is sufficient to transfer BMRF1-responsiveness to a heterologous promoter (Zhang et al., 1997). Given the overlap between the BMRF1-responsive BHLF1 promoter element and an essential domain of oriLyt, BMRF1-induced activation of BHLF1 transcription could potentially play a pivotal role in oriLyt replication.

The regions of BMRF1 required for transactivation of oriLyt have not yet been identified. In this study, we have mapped the domains in the BMRF1 protein required for transactivation of the BHLF1 promoter. We show that the two known DNA-binding domains of BMRF1 are required for efficient transactivation of the BHLF1 promoter. We also demonstrate that the carboxy-terminal 26 amino acids of BMRF1, although not required for DNA binding or in vitro polymerase processivity function, are required for BHLF1 promoter activation and nuclear localization. All mutants that...
had lost nuclear localization had also lost transactivator function, suggesting that transport to the nucleus is required for BMRF1-induced transactivation. However, by site-directed mutagenesis of the BMRF1 carboxy-terminal region, we constructed mutants that localized to the nucleus but had lost transactivator function. Thus, the transactivator and nuclear localization domains of BMRF1 are located within overlapping, but distinct, regions within the carboxy terminus.

To map the BMRF1 protein domains required for transactivation of the BHLF1 promoter, we constructed a series of in-frame deletions in BMRF1, as shown in Fig. 1. As previously described (Chen et al., 1995; Kiehl & Dorsky, 1995), within the first 300 amino acids of BMRF1 are two domains that are required for dsDNA binding in vitro, as well as for polymerase processivity function. However, the carboxy-terminal region of BMRF1 (amino acids 303–404) is not required in vitro for either DNA binding or polymerase processivity function (Chen et al., 1995; Kiehl & Dorsky, 1995).

Since the nuclear localization domain of BMRF1 has not previously been mapped, we first examined the stability and nuclear localization of the wild-type versus mutant proteins in vivo. The various BMRF1 constructs (or the control vectors, pSG5 and pCDNA3) were transfected into HeLa cells and 2 days later the transfected cells were examined by indirect immunofluorescence with the BMRF1 antibody R3, which recognizes an epitope immediately upstream of amino acid 371 (Kiehl & Dorsky, 1995; Pearson et al., 1983). As summarized in Fig. 1, the wild-type BMRF1 protein and the BMRF1 deletion plasmids ∆80–204, ∆238–276 and ∆278–306 all localized to the nucleus, although the ∆80–204 and ∆278–306 mutants were considerably less stable than the wild-type protein in both immunofluorescence and Western blot assays (data not shown). The stability and nuclear localization of the ∆304–404 and ∆316–378 mutants could not be determined with the R3 antibody, since these mutations each delete the R3 epitope. By using a different antibody, 90E2 (Tsai & Glaser, 1991), that recognizes an epitope between residues 379 and 404 (Kiehl & Dorsky, 1995), the ∆316–378 mutant was shown to be at least as stable as wild-type protein and to localize to the nucleus (data not shown). In contrast, the ∆378–404 mutant, although as stable as wild-type protein in both immunofluorescence assays and Western blot analysis, was localized to the cytoplasm. Thus, the last 26 amino acids of BMRF1 are clearly required for nuclear localization in vivo. As shown in Fig. 1, this region of BMRF1 contains the consensus bipartite nuclear localization motif, (K/R)(K/R)XXXXXXXKXXKK (Dingwall & Laskey, 1991).

To examine whether the BMRF1 transactivator effect is exerted through a transcriptional mechanism, we performed a ribonuclease protection assay to quantify the level of BHLF1 RNA in HeLa cells transfected either with a plasmid containing the BHLF1 promoter (and downstream sequences) and pSG5

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**Fig. 1.** Mapping the region of the BMRF1 protein required for nuclear localization. Deletion constructs of the BMRF1 protein were constructed, as shown, by cutting at convenient restriction enzyme sites and re-ligating. In the map at the top of the figure, the full-length BMRF1 protein containing 404 amino acids is shown, as well as the consensus bipartite nuclear localization motif (Dingwall & Laskey, 1991) (indicated by N). The expression plasmid pSG5-BMRF1 has been described previously (Zhang et al., 1996) and contains the 1341 bp Bgl–BglII fragment of the EBV BamHI M fragment subcloned in BamHI–BglII cut pSG5 (Stratagene) under the control of the SV40 early promoter. pCDNA3-BMRF1 plasmid was constructed by transferring the HindIII–BglII fragment from the pYES2-M1 vector (Kiehl & Dorsky, 1995) into the HindIII–BamHI fragment of the pCDNA3 vector (Invitrogen), such that the BMRF1 gene is driven by the CMV IE promoter. Similarly, BMRF1 deletion plasmids ∆80–204, ∆238–276 and ∆278–306 were constructed by inserting the HindIII–BglII fragment from pYES2-M1∆80–204, pYES2-M1∆238–276 and pYES2-M1∆278–306 into HindIII–BamHI-cut pCDNA3. Plasmids BMRF1∆304–404, BMRF1∆316–378 and BMRF1∆378–404 were constructed by digesting pSG5-BMRF1 with NotI and BglII, SmaI and NruI, and NruI and BglII, respectively, filling in with Klenow enzyme as appropriate and re-ligating. Immunofluorescence studies (IF) were performed with the R3 and/or 90E2 antibodies (Pearson & Kieff, 1983; Tsai & Glaser, 1991) 2 days after transfecting HeLa cells with each of the above constructs. The strength of nuclear (Nuc.) and cytoplasmic (Cyto.) BMRF1 staining is summarized from two separate experiments: staining was as strong as that from wild-type BMRF1 (+ + +), barely detectable (+) or intermediate (+ +), undetectable (–) or not determined (ND) due to the known absence of the antibody epitopes.

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<th>Deletion</th>
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<th>Localization</th>
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<tr>
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<td>∆278–306</td>
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<tr>
<td>∆316–378</td>
<td>−</td>
<td>+++</td>
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<tr>
<td>∆378–404</td>
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**Polymerase processivity**

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<th>Restriction Enzyme</th>
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<th>Nucleosome Binding</th>
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**IF**

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Functional dissection of EBV BMRF1

Fig. 2. Mapping the transactivator domain of BMRF1. (a) HeLa cells were transfected with a plasmid containing the BHLF1 promoter sequences (from −1026 to +406 relative to the RNA start site) and pSG5 vector DNA, with the BHLF1 plasmid and the BMRF1 expression vector or with the BHLF1 plasmid and a BZLF1 expression vector. RNA from each condition was hybridized with a [32P]CTP-labelled, single-stranded, antisense RNA probe spanning the BHLF1 mRNA start site (from −789 to +200) and ribonuclease protection assays were performed using the Direct Protect kit (Ambion) according to the manufacturer’s instructions. A ribonuclease-protected fragment of about 200 bp (the expected size based on the location of the BHLF1 TATA box and the construction of the probe) was obtained when the probe was hybridized with RNA from cells transfected with the BHLF1 promoter plasmid and the BMRF1 or BZLF1 transactivators, but not in cells transfected with the BHLF1 promoter plasmid and vector DNA. A labelled actin antisense RNA probe used in parallel assays demonstrated that approximately equal amounts of cellular RNA were present in each experiment. (b) BHLF1–CAT reporter plasmid (5 µg) was co-transfected into HeLa cells with either 5 µg vector DNA (pSG5 or pCDNA3, as appropriate) or 5 µg wild-type or mutant BMRF1 constructs. The percentage of 14C-labelled chloramphenicol acetylation in each experiment was measured 2 days after transfection as previously described (Gorman et al., 1982). Results were normalized so that the level of BHLF1–CAT transactivation induced by wild-type BMRF1 (approximately 20-fold) in each experiment was set at 100%.

vector DNA, with the BHLF1 promoter plasmid and the BMRF1 expression vector or with the BHLF1 promoter plasmid and a BZLF1 expression vector (Fig. 2a). As expected, both BMRF1 and BZLF1 increased the quantity of the BHLF1 transcript in HeLa cells.

We next examined the ability of the wild-type and deleted BMRF1 proteins to activate a BHLF1–CAT construct, which contains the oriLyt BHLF1 promoter driving the chloramphenicol acetyltransferase reporter gene (Zhang et al., 1996), in co-transfection assays. Preliminary mapping results (Fig. 2b) indicated that the only mutant which still activated the BHLF1 promoter efficiently was the ∆316–378 mutant. This mutant localizes to the nucleus and retains DNA-binding activity. The three deletions, ∆80–204, ∆238–276 and ∆278–306, that have previously been shown to abolish BMRF1 DNA-binding activity (Kiehl & Dorsky, 1995) all decreased BMRF1 transactivator function dramatically. However, it should be noted that each of these mutants also decreased BMRF1 stability in vivo, which could account at least partially for the decrease in transactivator function. The finding that BMRF1 mutants that cannot bind DNA are less stable in vivo suggests that stabilization of the protein through DNA interaction may contribute to the transactivator function of BMRF1.

Each of the mutants (∆304–404 and ∆378–404) that did not include the last 26 amino acids of BMRF1 also inhibited BMRF1-induced transactivation completely. Since this region of BMRF1 is required for nuclear localization, the loss of transactivator function in these mutants could reflect primarily the loss of nuclear localization and does not necessarily imply that the transactivator domain of BMRF1 is contained within the carboxy terminus of the protein.

To map the regions of BMRF1 required for transactivation and nuclear localization further, we constructed a series of smaller mutations within the carboxy-terminal domain, as shown in Fig. 3(a). A protein from which amino acids 379–383 (RKRTS) were deleted still localized in the nucleus (Fig. 3b), although this mutation removed a portion (RK) of the bipartite nuclear localization motif. However, the ∆379–383 mutant was unable to transactivate the BHLF1 promoter (Fig. 3c), even though it localized to the nucleus. A mutant BMRF1 protein from which amino acids 383–404 had been deleted, removing most of the bipartite nuclear localization motif, no longer
Fig. 3. Dissecting the BMRF1 carboxy-terminal nuclear localization and transactivation domains. (a) BMRF1 constructs containing small mutations or deletions in the carboxy-terminal domain were constructed as shown, transfected into HeLa cells and examined for nuclear localization by immunofluorescence staining with the R3 BMRF1 antibody. Plasmid BMRF1Δ379–383 was constructed by digesting pSG5-BMRF1 with NruI and TthIII, treating both ends with T4 polymerase and re-ligating. Plasmid BMRF1Δ383–404 was constructed by digesting pSG5-BMRF1 with TthIII and BglII, filling in with Klenow enzyme and re-ligating. Plasmids BMRF1Δ397–404 and BMRF1Δ385/387/388 were made by PCR. Restoration of normal reading frames was confirmed by DNA sequencing. (b) Representative immunofluorescent staining of wild-type and mutant BMRF1 constructs: A, wild-type BMRF1; B, BMRF1Δ383–404; C, BMRF1Δ397–404; D, BMRF1Δ379–383. (c) BHLF1–CAT plasmid (5 µg) was co-transfected into HeLa cells with either 5 µg pSG5 vector DNA or 5 µg wild-type BMRF1 or mutation constructs. The percentage acetylation of 14C-labelled chloramphenicol in each experiment was measured and the degree of activation was calculated. Results were normalized such that wild-type BMRF1 transactivation is set at 100%.
localized to the nucleus and could not function as a transactivator. Removal of the last eight amino acids in BMRF1 (397–404) did not affect nuclear localization or transactivation. Finally, we constructed a mutation within the bipartite nuclear localization motif (altering amino acids 385, 387 and 388) which kept the essential nuclear localization sequences and spacing intact. This mutation, as expected, still localized to the nucleus (Fig. 3 b). However, mutation of residues 385, 387 and 388 inhibited BMRF1 transactivator function (Fig. 3 c).

The nuclear localization and transactivator functions of BMRF1 are therefore contained within overlapping, but distinct, sequences within the carboxy terminus. The BMRF1 transactivator function appears to require the domain localized between residues 379 and 388 (and possibly also the DNA-binding domains). While nuclear localization is probably essential for BMRF1 transactivator function, since all mutants that lost the ability to localize to the nucleus also lost transactivator function, nuclear localization per se is not sufficient for transactivator function, as demonstrated by mutants Δ379–383 and Δ385/386/388. Nevertheless, the results presented in Fig. 3 (b) suggest that the precise pattern of BMRF1 nuclear localization may be altered in the Δ379–383 and Δ385/386/388 mutants, since the wild-type BMRF1 protein appeared not to enter the nucleolus whereas the mutant proteins did.

Currently, the precise mechanism by which BMRF1 transactivates the essential downstream component of oriLyt is not known. It has previously been shown that the last 100 amino acids of BMRF1 do not function as a transactivator when linked to the yeast GAL4 DNA-binding domain (Chen et al., 1995). Likewise, we show here that the amino-terminal portion of BMRF1, as well as the carboxy-terminal portion, is required for transactivation of the BHLF1 promoter. Thus, while the carboxy-terminal domain in BMRF1 is clearly required for its transactivator function, it does not appear to be sufficient in itself.

Although the amino-terminal portion of BMRF1 is clearly required for non-specific dsDNA-binding in vitro (Chen et al., 1995; Kiehl & Dorsky, 1995), we have been unable to demonstrate that BMRF1 binds specifically to the BMRF1-responsive region of the BHLF1 promoter in vitro (Zhang et al., 1997). A region in the downstream essential component of oriLyt that mediates BMRF1 transactivation is also bound specifically by an unidentified cellular protein complex (Zhang et al., 1997). The BMRF1 protein could potentially activate oriLyt transcription through direct protein–protein interactions with this cellular protein(s), in which case, BMRF1 residues between 379 and 388 could be required for direct interaction with this protein(s). Alternatively, there are two serines and one threonine residue between amino acids 379 and 388 and phosphorylation of one (or more) of these residues might be required for BMRF1 transactivator function.

We have previously speculated that BMRF1-induced transactivation of the oriLyt BHLF1 promoter during lytic EBV replication is an essential aspect of BMRF1 function that is distinct from its role as the DNA polymerase accessory factor (Zhang et al., 1996, 1997). In this report, we have constructed mutations in the BMRF1 carboxy-terminal domain that abolish BMRF1 transactivator function, without altering the nuclear localization or polymerase processivity domains. Therefore, it should now be possible to study the importance of BMRF1 transactivator function within the context of the intact viral genome.

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