Inhibition of Ran Guanosine Triphosphatase–Dependent Nuclear Transport by the Matrix Protein of Vesicular Stomatitis Virus

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Transport of macromolecules into and out of nuclei, essential steps in gene expression, are potential points of control. The matrix protein (M protein) of vesicular stomatitis virus (VSV) was shown to block transport of RNAs and proteins between the nucleus and cytoplasm of *Xenopus laevis* oocytes. The pattern of inhibition indicated that M protein interfered with transport that is dependent on the ras-like nuclear guanosine triphosphatase (GTPase) Ran-TC4 and its associated factors. This inhibition of nuclear transport by M protein explains several observations about the effects of VSV infection on host cell gene expression and suggests that RNA export is closely coupled to protein import.

Macromolecules are in constant flux between the nucleus and cytoplasm; RNAs are transported between their sites of synthesis, maturation, and function; and proteins are both imported into, and exported from, the nucleus (*1*). This intracellular traffic, essential to the expression of nuclear genes, is a potential target for control by cells and viruses (*2, 3*). We now show that a single protein of VSV can specifically shut down both RNA export and protein import, demonstrating a close coupling of nuclear transport in the two opposite directions.

RNAs and proteins are transported between the nucleus and cytoplasm by signal-mediated, energy-requiring processes that involve many components of the nuclear pore complex (NPC) (*4*) and a large number of soluble factors (*1, 5*). Export of RNA is dependent on bifunctional RNA binding proteins (such as the Rev protein of HIV-1) that recognize specific sequences in the RNA and interact with other export factors or components of the NPC (*1, 3*). RNA-binding proteins like Rev (*3*), CBP20, the 20-kD component of the 5′ cap binding complex (*6*) and heterogeneous nuclear ribonucleoprotein particle (hnRNP)-A1 (*7*) exit the nucleus with the RNA transport substrates; subsequently, these proteins are again imported (*8*) by means of soluble transport factors like importins-α and -β (*5, 6*) or homologs of importin-β (*7*). Both RNA export and protein import are blocked by the lectin wheat germ agglutinin (WGA) and by antibodies specific for proteins of the NPC (nucleoporins) (*4, 8, 9*).

Key components of protein import systems (*1, 5, 7*) are the GTPase Ran-TC4 (Ran), several Ran-binding proteins, and the Ran-specific effectors RCC1 (the nuclear GTP:GDP exchange factor for Ran) and RanGAP1 (the cytoplasmic Ran GTPase activating protein) (*10, 11*). Inactivation of this system blocks import of proteins (*12*) and small nuclear ribonucleoprotein particles (snRNPs) (*13*), and export of most RNAs with the notable exception of tRNAs (*14, 15*); movement of RNAs within the nucleus also is impaired (*15*). However, the precise function or functions of the Ran GTPase in RNA transport remains to be established (*11*).

Infection by VSV, an RNA virus that replicates in the cytoplasm, results in rapid inhibition of host cell gene expression (*16*). The earliest observed effect is the cessation of maturation of small nuclear RNAs (snRNAs or U RNAs) (*17*). We have proposed (*15*) that this block in snRNA metabolism results from inhibition of export of snRNA precursors out of the nucleus (*18*). The putative viral inhibitor of RNA export would likely be contained in the infecting virions, since the effects on snRNA metabolism are extremely rapid and do not require formation of functional viral mRNA (*17*). A prime candidate for such an inhibitor is the matrix (M) protein of VSV, present in 1600 to 1800 copies per virion (*16*); synthesis of this protein in transiently transfected cells interferes with nuclear gene expression (*19, 20*). We now present data showing that M protein can inhibit export of nuclear pre-snRNA and other cellular RNAs and import of proteins.

We studied the ability of M protein to block nuclear transport by synthesizing the protein in *Xenopus laevis* oocytes (*21–24*). After the oocytes were cytoplasmically injected with m^{7}G-capped, polyadenylated transcripts coding for M protein (M mRNA), the protein accumulated both in the nucleus and the cytoplasm for up to 48 hours, as assayed either by immunoblotting (Fig. 1A) or by direct labeling of injected oocytes with [^{35}S]methionine (*25*). Differences in the amounts of M protein in the two compartments reflect, at least in part, the approximately 10-fold difference between volumes of the nucleus and cytoplasm. After about 20 hours of synthesis of M protein, we assayed for RNA export by injection of various DNA templates or RNAs made in vitro (*21*).

Expression of M protein reduced the amounts (15 to 20 times less) of newly transcribed U1 and U2 RNAs in the cytoplasm (Fig. 1B). This reduction contrasts with only a two to three times lower accu-

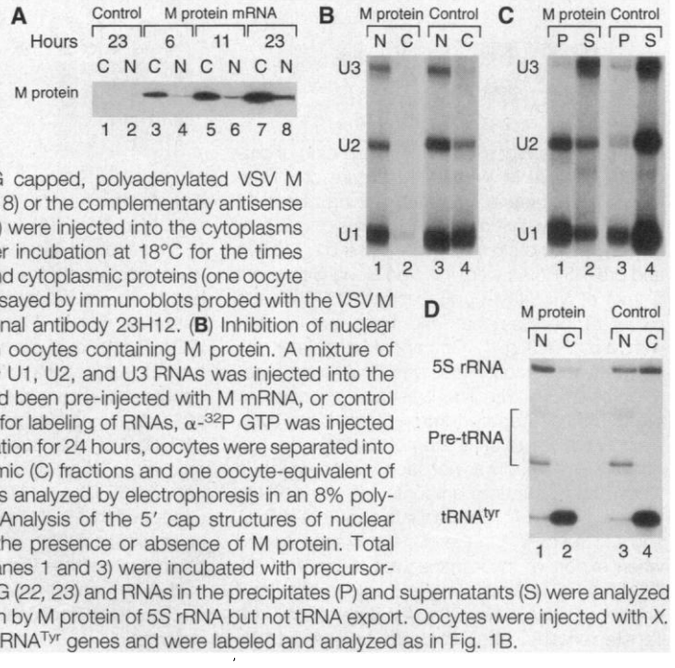

Fig. 1. Inhibition of export of small RNAs in *X. laevis* oocytes by VSV M protein. (**A**) Synthesis of VSV M protein in *X. laevis* oocytes injected with M mRNA. ~15 ng of m^{7}G capped, polyadenylated VSV M protein mRNA (lanes 3 to 8) or the complementary antisense sequence (lanes 1 and 2) were injected into the cytoplasms of *X. laevis* oocytes. After incubation at 18°C for the times indicated, total nuclear and cytoplasmic proteins (one oocyte equivalent, each) were assayed by immunoblots probed with the VSV M protein-specific monoclonal antibody 23H12. (**B**) Inhibition of nuclear export of pre-snRNAs in oocytes containing M protein. A mixture of genes encoding *X. laevis* U1, U2, and U3 RNAs was injected into the nuclei of oocytes that had been pre-injected with M mRNA, or control antisense RNA (Fig. 1A); for labeling of RNAs, α-^{32}P GTP was injected 2 hours later. After incubation for 24 hours, oocytes were separated into nuclear (N) and cytoplasmic (C) fractions and one oocyte-equivalent of RNA of each fraction was analyzed by electrophoresis in an 8% polyacrylamide gel (*21*). (**C**) Analysis of the 5′ cap structures of nuclear snRNAs synthesized in the presence or absence of M protein. Total nuclear RNAs (Fig. 1B, lanes 1 and 3) were incubated with precursor-specific antibodies to m^{7}G (*22, 23*) and RNAs in the precipitates (P) and supernatants (S) were analyzed as in Fig. 1B. (**D**) Inhibition by M protein of 5S rRNA but not tRNA export. Oocytes were injected with *X. laevis* 5S rRNA and pre-tRNA^{Tyr} genes and were labeled and analyzed as in Fig. 1B.

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mulation of newly made RNA, an indication that M protein inhibited export of the newly made transcripts, but had little on their synthesis (*26*). Blockage of the export of m^7G-capped precursor U1 and U2 RNAs by M protein was confirmed by immunoprecipitation with antibodies to m^7G (*22, 23*) that do not recognize the modified $m^{2,2,7}G$-cap that is generated on export of these RNAs to the cytoplasm (Fig. 1C) (*18*). The nuclear cap hypermethylation (*22*) and accumulation of U3 RNA were unaffected by the M protein, indicating that the inhibition of snRNA metabolism by M protein is specific for those snRNAs that must be transported across the nuclear envelope (*15, 18, 22*).

M protein also inhibited export of 5S ribosomal RNA (rRNA) made either from endogenous (*25*) or exogenously introduced genes, an indication that the inhibition of export was not limited just to m^7G-capped snRNAs (Fig. 1D, top). In contrast, M protein had no effect on the export of tRNAs, such as $tRNA^{Tyr}$ (Fig. 1D, bottom) or $tRNA^{Asn}$ (*25*), which utilize a transport system that functions even in the absence of functional RCC1 (*15*). Intranuclear maturation of tRNAs (splicing of $tRNA^{Tyr}$ and 5′ and 3′ end trimming of both tRNAs) also was not affected by M protein.

The effect of M protein on export of mRNA was assayed with a derivative of major late pre-mRNA from adenovirus (*21*). Although splicing of the injected pre-mRNA was normal, as indicated by reduction in precursor and a concomitant accumulation of the excised intron lariat (Fig. 2, lanes 2 and 6), export of the spliced mRNA product was severely compromised in oocytes containing M protein. At 1 hour, when about half of the mRNA was exported in control oocytes (lanes 6 and 8), no cytoplasmic mRNA was detected in oocytes treated with M protein (lane 4). Export of a small amount of the polyadenylated mRNAs in the treated oocytes was observed after a 4-hour incubation period (lane 5). However, even at 4 hours, export of the deadenylated spliced mRNA, which is formed when mRNAs are sequestered in the nucleus (*9*), was not detected; as expected (Fig. 1B), co-injected pre-$U1_{Sm^-}$ and pre-U5 RNAs were not exported.

The cytoplasmic accumulation of 18S and 28S rRNAs synthesized from endogenous oocyte rRNA genes was also sensitive to M protein (Fig. 3, lane 4) as was the maturation of these rRNAs. Both 45S precursor rRNA and abnormal processing intermediates (asterisks in Fig. 3) accumulated in the nucleus (lane 2), at the expense of mature forms. Both 18S and 28S rRNAs were occasionally detectable in the nuclei of some (Fig. 3) batches of oocytes (*25*); however, these mature rRNAs were never found in the cytoplasm, showing that M protein affects multiple steps in ribosome biogenesis. Because both maturation and export of rRNAs require transport of newly made ribosomal proteins into the nucleus (*27*), these results indicated that protein import was inhibited.

When tested directly, M protein was at least as effective as WGA in inhibiting the nuclear uptake of cytoplasmically injected *X. laevis* karyophilic proteins (*21*) (Fig. 4A, lanes 5 to 7). The M protein is also an extremely effective inhibitor of RNA import. U6 RNA and NL15 RNA are imported via the pathway used for NLS-containing proteins but U5 RNA uses a different pathway, specific for snRNPs (*24, 28*). Both of these Ran-dependent pathways (*1, 12, 13*) are blocked in the presence of M protein (Fig. 4B, lane 2).

Immunoblot analyses of several key transport factors indicated that M protein did not appreciably alter the amounts or the distributions between the nucleus and cytoplasm of RCC1, Ran and importin-β (Fig. 5A, lanes 1 to 4). However, it did increase the amount of importin-α that was associated with the nuclear fraction. Most of this importin-α co-migrated with a minor cytoplasmic form present in control oocytes (Fig. 5B, lanes 1 and 4) and both of these slowly migrating forms were phosphorylated (Fig. 5C) (*29*). Although phosphorylation of importin-α has been implicated in transport (*30*), it is unclear whether the changes that we observed play an obligate role in the inhibition of transport or are a consequence of the mechanism that is responsi-

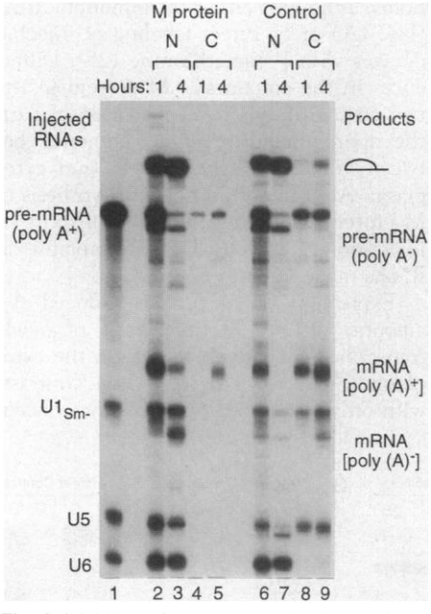

Fig. 2. Inhibition of export but not splicing of pre-mRNA by VSV M protein. A mixture of ^{32}P-labeled, m^7G-capped RNAs containing about 12 fmol of a derivative of adenovirus major late pre-mRNA plus about 5 fmol each of pre-$U1_{Sm^-}$ RNA and pre-U5 RNAs were injected along with about 5 fmol of γ-methyl-pppG-capped U6 RNA into nuclei of oocytes that had been pre-injected with M mRNA (Fig. 1). One and 4 hours later, the oocytes were fractionated and RNAs were analyzed as in Fig. 1B. The presence of unspliced pre-mRNA and spliced intron lariat in the cytoplasm was due to saturation of nuclear retention of these RNAs and was not seen in experiments in which nonsaturating amounts of pre-mRNAs were injected (*9*). Deadenylation of pre-mRNA and spliced mRNA in the nucleus is accentuated when export of these molecules is blocked (*9*). U6 RNA is retained in the nucleus (*21*) and pre-$U1_{Sm^-}$ RNA, which lacks an Sm protein binding site, remains in the cytoplasm upon export (*18*).

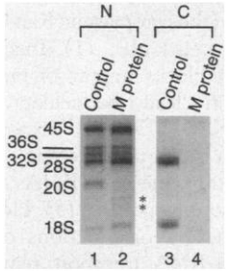

Fig. 3. Inhibition of maturation and export of rRNA by VSV M protein. Oocytes that had been injected with M mRNA or control mRNA (Fig. 1B) were then injected in the cytoplasm with about 0.5 μCi α[^{32}P]GTP and incubated for 24 hours. After fractionation, the newly synthesized endogenous rRNAs were analyzed by electrophoresis in a 1.2% agarose gel containing formaldehyde.

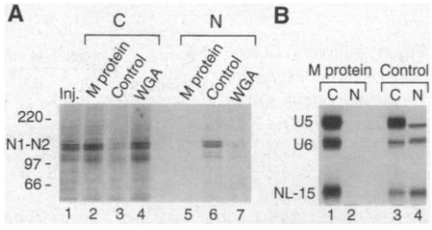

Fig. 4. Inhibition of import into oocyte nuclei by VSV M protein. (**A**) Inhibition of protein import. [^{35}S]methionine-labeled *X. laevis* nuclear proteins were injected into the cytoplasms of oocytes that had been pre-injected with M mRNA or control antisense mRNA (Fig. 1B) or with ~200 ng of WGA (2 to 3 hours previously). Twenty hours later, the labeled proteins in the cytoplasmic or nuclear fractions were analyzed by electrophoresis in a 10% polyacrylamide gel containing 0.1% SDS. (**B**) Inhibition of ribonucleoprotein (RNP) import. A mixture of approximately 5 fmol each of m^7G-capped U5 RNA. γ-Methyl-capped U6 RNA and uncapped NL15 RNA (an RNA selected for nuclear localization) (*24*) were injected into the cytoplasms of oocytes that had been injected with M mRNA or control antisense RNA (Fig. 1B). The oocytes were fractionated 24 hours later for RNA analysis.

ble (*31*). Clearly, the inhibition of transport is unlikely to be caused by depletion of the major form of importin-α from the cytoplasm (Fig. 5B, compare lanes 1 and 3). Because M protein inhibits export of mRNA, it is unlikely that the slowly migrating form of importin-α is due to induction of an isoform of this protein.

In contrast to M protein, WGA increased the amount of the major, rapidly migrating form of importin-α in the nuclear fraction (Fig. 5B, lanes 5 to 8), perhaps reflecting accumulation of docked import complexes at the cytoplasmic side of the NPC (*32*). The association of importin-α with the nuclear fraction in the presence of M protein might also result from non-productive binding of transport complexes at the NPC rather than from accumulation within the nucleus; however, the two inhibitors appear to work by different mechanisms in that different forms of importin-α accumulate, and WGA inhibits export of tRNAs (*8*) but M protein does not (*9*) (Fig. 1D).

How might M protein affect both the import of proteins (and snRNPs) and the export of several classes of RNAs? These features, including the continued export of tRNAs, are identical to those observed when the Ran system is inactivated (*12, 15, 31*). Thus, we propose that the pleiotropic effects of M protein on active transport between the nucleus and cytoplasm (*33*) result from interference with transport that is dependent on the Ran GTPase system. It is not known if M protein has enzymatic activity or affects cellular enzymes that impact on the Ran system. Injection of 20 times less M mRNA into oocytes still leads to inhibition of transport; the resulting number of M protein molecules, as detected by immunoblotting (*34*), is comparable to the number of NPCs per oocyte nucleus and well below the number of molecules of Ran or RCC1. Similarly, VSV infection at a multiplicity of 10 would introduce less than 2×10^4 molecules of M protein per cell (*16*), as compared to 10^7 molecules of Ran.

Inhibition of Ran-dependent protein import could block RNA export secondarily by depleting the nucleus of shuttling factors that are required for RNA export (*8*). Alternatively, a change in the activity of Ran or a Ran-specific factor may inactivate other components of the transport machinery that are required for mobilization of RNP export complexes prior to translocation through the NPC (*15*). The differential effects of M protein on the cytoplasmic appearance of snRNAs, mRNAs, and tRNAs (Figs. 1D and 2) also raises the possibility that additional Ran-independent RNA export pathways exist (*8, 33*).

It is possible that initiation of VSV transcription releases M protein from the nucleocapsids of infecting virions (*16*), thereby causing the inhibition of export of host snRNAs and mRNAs. In this model, synthesis of the 47-nucleotide VSV leader RNA would release the virion-bound M protein (*35*), which could explain the apparent paradox that blockage of snRNA maturation depends on VSV RNA synthesis but not on formation of functional VSV mRNA (*17*).

Inhibition of nucleocytoplasmic transport could account for changes in protein and RNA synthesis observed upon VSV infection (*16*) or introduction of M protein into cells (*19, 36*). Reduced accumulation of mRNA, due to turnover of transcripts that are sequestered in the nucleus, would cause an apparent inhibition of both mRNA and protein synthesis (*37*). The immediate blockage of export of host cell mRNAs would contribute to the rapid and efficient establishment of infection. Also, the increase (10 to 20 times greater) in translation of a transfected reporter mRNA observed in the presence of M protein mRNA (*36*) might be due to a lack of competing endogenous mRNAs.

Uninfected cells may use molecules similar to M protein to regulate transport between the nucleus and cytoplasm. Thus, elucidation of the mechanism of action of M protein may reveal novel ways by which gene expression could be controlled through the import and export of nuclear factors and RNAs.

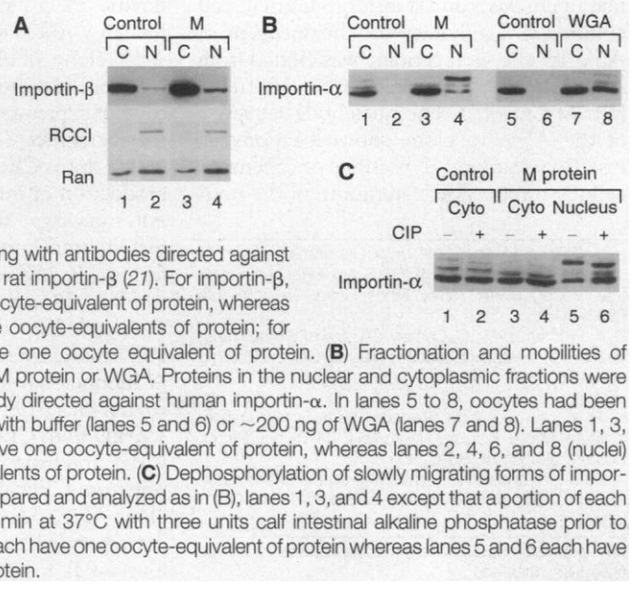

Fig. 5. Effects of VSV M protein on cellular transport factors. (**A**) Intracellular distributions of Ran, RCC1 and importin-β. Twenty-four hours after injection of M mRNA or control antisense RNA into oocyte cytoplasms, the nucleo-cytoplasmic distributions of several transport factors were analyzed by immunoblotting with antibodies directed against *X. laevis* RCC1, human Ran, or rat importin-β (*21*). For importin-β, lanes 1 and 3 each have one oocyte-equivalent of protein, whereas lanes 2 and 4 each have three oocyte-equivalents of protein; for RCC1 and Ran all lanes have one oocyte equivalent of protein. (**B**) Fractionation and mobilities of importin-α in the presence of M protein or WGA. Proteins in the nuclear and cytoplasmic fractions were analyzed as in (A), with antibody directed against human importin-α. In lanes 5 to 8, oocytes had been pre-injected in the cytoplasm with buffer (lanes 5 and 6) or ~200 ng of WGA (lanes 7 and 8). Lanes 1, 3, 5, and 7 (cytoplasms) each have one oocyte-equivalent of protein, whereas lanes 2, 4, 6, and 8 (nuclei) each have three oocyte-equivalents of protein. (**C**) Dephosphorylation of slowly migrating forms of importin-α. Oocyte proteins were prepared and analyzed as in (B), lanes 1, 3, and 4 except that a portion of each sample was incubated for 25 min at 37°C with three units calf intestinal alkaline phosphatase prior to electrophoresis. Lanes 1 to 4 each have one oocyte-equivalent of protein whereas lanes 5 and 6 each have three oocyte-equivalents of protein.

REFERENCES AND NOTES

1. E. Izaurralde and I. W. Mattaj, *Cell* **81**, 153 (1995); L. Gerace, *ibid.* **82**, 341 (1995); D. Görlich and I. W. Mattaj, *Science* **271**, 1513 (1996).
2. C. Feldherr and D. Akin, *Mol. Cell. Biol.* **15**, 7043 (1995); Y. Qui and R. M. Krug, *J. Virol.* **68**, 2425 (1994); M. Bray *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1256 (1994); C. Saavedra, K.-S. Tung, D. C. Amberg, C. N. Cole, *Genes Dev.* **10**, 1608 (1996).
3. B. R. Cullen and M. H. Malim, *Trends Biochem. Sci.* **16**, 346 (1991); U. Fischer, J. Huber, W. C. Boelens, I. W. Mattaj, R. Lührmann, *Cell* **82**, 475 (1995); R. A. Fridell, H. P. Bogerd, B. R. Cullen, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4421 (1996); C. C. Fritz and M. R. Green, *Curr. Biol.* **6**, 848 (1996); F. Stutz, E. Izaurralde, I. W. Mattaj, M. Rosbash, *Mol. Cell. Biol.* **16**, 7144 (1996).
4. E. Fabre and E. C. Hurt, *Curr. Opin. Cell. Biol.* **6**, 335 (1994); L. I. Davis, *Annu. Rev. Biochem.* **64**, 865 (1995); N. Panté and U. Aebi, *Int. Rev. Cytol.* **162B**, 225 (1995).
5. S. A. Adam and L. Gerace, *Cell* **66**, 837 (1991); M. A. Powers and D. J. Forbes, *ibid.* **79**, 931 (1994); F. Melchior and L. Gerace, *Curr. Opin. Cell Biol.* **7**, 310 (1995); N. Panté and U. Aebi, *ibid.* **8**, 397 (1996).
6. E. Izaurralde *et al.*, *Nature* **376**, 709 (1995); N. Visa, E. Izaurralde, J. Ferreira, B. Daneholt, I. W. Mattaj, *J. Cell Biol.* **133**, 5 (1996); D. Görlich *et al.*, *Cell* **87**, 21 (1996).
7. S. Pinol-Roma and G. Dreyfuss, *Trends Cell Biol.* **315**, 730 (1993); W. M. Michael, M. Choi, G. Dreyfuss, *Cell* **83**, 415 (1995); V. W. Pollard *et al.*, *ibid.* **86**, 985 (1996); N. Visa *et al.*, *ibid.* **84**, 253 (1996); J. D. Aitchison, G. Blobel, M. P. Rout, *Science* **274**, 624 (1996); E. Izaurralde *et al.*, *J. Cell Biol.* **137**, 27 (1997).
8. J. E. Dahlberg and E. Lund, *Semin. Cell Dev. Biol.* **8**, 65 (1997).
9. E. Lund and J. E. Dahlberg, unpublished results.
10. F. R. Bischoff and H. Ponstingl, *Nature* **354**, 80 (1991); E. Coutavas, M. Ren, J. D. Oppenheim, P. D'Eustachio, M. G. Rush, *ibid.* **366**, 585 (1993); N. Yokoyama *et al.*, *ibid.* **376**, 184 (1995); K. M. Lounsbury, A. L. Beddow, I. G. Macara, *J. Biol. Chem.* **269**, 11825 (1994); J. Wu, M. J. Matunis, D. Kraemer, G. Blobel, E. Coutavas, *ibid.* **270**, 14209 (1995); F. R. Bischoff, F. Krebber, T. Kempf, I. Hermes, H. Ponstingl, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1749 (1995); B. N. Paschal, C. Delphin, L. Gerace, *ibid.* **93**, 7679 (1996).
11. M. S. Moore and G. Blobel, *Trends Biochem. Sci.* **19**, 211 (1994); D. S. Goldfarb, *Curr. Biol.* **4**, 57 (1994); S. Sazer, *Trends Cell Biol.* **6**, 81 (1996); D. M. Koepp and P. A. Silver, *Cell* **87**, 1 (1996); M. G. Rush, G. Drivas, P. D'Eustachio, *BioEssays* **18**, 103 (1996).
12. M. S. Moore and G. Blobel, *Nature* **365**, 661 (1993); F. Melchior, B. Paschal, E. Evans, L. Gerace, *J. Cell Biol.* **123**, 1649 (1993); D. Görlich, N. Panté, U. Kutay, U. Aebi, F. R. Bischoff, *EMBO J.* **15**, 5584

(1996); G. Schlenstedt, D. H. Wong, D. M. Koepp, P. A. Silver, *ibid.* **14**, 5367 (1995); T. Tachibana, N. I. Imamoto, H. Seino, T. Nishimoto, Y. Yoneda, *J. Biol. Chem.* **269**, 24542 (1994); A. H. Corbett *et al.*, *J. Cell Biol.* **130**, 1017 (1995).
13. I. Palacios, K. Weis, C. Klebe, C. Dingwall, *J. Cell Biol.* **133**, 485 (1996). For a different conclusion see C. Marshallsay *et al.*, *Nucleic Acids Res.* **24**, 1829 (1996).
14. W. Forrester, F. Stutz, M. Rosbash, M. Wickens, *Genes Dev.* **6**, 1914 (1992); T. Kadowaki, D. Goldfarb, L. M. Spitz, A. M. Tartakoff, M. Ohno, *EMBO J.* **12**, 2929 (1993); D. C. Amberg, M. Fleischmann, I. Stagljar, N. C. Cole, M. Aebi, *ibid.*, p. 233; G. Schlenstedt, C. Saavedra, J. D. J. Loeb, C. N. Cole, P. A. Silver, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 225 (1995).
15. Y. Cheng, J. E. Dahlberg, E. Lund, *Science* **267**, 1807 (1995).
16. R. R. Wagner, J. R. Thomas, J. J. McGowan, *Compr. Virol.* **19**, 223 (1984); P. K. Weck and R. R. Wagner, *J. Virol.* **30**, 410 (1979); F.-S. Wu and J. M. Lucas-Lenard, *Biochemistry* **19**, 804 (1980); D. Thomas, *J. Virol.* **54**, 598 (1985).
17. L. D. Fresco, M. G. Kurilla, J. D. Keene, *Mol. Cell. Biol.* **7**, 1148 (1987); D. E. Crone and J. D. Keene, *J. Virol.* **63**, 4172 (1989).
18. I. W. Mattaj, *Cell* **46**, 905 (1986).
19. B. L. Black and D. S. Lyles, *J. Virol.* **66**, 4058 (1992).
20. It has been suggested that the short (47-nucleotide) leader RNA that is made early in VSV infection plays a role in host cell shutoff (*17*), but evidence that it directly affects nuclear gene expression is lacking.
21. The mechanisms of transport in *X. laevis* oocytes are the same as those of other eukaryotic cells (*1*). Oocytes were injected and RNA and protein transport was analyzed as described [(*22, 23*); E. Lund and J. E. Dahlberg, *EMBO J.* **8**, 278 (1989)]. ^{32}P-labeled RNA export substrates were synthesized in vitro [A. Pasquinelli, J. E. Dahlberg, E. Lund, *RNA* **1**, 957 (1995)] with the use of DNA templates as described (*21–24*). The template for synthesis of M mRNA was made by inserting into pSP64-poly(A) (Promega Inc., Madison, WI) a Hind III fragment of pSV-OM (*19*) that corresponds to the full-length cDNA of VSV Orsay M protein. Preparation and analysis of ^{35}S-labeled nuclear proteins for import assays have been described (*23*). The immunoblot analysis was done by standard techniques with monoclonal antibodies to M protein [L. Lefrancois and D. S. Lyles, *J. Virol.* **121**, 157 (1982)] and monospecific antibodies to transport proteins as noted in the legend to Fig. 5; bound antibodies were detected by ECL (Kirkegaard & Perry Laboratories, Inc., Gaithersberg, MD).
22. M. P. Terns and J. E. Dahlberg, *Science* **264**, 959 (1994).
23. M. A. Powers, D. J. Forbes, J. E. Dahlberg, E. Lund, *J. Cell Biol.* **136**, 241 (1997).
24. C. Grimm, E. Lund, J. E. Dahlberg, *EMBO J.* **16**, 793 (1997).
25. L.-S. Her, E. Lund, J. E. Dahlberg, unpublished results.
26. In the presence of inhibitors of export pre-snRNAs are unstable in the nucleus (*9, 23*), thereby accounting for the lower amounts of these RNAs in the presence of M protein.
27. A. A. Hadjilov, *The Nucleolus and Ribosome Biogenesis* (Springer-Verlag, New York, 1985).
28. Unlike protein import, snRNP import is quite insensitive to other general inhibitors of NPC function such as WGA [U. Fisher *et al.*, *J. Cell Biol.* **113**, 705 (1991); N. Michaud and D. S. Goldfarb, *ibid.* **112**, 215 (1991)] and monoclonal antibody mAb414 (*9*).
29. Ratios, rather than absolute amounts, of the proteins in the nuclear and cytoplasmic fractions are significant. Both the quantitative and qualitative changes in the distribution of importin-α in the treated oocytes were highly reproducible (*25*) and minor variations in amounts of Ran and importin-β between control and treated cells were not observed with other batches of oocytes. The persistance of multiple forms of importin-α even after phosphatase treatment (Fig. 5C) indicates that inaccessible phosphates or additional modifications may be involved. By co-immunoprecipitation we were unable to detect any interaction between M protein and Ran, or importins-α or -β (*25*).
30. Y. Azuma, M. M. Tabb, L. Vu, M. Nomura, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5159 (1995); I. Török *et al.*, *J. Cell Biol.* **129**, 1473 (1995).
31. In *Saccharomyces cerevisiae*, importin-α accumulates in the nucleus under conditions when the GDP form of Ran is expected to predominate; D. M. Koepp, D. H. Wong, A. H. Corbett, P. A. Silver, *J. Cell Biol.* **133**, 1163 (1996).
32. N. Panté and U. Aebi, *Science* **273**, 1729 (1996).
33. M protein does not affect passive diffusion of small RNAs or nucleotides across the nuclear envelope; C. Grimm, A. E. Pasquinelli, E. Lund, J. E. Dahlberg, unpublished results.
34. V. Varvel, E. Lund, J. E. Dahlberg, unpublished results.
35. Although M protein alone is sufficient to inhibit RNA export, a role for the VSV leader RNA in shut-off of nuclear gene expression of virus infected cells cannot be excluded (see *19*).
36. B. L. Black, G. Brewer, D. S. Lyles, *J. Virol.* **68**, 555 (1994); D. S. Lyles, M. O. McKenzie, M. Ahmed, S. C. Woolwine, *Virology* **225**, 172 (1996).
37. Inhibition of protein import by M protein may secondarily inhibit intranuclear functions such as transcription (for example, through depletion of transcription factors), but these effects are unlikely to be as immediate as a direct block of RNA export.
38. We thank members of our laboratory for helpful criticisms, D. Lyles for providing clones of the VSV M protein gene and monoclonal antibodies to M protein; M. Moore for antibodies to importins-α and -β; M. Dasso for antibodies to Ran and RCC1; T. Munns for antibody to m^{7}G-caps; and A. Grandjean and M. Barr for technical assistance. Supported by NIH grant GM30220.

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Transformation of Chicken Cells by the Gene Encoding the Catalytic Subunit of PI 3-Kinase

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The avian sarcoma virus 16 (ASV 16) is a retrovirus that induces hemangiosarcomas in chickens. Analysis of the ASV 16 genome revealed that it encodes an oncogene that is derived from the cellular gene for the catalytic subunit of phosphoinositide 3-kinase (PI 3-kinase). The gene is referred to as v-*p3k*, and like its cellular counterpart c-*p3k*, it is a potent transforming gene in cultured chicken embryo fibroblasts (CEFs). The products of the viral and cellular *p3k* genes have PI 3-kinase activity. CEFs transformed with either gene showed elevated levels of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate and activation of Akt kinase.

Retroviruses found in spontaneous animal tumors can be sources of oncogenes that reveal important aspects of cellular growth control (*1*). The avian sarcoma virus 16 has recently been isolated from a spontaneous chicken tumor. It induces hemangiosarcomas in chickens and transforms CEFs in cell culture (*2*). To characterize the oncogene of ASV 16, the viral genome was cloned from a λZAP cDNA library of ASV 16–transformed CEFs (*3*). The nucleotide sequence of the ASV 16 clone showed a nonviral insertion marking a possible oncogene of cellular origin. The 5′-terminus of the nonviral sequence was fused to viral *gag*, and the 3′-recombination junction was located within the viral *env* gene. A computer-assisted comparison revealed that the putative oncogene was homologous to the gene encoding the catalytic subunit, p110, of bovine PI 3-kinase (*4*). It was therefore named v-*p3k* (Fig. 1).

A clone of ASV 16 minus the 3′ *env* sequence was introduced into the avian retroviral expression vector RCAS (*5*), and the construct (RCAS-v-P3k) was transfected into CEFs, which resulted in the production of infectious retroviral progeny. After passage, the cultures became completely transformed and released a focus-forming RCAS retrovirus (Fig. 2, A and B).

The RCAS-v-P3k–transformed CEFs were tested for the presence of the Gag-v-P3k fusion protein by immunoprecipitation. A monoclonal antibody against avian retroviral Gag p19 (*6*) precipitated a protein of 150 kD from CEFs transfected with RCAS-v-P3k and from ASV 16–infected CEFs but not from CEFs transfected with the vector alone (Fig. 3A). The size of the protein corresponded to the predicted size of the Gag-v-P3k fusion. The 150-kD protein was

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