Letter to the Editor

The Nuclear Export Signal within the Adenovirus E4orf6 Protein Contributes to Several Steps in the Viral Life Cycle

The concept of a Rev-like nuclear export pathway influencing adenovirus late gene expression has recently been challenged by a note in the *Journal of Virology* entitled “Adenovirus Late Gene Expression Does Not Require a Rev-Like Nuclear RNA Export Pathway” (5). The authors summarize that they “have tested the role of NES-mediated RNA export during adenovirus infection, and find that it is not essential for the expression of adenovirus late genes.” For the reasons outlined below, this conclusion seems misleading and not fully justified by the data.

We found that the E4orf6 protein of adenovirus type 5 contains a nuclear export signal (NES), allowing the protein to shuttle between the nucleus and cytoplasm (2). More recently, we observed that a functional NES of E4orf6 is required for multiple functions during infection, such as virus production, DNA replication, accumulation and transport of viral mRNA, and late expression of viral proteins (6). Nucleo-cytoplasmic shuttling of E4orf6 was now confirmed by Eileen Bridge and coworkers (5). However, the authors did not observe a significant reduction in the late expression of viral proteins when comparing wild-type E4orf6 with a mutant lacking the NES. They conclude that the export function of E4orf6 is not critical for late gene expression. However, the experimental approach leading to this conclusion requires critical evaluation. The authors transiently transfected expression plasmids for E4orf6 and mutants, followed by superinfection with E4-deficient adenovirus. They then stained the cells with antibodies to E4orf6 and simultaneously with antibodies against late proteins. The proportion among the E4orf6-positive cells that also expressed late proteins was scored and compared for various E4orf6 mutants. This score did not significantly differ between wild-type E4orf6 and the NES mutant. The problem with this approach is that it selects for those cells that express high levels of E4orf6—at least high enough to allow detection by immunofluorescence. The experiment does not score those cells that express the protein at levels that are too low for immunodetection but high enough to support virus replication. However, such a low level of expression would be what resembles the physiological situation during infection. E4orf6 is expressed by the virus at levels that barely allow immunodetection at late times after infection and essentially preclude detection at earlier times or at low multiplicities of infection (references 1 and 4 and our unpublished observations). Therefore, Rabino et al. (5) have almost certainly observed the effects of E4orf6 and mutants at supraphysiological intracellular concentrations. Our unpublished observations show that indeed, strong overexpression of E4orf6 using newly developed transfection lipids makes the NES function unnecessary for complementation of virus growth. However, this does by no means reflect the situation of an infected cell. In the experiments described in our previous report (6), we expressed E4orf6 using electroporation, which allows a relatively even, albeit weak expression of transfected plasmids. Under these conditions, the NES strongly contributed to virus growth. Instead of counting cells that stain positive for late proteins, we have documented several hallmarks of the viral life cycle. Our results consistently

show that DNA replication, virus production, and late protein synthesis (assayed by immunoprecipitation of hexon to allow overall quantitation) as well as RNA levels and distribution all depend to a large extent on the NES within E4orf6 (6).

Second, Rabino et al. (5) report that leptomycin B (LMB) fails to inhibit the synthesis of late viral proteins when added 12 h postinfection, and they conclude that adenovirus late gene expression does not require a Rev-like nuclear RNA export pathway. Again, this conclusion is not fully justified by the data. First, the authors do not provide an unequivocal test for LMB function in their assays. They report an inhibitory effect on cell growth, but this may be related to the general toxicity of LMB and does not provide a proof that nuclear export was abolished by LMB at this concentration. Indeed, the concentration used by the authors (10 nM) was previously shown to inhibit nuclear export of the Rev protein only partially but not entirely (3). Furthermore, the authors do observe a reduction of late protein synthesis when LMB was added shortly after infection. Thus, the data provided by Rabino et al. (5) merely suggest that a nuclear export function of E4orf6 may be of greater importance during the first 12 h after infection than afterward.

Taken together, these considerations and the currently available data suggest that the NES of E4orf6 significantly contributes to several steps in the viral life cycle. One of our goals is the construction of a recombinant adenovirus with a mutated NES in the E4orf6 protein. This is expected to further clarify the role of this export signal in the physiological context of an adenovirus infection.

REFERENCES

1. **Cutt, J. R., T. Shenk, and P. Hearing.** 1987. Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. *J. Virol.* **61**:543–552.
2. **Dobbelstein, M., J. Roth, W. T. Kimberly, A. J. Levine, and T. Shenk.** 1997. Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a Rev-like signal sequence. *EMBO J.* **16**:4276–4284.
3. **Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj.** 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**:1051–1060.
4. **König, C., J. Roth, and M. Dobbelstein.** 1999. Adenovirus type 5 E4orf3 protein relieves p53 inhibition by E1B-55-kilodalton protein. *J. Virol.* **73**:2253–2262.
5. **Rabino, C., A. Aspegren, K. Corbin-Lickfett, and E. Bridge.** 2000. Adenovirus late gene expression does not require a Rev-like nuclear RNA export pathway. *J. Virol.* **74**:6684–6688.
6. **Weigel, S., and M. Dobbelstein.** 2000. The nuclear export signal within the e4orf6 protein of adenovirus type 5 supports virus replication and cytoplasmic accumulation of viral mRNA. *J. Virol.* **74**:764–772.

Matthias Dobbelstein
Institut für Virologie
Universität Marburg
Robert Koch Str. 17
35037 Marburg, Germany

Phone: 49 6421 2864318

Fax: 49 6421 2868962

E-mail: dobbelst@mail.uni-marburg.de

Author's Reply

The precise role of the 34-kDa nuclear export signal (NES) during adenovirus infection is of great interest. Dobbelstein et al. (1), proposed a provocative and interesting model for the

export of adenovirus late mRNAs in which the 34-kDa NES would direct viral mRNA to be exported via a Rev-like RNA export pathway. In our experiments, viral late gene expression did not require either the 34-kDa NES or the Rev-like nuclear export pathway that is mediated by exportin-1 and inhibited by the drug leptomycin B (2). In contrast, complementation experiments performed by Weigel and Dobbelstein suggested that the 34-kDa NES was important for virus yield, late protein production, late mRNA levels, and importantly, for the accumulation of viral DNA (3). Since subsequent steps in the viral life cycle depend on efficient DNA replication, it is very possible that the role of the 34-kDa NES in this complementation system stemmed from its ability to promote the efficient onset of DNA replication. Why then, did we fail to detect a similar defect in DNA accumulation and late gene expression in our transfection/infection experiments? As we stated in our publication, we think the most likely explanation for the differences in our results stems from the multiplicity-dependent phenotype of E4 deletion mutants in viral DNA replication (see reference 2 for this discussion). We do not dispute Weigel and Dobbelstein's observation that the 34-kDa NES can play a role in the adenovirus life cycle (3), especially at low multiplicities of infection. While these results raise the interesting possibility that 34-kDa NES-mediated protein shuttling may promote viral DNA replication, neither of our groups has compelling evidence to support a role for the 34-kDa Rev-like NES in the export of adenovirus late mRNA.

In the accompanying letter to the editor, Matthias Dobbelstein raises a number of points to question the validity of our experimental system. First, he claims that by assaying late protein production in transfected cells identified by microscopy, we have studied only that fraction of the transfected cell population expressing levels of the 34-kDa NES that can be detected by immunofluorescence. However, Western blotting experiments confirmed that equal levels of wild-type and the NES 34-kDa mutant resulted in similar levels of late proteins in extracts prepared from transfected/infected cultures and suggest that the results we obtained by microscopy reflect the level of complementation present in our cultures (2). Second, he claims that the 34-kDa protein produced from our transfected expression constructs was not at physiological concentrations since we used a commercial lipid-based transfection system rather than electroporation. We do observe a range in the intensity of 34-kDa protein staining in cells from our transfection/infection experiments. Interestingly, the cells that are most intensely stained for the 34-kDa protein are not usually expressing viral late proteins. In cells which are both 34-kDa

protein positive and late protein positive and have therefore complemented late gene expression, the level of 34-kDa protein is similar to that seen after a 24-h infection with wild-type Ad5 (data not shown). Although we have no reason to believe that a "supraphysiological" concentration of the 34-kDa NES mutant protein was required for complementation in our system, we do agree that it will be critically important to determine the role of the 34-kDa NES when the mutant gene is expressed from the adenovirus genome in its proper context during infection. Finally, Dobbelstein raises the question of whether the leptomycin B treatment we used inhibited nuclear export. In our experiments, leptomycin B treatment reduced 34-kDa protein shuttling in cell fusion experiments by about threefold, indicating that it could interfere with the export of a protein containing a Rev-like NES (2). We also studied the effect of leptomycin B on the shuttling of 34-kDa proteins produced in transiently transfected cells fused to untransfected cells. These experiments were complicated by the fact that we observed some "shuttling" of transiently expressed 34-kDa proteins in our assay that was not NES dependent. This may be due to the presence of 34-kDa protein in the cytoplasm of some transfected cells overexpressing the protein prior to making the cell fusions. Nevertheless, when we compared shuttling of a 34-kDa protein lacking an intact nuclear retention signal (NRS) and a double mutant carrying lesions in both the NES and NRS, shuttling was only inhibited by leptomycin B (threefold) when the protein had an intact NES (data not shown). This indicates that mutations in the 34-kDa NES abolished leptomycin B-sensitive shuttling and shows that leptomycin B treatment inhibits 34-kDa NES-mediated export.

REFERENCES

1. Dobbelstein, M., J. Roth, W. T. Kimberly, A. J. Levine, and T. Shenk. 1997. Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a Rev-like signal sequence. *EMBO J.* **16**:4276-4284.
2. Rabino, C., A. Aspegren, K. Corbin-Lickfett, and E. Bridge. 2000. Adenovirus late gene expression does not require a Rev-like nuclear RNA export pathway. *J. Virol.* **74**:6684-6688.
3. Weigel, S., and M. Dobbelstein. 2000. The nuclear export signal within the E4orf6 protein of adenovirus type 5 supports virus replication and cytoplasmic accumulation of viral mRNA. *J. Virol.* **74**:764-772.

Eileen Bridge
Department of Microbiology
Miami University
Oxford, Ohio 45056

Phone: 513-529-7264
Fax: 513-529-2431
E-mail: BridgeE@muohio.edu