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Nucleolar and Nuclear Localization Properties of a Herpesvirus bZIP Oncoprotein, MEQ

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Marek's disease virus (MDV) is one of the most oncogenic herpesviruses and induces T lymphomas in chickens within weeks after infection. Only a limited number of viral transcripts are detected in MDV tumor samples and cell lines. One of the major transcripts encodes MEQ, a 339-amino-acid bZIP protein which is homologous to the Jun/Fos family of transcription factors. The C-terminal half of MEQ contains proline-rich repeats and, when fused to the DNA-binding domain of a yeast transcription factor, Gal4 (residues 1 to 147), exhibits transactivation function. MEQ can dimerize with itself and with c-Jun. The MEQ–c-Jun heterodimers bind to an AP-1-like enhancer within the MEQ promoter region with greater affinity than do homodimers of either protein, and they transactivate MEQ expression. Here we show that MEQ is expressed in the nucleus but, interestingly, with a predominant fraction in the nucleoli and coiled bodies. This makes MEQ the first bZIP protein to be identified in the nucleoli. MEQ contains two stretches of basic residues, designated basic region 1 (BR1) and basic region 2 (BR2). Using a series of deletion mutants, we have mapped the primary nuclear localization signal (NLS) and the sole nucleolar localization signal (NoLS) to the BR2 region. BR1 was shown to provide an auxiliary signal in nuclear translocation. To demonstrate that BR2 is an authentic NoLS, BR2 was fused to cytoplasmic v-Raf (Δ*gag*) kinase. The BR2-Raf fusion protein was observed to migrate into the nucleoplasm and the nucleolus. The BR2 region can be further divided into two long arginine-lysine stretches, BR2N and BR2C, which are separated by the five amino acids Asn-Arg-Asp-Ala-Ala (NRDAA). We provide evidence that the requirement for nuclear translocation is less stringent than that for nucleolar translocation, as either BR2N or BR2C alone is sufficient to translocate the cytoplasmic v-Raf (Δ*gag*) into the nucleus, but only in combination can they translocate v-Raf (Δ*gag*) into the nucleolus. Our studies demonstrate that MEQ is both a nuclear and nucleolar protein, adding MEQ to the growing list of transactivators which localize to the nucleolus.

Marek's disease virus (MDV), an avian alphaherpesvirus, can induce malignant T lymphomas in chickens within several weeks after infection (reviewed in references 9 and 39). Like other herpesviruses, MDV initially induces cytolysis in the host. The early cytolytic infection involving B cells peaks by 4 to 5 days postinfection and then subsides by 6 to 7 days postinfection. Meanwhile, T cells are activated in response to B-cell lysis and become susceptible to MDV infection. The MDV-infected T cells also go through a cytolytic phase, but a fraction of them eventually switch to a latent phase. Within 4 to 6 weeks, some of these latently infected T cells develop into frank lymphomas at multiple sites.

MDV has served as an excellent model to study herpesvirus oncogenesis. The rapid onset and polyclonal nature of MDV-induced lymphomas resemble those of acutely transforming retroviruses and suggest the presence of a virally encoded oncogene(s). The nature of the putative oncogene(s), however, remains poorly understood. Expression of the MDV genome in tumor cells is restricted to the long repeat region, namely, the *Bam*HI D, H, I_2, L, and Q_2 fragments (6, 66, 71, 75). We have recently identified a protein, MEQ, from the MDV *Eco*RI Q fragment (for which it is named) which spans the *Bam*HI I_2 and Q fragments and is the major protein derived from these fragments (36). MEQ encodes a 339-amino-acid (aa) bZIP (basic region leucine zipper) protein at the N terminus that is homologous to other bZIP proteins, particularly, the Jun/Fos family of transcription factors (Fig. 1). The C-terminal half of MEQ contains proline-rich sequences and, when fused to the DNA-binding domain of a yeast transcription factor, Ga14 (residues 1 to 147), exhibits transactivation function (60). MEQ is capable of forming dimers with itself and with c-Jun. The MEQ-Jun heterodimer binds to an AP-1-like enhancer within the MEQ promoter region and transactivates expression from the MEQ promoter. MEQ-MEQ or c-Jun–c-Jun homodimers also bind to a similar motif, but with a much lower affinity (60). Interestingly, MEQ-MEQ homodimers have their own target DNA sequences which are not shared by MEQ-Jun or Jun-Jun (61). Recent evidence suggests that MEQ is essential in the maintenance of the transformed phenotype of the MDV tumor cell line MSB-1 (78). These studies demonstrated that MEQ is a versatile transcription factor with the potential to regulate MDV as well as host genes. Since substantial evidence suggests that MEQ functions principally as a transactivator, its subcellular localization was presumed to be in the nucleus. In this report, we demonstrate that MEQ is not only a nuclear protein but also a nucleolar protein. Between the two long Arg/Lys stretches found at the N terminus of MEQ, basic region 1 (BR1) and BR2, the BR2 domain has been mapped to be the primary nuclear localization signal (NLS) and the sole nucleolar localization signal (NoLS). In addition, while either the BR2N or the BR2C peptide is sufficient for nuclear trans-

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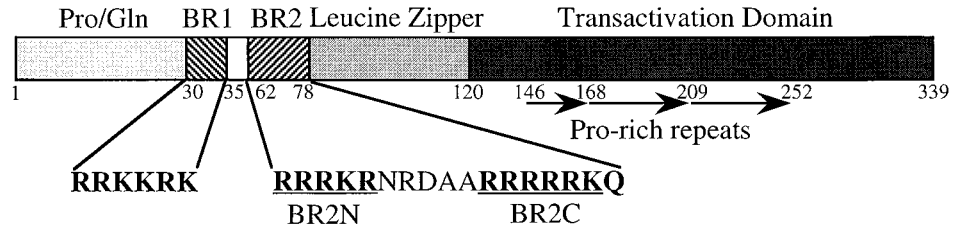

FIG. 1. Molecular structure of MEQ protein. MEQ protein is composed of an N-terminal proline-glutamine-rich domain (Pro/Gln), the basic region, and the leucine zipper, as well as the transactivation domain. The basic region contains two clusters of basic residues, BR1 and BR2. BR2 can be further divided into two subregions, BR2N and BR2C, which are separated by 5 aa, NRDAA. In addition, there are two and one-half proline-rich repeats (indicated by arrows) in the transactivation domain.

location of cytoplasmic v-Raf (Δ*gag*) kinase, the entire BR2 sequence is required for efficient nucleolar localization. Our data provide direct evidence that MEQ is a nuclear/nucleolar protein and carries an active NoLS. The significance of these findings will be discussed.

MATERIALS AND METHODS

Cells. Rat-2 cells and COS-1 cells were maintained in Dulbecco modified Eagle medium (high glucose) supplemented with 10% calf serum.

Virus and infection. Packaging cell line ψ2 was transfected with MEQ in the context of pBabe vector (54) by the Ca_2PO_4 method. Puromycin (1 μg/ml) selection was imposed 2 days after transfection. After stable transfectants were established, the supernatant (without puromycin) containing viral particles was collected and filtered to infect Rat-2 cells.

Plasmids. pBS (KS+)-MEQ (ΔBR1) was generated by the deletion of the first 53 aa (digested with *Nco*I and *Eco*NI first, blunt ended by the Klenow fragment of DNA polymerase I, and then religated). pBS (KS+)-MEQ (ΔBR2) was generated by deletion of the *Eco*NI-*Acc*I fragment (aa 53 to 82) followed by blunt-end ligation. pBS (KS+)-MEQ (ΔBR1&2) was generated first by introducing a *Sal*I site to create a Thr-79→Val mutant, digested with *Sal*I, and then blunt-end ligated (deletion of aa 28 to 79). pBS (KS+)-MEQ (bZIP) was digested with *Kpn*I to delete aa 128 to 329, and stop codons were introduced. In all cases, a T7 tag (MASMTGGQQMGR) was inserted in frame into pBS (KS+) immediately in front of the start codon. Wild-type (WT) MEQ and mutant MEQ cDNAs were subsequently cloned into pSVL at *Xba*I and *Ban*I sites or cloned into pBabe vector at *Xba*I-*Bam*HI (blunt end) and *Eco*RI sites. Simian virus 40 (SV40) large T antigen NLS (PRKKKRV), BR2N (RRRKR), BR2C (RRRRRK), and BR2 (RRRKRNRDAARRRRRKQ) peptides of MEQ were fused to the N-terminal end of v-Raf (Δ*gag*) kinase via PCR and then subcloned into a T7-tagged pSVL vector.

Transfection. The transfection was accomplished with either Ca_2PO_4 (Promega) or Lipofectamine (Gibco/BRL) as suggested by the manufacturers.

Bacterial expression of MEQ proteins and generation of rabbit antisera against MEQ. The N-terminal portion of MEQ (aa 1 to 169) was cloned into pET21b vector, which produced a fusion protein with a T7 tag at the N terminus and a $(His)_6$ tag at the C terminus. The expression and purification of MEQ proteins were performed as previously described (60). The purified MEQ fusion proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to achieve homogeneity before being injected into rabbits to generate antisera.

Preparation of mouse MAb to MEQ. Recombinant fowlpox virus (rFPV) (79) containing the MEQ open reading frame was used for immunization of mice for generating hybridoma cell lines secreting monoclonal antibodies (MAb) against MEQ. The procedure for immunization and selection of hybridoma has been described (44). Briefly, female BALB/c mice were immunized with rFPV-MEQ. Fusion of immunized spleen cells with NS-1 myeloma cells was at a ratio of five spleen cells to one NS-1 cell. Hybridoma supernatants were screened for MEQ-specific antibodies by indirect enzyme-linked immunosorbent assay with rFPV-MEQ-infected chicken embryo fibroblasts (CEF) and rFPV-infected CEF according to a previously described procedure (12). Hybridomas positive for MEQ-specific proteins were cloned by limiting dilution, and ascitic fluids of high titer were produced in BALB/c mice. MAb 23B46 was characterized and reacted specifically with rFPV-MEQ-infected CEF and not with WT FPV in enzyme-linked immunosorbent assay, immunofluorescence staining, and immunoprecipitation studies.

Indirect immunofluorescence. Cells were seeded at 5×10^5 cells/well in 6-well plates the day before immunofluorescence staining. Media were aspirated, and the cells were washed twice with phosphate-buffered saline (PBS) before the cells were fixed with 3.7% formaldehyde–PBS for 20 min. After another PBS wash, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min followed by blocking with 3% bovine serum albumin (BSA)–0.1% Tween 20–PBS for 1 h. The cells were then incubated with primary antibodies for 1 h. After two washes with PBS (0.1% Tween 20), the secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or Texas Red (Vector Labs) were applied for another hour, and the cells were examined under the fluorescence microscope (Nikon).

DAPI staining. The procedure for 4′,6-diamidino-2-phenylindole (DAPI) staining is modified from the method of Matera et al. (52). Briefly, after the immunofluorescence staining is complete, cells in 6-well plates are counterstained with DAPI (200 ng/ml in PBS; Sigma) for 2 min and then destained with 0.1% Tween 20–PBS for 10 min.

RESULTS

MEQ is expressed in the nucleoplasm, the nucleolus, and the coiled bodies. MEQ shares extensive homology with other bZIP proteins in the basic region and has been shown to be a transactivator (60). Like EBNA2 of the Epstein-Barr virus (2, 29), MEQ is both an immediate-early gene product and a latent protein; presumably MEQ functions in both replication and transformation. Since fibroblasts are natural permissive hosts for MDV replication, as a first step toward understanding the biological function of MEQ, we established MEQ-Rat2 fibroblast cell lines through infection by a retrovirus carrying the MEQ gene. In addition, we also developed rabbit antisera against $(His)_6$-tagged MEQ proteins and a mouse MAb against rFPV MEQ proteins (see Materials and Methods). No staining was detected in parental Rat-2 cells with either MEQ-specific antisera or MAb, attesting to the specificity of these antibodies (data not shown), whereas in MEQ-Rat2 cells, both antibodies detect a preferential subcellular localization of MEQ proteins in the nucleus, although the staining patterns are slightly different between rabbit polyclonal antibodies and mouse MAb (Fig. 2A and C and see below). Interestingly, there are intense spots in the subnuclear structures resembling nucleoli or nucleolus organizing regions, which are recognized by their exclusion to counterstaining by DAPI, a dye that specifically reacts with double-stranded DNA (Fig. 2B and D). This is especially evident with anti-MEQ MAb, as the MAb seems to preferentially recognize MEQ proteins expressed in those organelles (Fig. 2C and D). On the other hand, MEQ antisera appear to detect MEQ proteins in the nucleoplasm in addition to those subnuclear structures (Fig. 2A and B). To conclusively demonstrate that the nucleolus is the subnuclear structure in which MEQ proteins are expressed, we conducted a double-labeling experiment with rabbit anti-MEQ sera and a fibrillarin-specific MAb. Fibrillarin is a protein of the dense fibrillar component of the nucleolus (55) which is also found in the coiled bodies (62). As shown in Fig. 3A, the larger subnuclear structures are the nucleoli to which fibrillarin proteins localize. Conversely, those smaller nuclear bodies (Fig. 3A) represent the coiled bodies which are present either in the nucleoplasm or in tight association with the nucleolar periphery. When the same cells were double labeled with MEQ antisera (Fig. 3B), the overlapped images of Fig. 3A and B show that, indeed, MEQ colocalizes with fibrillarin (Fig. 3C). It should be noted,

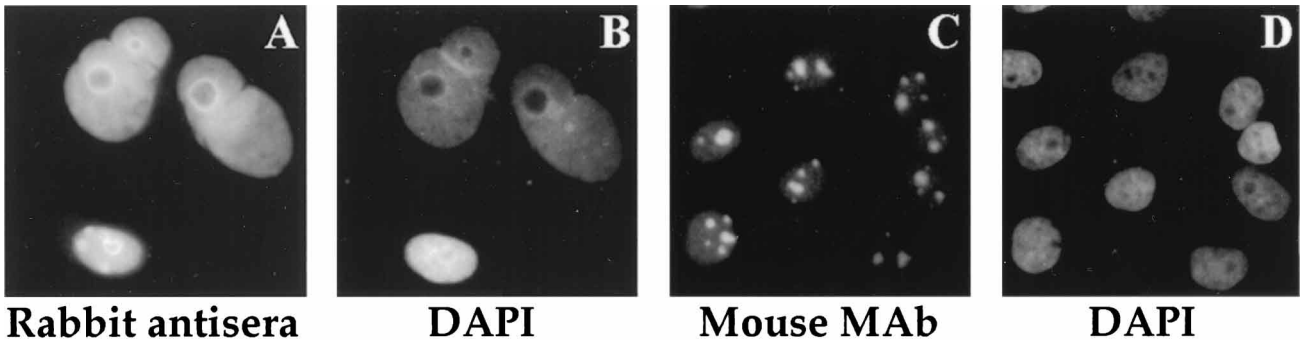

FIG. 2. Nuclear localization of MEQ proteins in MEQ-Rat2 cells. MEQ-Rat2 cells were stained either with rabbit anti-MEQ serum (1:100 dilution) (A) or with mouse anti-MEQ MAb (1:50 dilution) (C) followed by FITC-conjugated secondary antibodies (1:400 dilution) and counterstained with DAPI (B and D, respectively) as described in Materials and Methods. Magnification, ×368.

however, that the localization of MEQ proteins is not limited to the dense fibrillar component but, rather, involves the entire nucleolus. These results demonstrate that MEQ localizes to the nucleolus and the coiled bodies. To our knowledge, it is the first instance in which nucleolar localization has been demonstrated for a bZIP protein and in which a viral protein has been found in the coiled bodies (Table 1).

Identification of the NLS and the NoLS in MEQ protein. There are two clusters of Arg/Lys-rich sequences present in the basic region of MEQ (aa_{30}RRKKRKaa_{35} and aa_{62}RRRKRNR DAARRRRRKQaa_{78}), designated BR1 and BR2 (Fig. 1). For other bZIP proteins, the basic regions have been demonstrated to serve the dual roles of NLS and DNA-binding domain. The DNA-binding activity of MEQ has similarly been shown to map to this region (60, 61). To substantiate the findings in MEQ-Rat2 cells and to provide a sensitive assay for mapping the NLS and the NoLS within the MEQ protein, we took advantage of the mammalian overexpression system in COS-1 cells. WT MEQ and a series of deletion mutants (Fig. 4) were constructed in the context of the pSVL vector and transiently transfected into COS-1 cells under the control of the SV40 late promoter. MEQ (ΔBR1) was constructed by removing aa 1 to 53, including the entire BR1 region. MEQ (ΔBR2) carries a deletion of BR2 from aa 53 to 82. MEQ (ΔBR1&2) contains a deletion of both basic regions from aa 28 to 78, whereas, MEQ (bZIP) is devoid of the transactivation domain from aa 128 to 339. To ensure that the detection was specific for MEQ and not due to cross-reactivity of the MEQ antisera, the MEQ gene was also tagged by an epitope sequence recognized by a MAb (against T7 peptide). An indirect immunofluorescence assay was performed 2 days after transfection. The patterns were virtually identical when detected by anti-T7-tag MAb or rabbit anti-MEQ sera. Figure 5A shows that WT MEQ is expressed predominantly in the nucleus and nucleolus, with only a small fraction present in the cytoplasm. As shown in Fig. 5 and summarized in Fig. 4, the pattern of subcellular localization of MEQ (ΔBR1) is very similar to that of WT MEQ; they are expressed both in the nucleoplasm and in the nucleolus (Fig. 5B). By contrast, MEQ (ΔBR2) is expressed in the nucleoplasm and to a lesser extent in the cytoplasm (Fig. 5C and 5C′); the most striking feature is that MEQ (ΔBR2) is no longer detected in the nucleolus (Fig. 5C). On the other hand, the double deletion mutant, MEQ (ΔBR1&2) is present primarily in the cytoplasm and to a much lesser extent in the nucleoplasm, but not at all in the nucleolus (Fig. 5D). In addition, MEQ (bZIP) also shows a nuclear-nucleolar distribution similar to that of WT MEQ (Fig. 5E). These findings suggest that (i) BR2 is the major NLS and the sole NoLS, as deletion of this region abolishes the nucleolar localization of MEQ; (ii) BR1 serves an auxiliary function in nuclear translocation of MEQ;

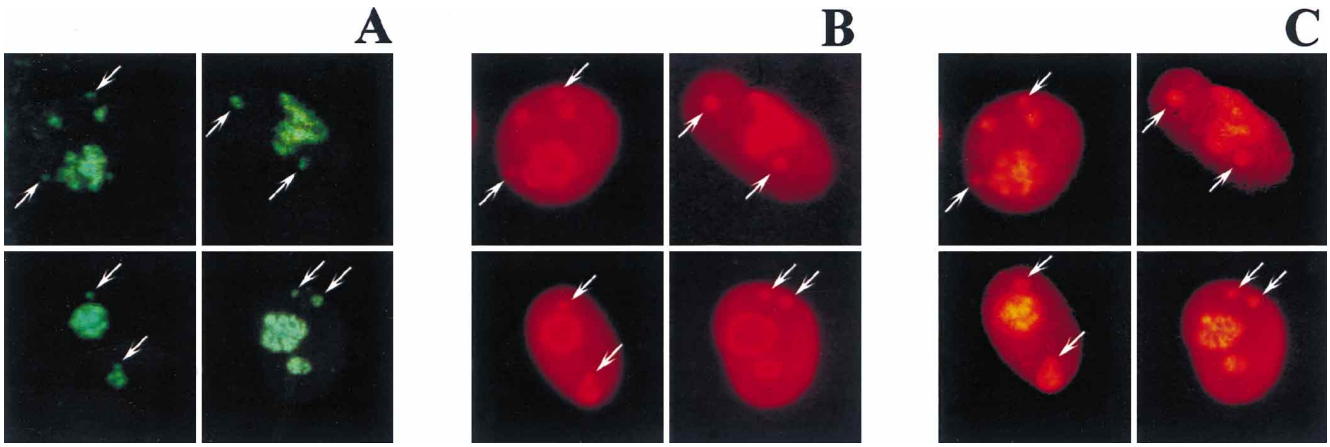

FIG. 3. Colocalization of MEQ proteins with fibrillarin in the nucleolus and the coiled body. MEQ-Rat2 cells were double labeled with mouse anti-fibrillarin MAb (1:200 dilution) (detected by FITC-conjugated horse anti-mouse immunoglobulins G [IgGs] [1:400 dilution], green fluorescence) (A) and rabbit anti-MEQ serum (1:100 dilution) (detected by Texas Red-conjugated goat anti-rabbit IgGs [1:200 dilution], red fluorescence) (B). (C) Overlapped images of panels A and B. The yellow dots represent the colocalization of MEQ proteins with fibrillarin in the nucleoli and the coiled bodies (coiled bodies are indicated by arrows). Magnification, ×400.

TABLE 1. The multiple subnuclear compartmentalization of cellular and viral proteins

Protein	Localization in:					Reference(s)
	Nucleoplasm	Nucleolus	Coiled body	Spliceosome (speckles)	ND10 (PML)	
Cellular						
WT-1	+		+	+		42
Rb	+	+				11
YY1	+	+				28
PCNA[a]	+	+				77
HSP70	+	+				57
TBP	+	+				37, 63
HOXB7, C6, D4	+	+				14
LYAR	+	+				70
IFI 16	+	+				17
DSSRP[b]	+	+				35
Viral						
MEQ (MDV)	+	+	+			
Rev (HIV)	+	+		+		38, 41, 49, 58
Tat (HIV)	+	+				22, 33
Rex (HTLV-1)	+	+				68
Us11 (HSV-1)	+	+				48
ICP27 (HSV-1)	+	+		+		53, 59
EBNA5 (EBV)[c]	+	+			+	73, 74
IVa2 (adenovirus)	+	+				47
E1A (adenovirus)	+				+	10
E4-ORF3 (adenovirus)	+				+	10
ICP0 (HSV-1)	+				+	21
Tax (HTLV-1)	+			+		67

[a] PCNA, proliferating cell nuclear antigen.
[b] DSSRP, *Drosophila* single-strand DNA/RNA-binding factor.
[c] EBV, Epstein-Barr virus.

and (iii) the N-terminal region and the C-terminal transactivation domain are not involved in the nuclear-nucleolar localization. Furthermore, these results show that the nucleolar staining is not an artifact resulting from the overexpression of a nuclear protein, since MEQ (ΔBR2) and MEQ (ΔBR1&2) are excluded from the nucleoli, despite their expression at levels similar to those of the nucleolar MEQ (ΔBR1) and WT MEQ. Our results suggest that BR2 exerts a dominant role in nucleolar transportation of MEQ.

The BR2 sequence of MEQ is sufficient for the translocation of cytoplasmic v-Raf (Δ*gag*) kinase into the nucleolus. Having demonstrated that BR2 is essential for MEQ's translocation into the nucleus, we wished to determine whether BR2 represents a sufficient and active NoLS. We also sought to better define BR2 sequences responsible for the nuclear versus nucleolar translocation. To this end, the BR2 sequence was fused to the N terminus of a cytoplasmic v-Raf (Δ*gag*) kinase, in the context of a pSVL vector with an N-terminal T7-tag (Fig. 6). The Gag–v-Raf protein is associated with the plasma membrane due to glycosylation of the Gag sequence; if the Gag sequence is deleted, v-Raf (Δ*gag*) becomes cytosolic (Fig. 7A). As a control, the well-studied NLS (PRKKKRV) of the SV40 large T antigen was fused to the N terminus of v-Raf (Δ*gag*). As shown in Fig. 7B, the T-Ag (NLS)–v-Raf (Δ*gag*) fusion proteins localize to the nucleoplasm but not to the nucleolus. By stark contrast, BR2–v-Raf (Δ*gag*) proteins localize to both the nucleoplasm and the nucleolus (Fig. 7C). This provides evidence that BR2 is an active NLS as well as NoLS. To further delineate the NLS and NoLS of BR2, we noticed that the basic region of BR2 is interrupted by 5 aa, NRDAA. We refer to the basic region N terminal to NRDAA as BR2N and to the C-terminal region as BR2C. BR2N and BR2C each contain more than 5 consecutive Arg/Lys residues, a hallmark of authentic type I (highly basic) NLS (5). Figure 7D and E show that either subregion of BR2, BR2N or BR2C, is sufficient to translocate v-Raf (Δ*gag*) kinase into the nucleus. However, only when the entire BR2 is present can the fused v-Raf (Δ*gag*) protein be translocated into the nucleolus (Fig. 7C). The data presented above suggest that the entire BR2 region is required to serve as an efficient NoLS. Indeed, the usually long stretch of basic residues of BR2 appears to be a characteristic of the NoLS, shared by some proteins preferentially localized to the nucleolus (Table 2).

DISCUSSION

MEQ was identified as a protein persistently expressed in MDV-induced tumor and T-cell lines. As such, it may function in viral latency and/or in viral oncogenesis. That MEQ is required for the maintenance of the transformed state of an MDV tumor cell line was recently shown by Xie et al. (78). MEQ has a structure resembling a fusion of the bZIP domain of the Jun/Fos family of oncoproteins and the proline-rich domain of the WT-1 tumor suppressor protein (8). Like Jun/Fos and WT-1, MEQ has been shown to behave as a transcription factor. It is capable of binding to specific DNA elements and can transactivate or repress target genes accordingly (61). As a transcription factor, MEQ is expected to localize to the nucleus, but this has not been experimentally determined. The distinct staining patterns between anti-MEQ sera and MAb in the MEQ-Rat2 cells could be due to different secondary or tertiary structures of MEQ proteins expressed in the nucleoplasm and the nucleolus, as was demonstrated for other proteins (77). Alternatively, the epitope for MAb may be masked by MEQ-binding proteins in the nucleoplasm. When MEQ proteins are translocated into the nucleoli, those binding proteins could be dissociated from MEQ proteins, resulting in the epitope being exposed.

Recently, growing attention has been focused upon the interrelationship between nuclear structure and nuclear functions. Different macromolecular structures in the nucleus, such as the nucleolus, spliceosome (speckles), coiled body, and nuclear domain 10 (ND10, also known as promyelocytic leukemia-associated nuclear body [PML]), as well as the foci for DNA replication and mRNA transcription, represent the compartments for discrete nuclear functions (reviewed in reference 69). As illustrated in Table 1, many cellular and viral proteins are expressed not only in the nucleoplasm but also in specific subnuclear compartments. Examples are the +KTS alternatively spliced form of the tumor suppressor WT-1, a transcriptional repressor of RNA polymerase II, which has been shown to localize to the spliceosomes (speckles) and the coiled bodies (42); proliferating cell nuclear antigen, which is involved in DNA replication and is also expressed in the nucleolus (77); human immunodeficiency virus (HIV) Rev, which in addition to its nucleolar localization has also been found to colocalize with RNA splicing factors in the spliceosomes (speckles) (38); EBNA5, which upon heat shock treatment is translocated from the nucleoplasm and ND10 into the nucleolus (73); and human T-cell leukemia virus type 1 (HTLV-1) transactivator Tax, which has also been detected in the spliceosomes (speckles). Indeed, viral proteins such as Rev and Tax serve multiple roles in the viral life cycle and are likely to participate in multiple nuclear functions. In this study, we examined the subcellular localization of MEQ to explore its known and unknown func-

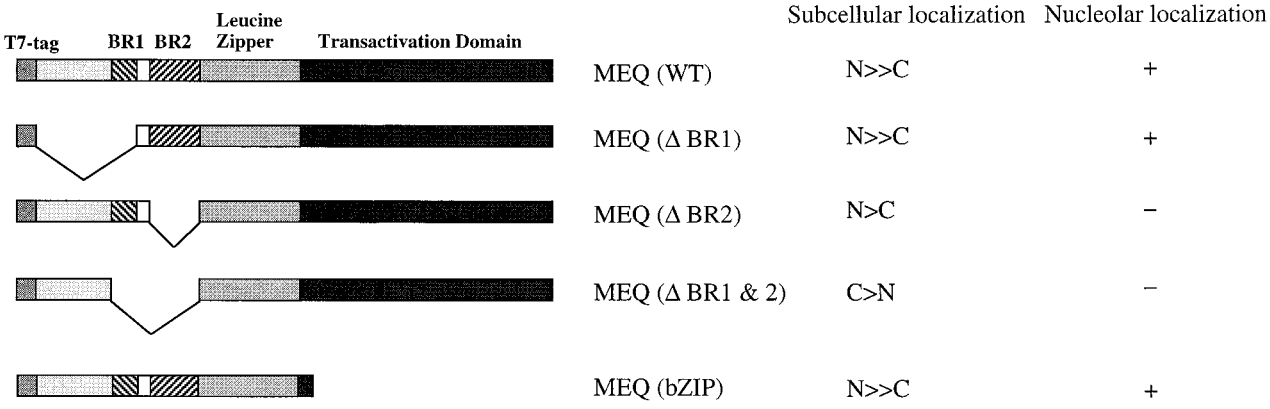

FIG. 4. A summary of subcellular locations of MEQ and its deletion mutants. pSVL vector was used to express all the constructs. MEQ (ΔBR1), deletion of the N-terminal region, including BR1; MEQ (ΔBR2), deletion of BR2 only; MEQ (ΔBR1&2), deletion of both BR1 and BR2; MEQ (bZIP), deletion of the transactivation domain; N, nucleus; C, cytoplasm; ∨, deletion.

tions. We found that MEQ protein localizes primarily to the nucleoplasm and to the nucleolus. The signal sequences responsible for transporting MEQ into the nucleus and the nucleolus were also determined.

Nuclear proteins with molecular masses larger than 40 to 60 kDa, such as MEQ, are transported into the nucleus presumably through nuclear pores in an energy-dependent manner. Simple karyophilic clusters of Arg and Lys residues of nuclear proteins are the most common type of NLS (reviewed in references 4 and 27). The NLSs are usually hidden or cryptic and need to be exposed on the protein surface by a variety of means, including protein phosphorylation-dephosphorylation or dissociation of an inhibitor that masks the NLS. The exposed and active NLS will then be bound by cytosolic transporter proteins and subsequently transported into the nucleus.

The two clusters of Arg/Lys-rich sequences in the basic re-

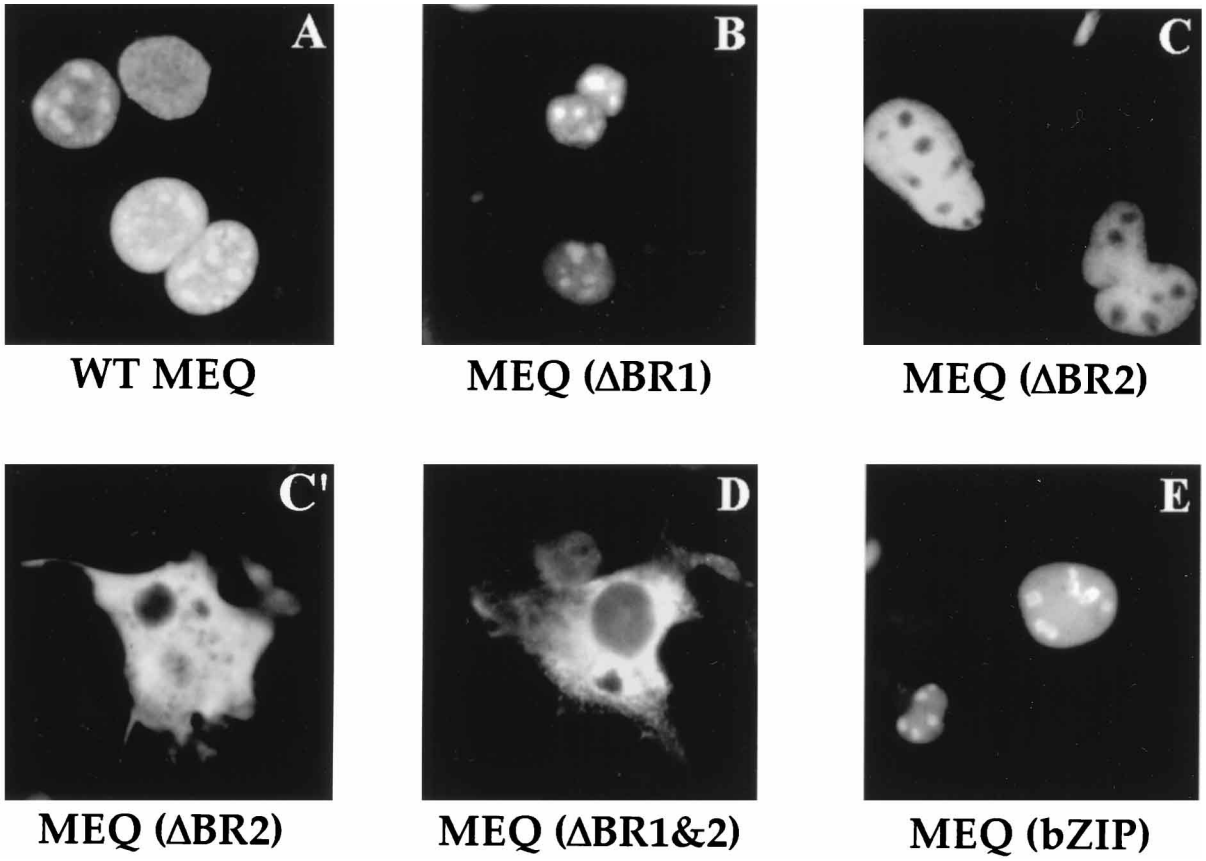

FIG. 5. Subcellular localization of WT MEQ protein and its mutants in COS-1 cells. WT MEQ and its mutants in the context of pSVL vector were transfected into COS-1 cells transiently. Immunofluorescence staining with rabbit anti-MEQ serum (1:100 dilution) and then with FITC-conjugated goat anti-rabbit IgGs (1:400 dilution) was performed 2 days posttransfection. Magnification, ×400.

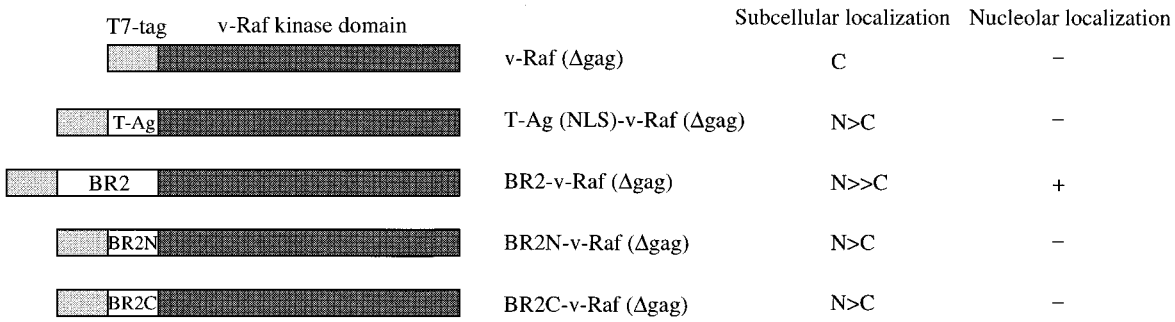

FIG. 6. A summary of subcellular locations of v-Raf (Δ*gag*) and its fusion derivatives. pSVL vector was used to express all the constructs. T-Ag, SV40 large T antigen NLS; BR2, the basic region 2 of MEQ; BR2N, the N-terminal half of BR2; BR2C, the C-terminal half of BR2; C, cytoplasm; N, nucleus.

gion (BR1 and BR2) represent the potential NLS for MEQ. Our deletion-mutation analysis confirmed such an assignment. Constructs retaining either one of the basic regions localize almost exclusively to the nucleus. Those lacking both of them have a major fraction present in the cytoplasm; a minor fraction, however, remains in the nucleoplasm, presumably due to the small size (<40 kDa) of this mutant, which was able to passively diffuse into the nucleus (25, 56). Presently, we cannot rule out the possibility of the existence of other "weak" NLSs located elsewhere in MEQ. It is, however, clear that BR2 and BR1 both represent the predominant NLS of MEQ.

Given MEQ's role as a transcription factor, its nucleolar localization is somewhat unexpected. However, a number of transcription factors such as YY1 (28); HOX B7, C6, and D4 (14); the tumor suppressor Rb (11); and the TATA-binding protein TBP (36, 62) have also been found in the nucleolus.

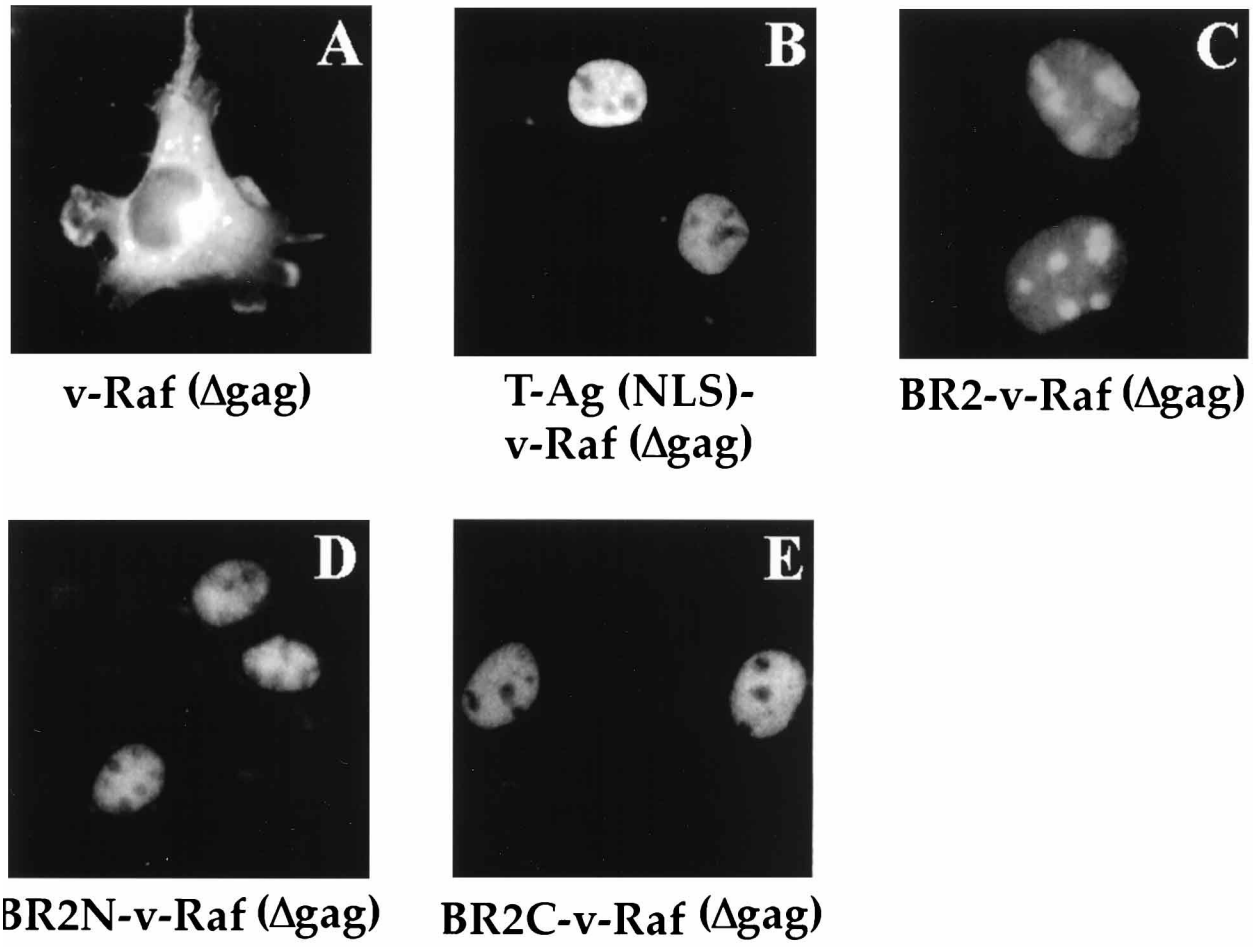

FIG. 7. Subcellular localization of v-Raf (Δ*gag*) fusion proteins in COS-1 cells. The indicated constructs in the context of pSVL vector were transfected into COS-1 cells transiently. Two days after transfection, the cells were stained with anti-T7-tag MAb (1:300 dilution) and then with FITC-conjugated horse anti-mouse IgGs (1:400 dilution). Used were v-Raf (Δ*gag*) alone (A) and fusion constructs with SV40 T-antigen NLS (B), MEQ BR2 (C), BR2N (D), and BR2C (E). Magnification, ×400.

TABLE 2. Alignment of NoLS sequences[a] of identified nucleolar proteins and MEQ

Protein	NoLS	Reference(s)
HTLV-1 Rex	MP**KTRRR**P**RR**SQ**RKR**PPTP	68
HIV-1 Tat	G**RKKRR**Q**RRR**P	16, 33
HIV-1 Rev	**RQ**A**RRN**R**RRR**W**RERQR**	41, 49, 58
Adenovirus PTP1 and PTP2	**RLPVRRRRRR**VP	81
Newcastle disease virus matrix protein	**KKGKK**VTFD**K**LE**RKIRR**	13
Semliki Forest virus capsid protein	**KPKKKK**TT**K**P**K**P**K**TQP**KK** (1) **KKKD**KQAD**KKKKK**P (2)	24
c-Myc (artificial sequence)[b]	PAA**KRVK**LDQ**RRR**P	15
HSP70	F**KRKHKK**DISQN**KR**AV**RR**	16
P120 nucleolar protein	S**KR**LSS**R**A**RKR**AA**KRR**LG	76
PTHrP[c]	G**KKKGKPGKRR**EQE**KKKRR**T	34
FGF3[d]	QP**RQRRQKK**QSPG	40
MDV MEQ	**RRRKR**N**R**DA**ARRRRK**Q	

[a] The Arg and Lys residues are indicated by boldface type.
[b] c-Myc is not expressed in the nucleolus. When this artificial sequence was fused to pyruvate kinase, however, it was able to translocate pyruvate kinase into the nucleolus.
[c] PTHrP, parathyroid hormone-related peptide.
[d] FGF3, fibroblast growth factor 3.

Other potential transcription factors such as Ly1 antibody-reactive clone (LYAR) (70), interferon-inducible protein (IFI 16) (17), and *Drosophila melanogaster* single-strand DNA/RNA-binding factor (35) are also shown to be expressed in the nucleolus. Among these proteins, TBP and Rb, in addition to their roles in RNA polymerase II transcription, also regulate rRNA transcription. While it is conceivable that MEQ may participate in ribosomal biogenesis, there is presently no evidence for or against this assertion. On the other hand, viral proteins may serve pleiotropic roles in regulating viral and host functions. As described above, a number of viral proteins involved in gene regulations, including HTLV-1 Rex (68), HIV Rev (49), and HIV Tat (22), have been identified in the nucleolus. For Rev and Rex, the nucleolar localization is required for their functions in posttranscriptional regulation of viral mRNA (20). Several herpesvirus proteins, herpes simplex virus type 1 (HSV-1) Us11 (48), infected cell protein 27 (ICP27) (53), and EBNA5 (73), also localize to the nucleolus. For Us11, an RNA binding protein is involved in regulating mRNA accumulation in the cytoplasm and can functionally substitute for Rev and Rex (19). The nucleolar localization of MEQ may suggest a new function for this protein.

At present, we do not understand how MEQ is translocated into the nucleolus. It is clear, though, that the arginine-rich BR2 is responsible for this translocation. BR2 is both a necessary and a sufficient signal for nucleolar localization, based on our analysis of MEQ deletion mutants and Raf fusion proteins. BR2 has a 12-basic-residue cluster, which is longer than most NLSs but similar in length to the NoLS of viral proteins such as Rex, Tat, Rev, and a number of other proteins listed in Table 2 (reviewed in references 4 and 31). The alignment of identified NoLSs reveals a hallmark of a long stretch of basic residues. It has been shown that B23, a nucleolar shuttle protein, interacts with Rev (23), Rex (1), and Tat (50). The interaction domain for B23 is mapped to its highly acidic domain (1, 72), whereas those for Rev (72) and Rex (1) are in their respective NoLSs. Experiments are in progress to study whether BR2 of MEQ interacts with B23. B23 usually interacts strongly with a long stretch of basic residues and much less so with a shorter stretch such as the SV40 T antigen NLS (PRKKKRV) (72). This perhaps can explain why SV40 T antigen is inefficiently translocated into the nucleolus and why the nucleolar localization of MEQ requires the entire stretch of BR2, whereas the nuclear localization can be accomplished by either the N- or C-terminal half of BR2 alone.

Until a function of MEQ in the nucleolus is defined, it remains possible that MEQ's nucleolar localization is coincidental, due to fortuitous association of BR2 with a nucleolar protein or rRNA. However, we think that this is unlikely, since mere association with nucleolar proteins and/or rRNA does not guarantee the nucleolar translocation. For instance, p53 has been found to interact with rRNA (26, 51, 65), yet p53 has never been detected in the nucleolus. Likewise, HSV-1 transcription factor ICP4 was shown to specifically interact with a nucleolar-ribosomal protein, L22 (EAP), but it did not localize to the nucleolus (46). By analogy to Rev, Rex, and Us11, MEQ may be involved in the regulation of viral RNA processing or transport. We previously noted that MEQ contains an RNA-recognition motif (RNP-1) at the C terminus, in addition to an arginine-rich region (arginine fork) which is highly homologous to the RNA-binding domains identified in many viral proteins (7, 18, 30, 32, 43, 45, 64, 80). Preliminary results indicate that MEQ indeed associates with RNA species (46a), although its specificity has not been characterized. We also observed that MEQ colocalizes with fibrillarin to the coiled bodies, in addition to the nucleolus. The localization of MEQ proteins in the coiled bodies was confirmed by double-labeling immunofluorescence assay with rabbit polyclonal antibodies against p80 coilin and anti-T7-tag MAb (data not shown). Coiled bodies belong to another class of nuclear structures with a size smaller than the nucleolus (average diameter, around 0.5 μm). Like the nucleoli, the presence of coiled bodies is correlated with transformation and increased metabolic activity. They are usually found in the nucleoplasm or in contact with the nucleolar periphery (3). It has been suggested that coiled bodies are involved in some aspects of RNP assembly, transport, or recycling and that they may provide enzymatic machinery for modifying nucleolar rRNA. In this regard, it is of interest that a fraction of WT-1 (the alternatively spliced form, +KTS), which interacts with p80 coilin and splicing factors, is colocalized in the coiled bodies (42).

To clearly define the role of MEQ's nucleolar localization in viral replication or oncogenesis, it would be helpful if mutants that dissociate transcriptional function from nucleolar localization could be developed. However, this may not be a straightforward experiment, since BR2 is essential for DNA binding and dimerization. More subtle point mutations may be needed to help delineate these functions. Taken together, our studies presented here suggest that MEQ's function as a viral or host gene regulator may not be limited to its transcriptional potential. Its association with the nucleolus and the coiled bodies may provide new leads to uncovering other novel activities.

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