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12. TA17Δ80 was constructed from pRS104 (*11*), which contains TRP1ARS1 sequences from 615 to 1208 map units cloned into pUC18. The near-consensus 17-bp GAL4 binding site 5′-CGGAA-GACTCTCCTCCGATC-3′ [E. Giniger, S. M. Varnum, M. Ptashne, *Cell* **40**, 767 (1985)] was introduced into this plasmid between 857 and 858 map units with the polymerase chain reaction (PCR) [R. Higuchi, B. Krummel, R. K. Saiki, *Nucleic Acids Res.* **16**, 7351 (1988)]; the 80-bp deletion (from 872 to 951 map units), which leads to incorporation of the GAL4 binding site into nucleosome I (*10*), was also made by PCR. Digestion of the resulting plasmid, pRS104-17Δ80, along with pRS110, which bears the complementary part of the TRP1 gene and a copy of the ARS1 sequence (*11*), with Hind III and Sst I, followed by ligation yields TA17Δ80. This construction eliminates the GAL4 binding site from the *GAL3* promoter centered at 1415 map units in TRP1ARS1 [W. Bajwa, T. E. Torchia, J. E. Hopper, *Mol. Cell. Biol.* **8**, 3439 (1988)]. TA17Δ80 was introduced directly into the yeast strain FY24 (*matα*, *ura3-52, trp1Δ63, leu2Δ1*), which is isogenic to the strain S288C except that it is *GAL2*$^+$ (*27*), by a modification [J. Hill, K. A. Ian, G. Donald, D. E. Griffiths, *Nucleic Acids Res.* **19**, 5791 (1991)] of the method of Ito *et al.* [H. Ito, Y. Fukuda, K. Marata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983)]. The resulting transformants were transformed with the GAL4 expression vector pRS425-GAL4, which comprises the 3.6-kb Bam HI fragment containing the *GAL4* gene from the plasmid pG525 [A. Laughon and R. F. Gesteland, *Mol. Cell. Biol.* **4**, 260 (1984)] carried on the 2-μm-based shuttle vector pRS425 (*28*). TAΔ80 was made identically to TA17Δ80 except that the initial introduction of the GAL4 binding site was omitted.
13. Yeast cells (400 ml) were grown at 30°C in media supplemented with 2% glucose or 2% galactose and amino acids plus uracil as appropriate [M. D. Rose, F. Winston, P. Hieter, *Methods in Yeast Genetics: A Laboratory Course Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990)] to an absorbance at 600 nm between 0.6 and 1.6. Spheroplast lysates were prepared according to Fedor *et al.* [in (*26*)], and 300-μl samples of the supernatant containing minichromosomes were incubated at 37°C for 5 min before treatment with micrococcal nuclease for 10 min at 37°C at concentrations varying from 0 to 20 U/ml. For digestion of naked DNA, samples were first treated with proteinase K, extracted with phenol and chloroform, then taken up in 300 μl of 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 20 mM tris (pH 8.0) before digestion as above. Digestions were stopped by the addition of 55 μl of 5% SDS, proteinase K (5 mg/ml), and after >2 hours at 37°C, samples were extracted with phenol and chloroform, and the DNA was precipitated. The sedimented material was taken up in 100 μl of 10 mM tris acetate (pH 8.0), 0.05 mM EDTA, treated with RNase A (40 μg), and cleaned over 1-ml G50 spin columns (*29*). For analysis by indirect end-labeling [S. A. Nedospasov and G. P. Georgiev, *Biochem. Biophys. Res. Commun.* **92**, 532 (1980); C. Wu, *Nature* **286**, 854 (1980)], samples (25 to 40 μl) were digested with Eco RV for 3 to 4 hours, and the DNA was precipitated and then electrophoresed on 1.2% agarose gels at 4 V/cm for 5 to 6 hours. Bromophenol blue was omitted from sample buffers. DNA was transferred to nylon membranes (Duralon UV; Stratagene) by capillary action and hybridized with random-primer labeled probes (*29*). Hybridization and washing were as described [G. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984)]. Blots were probed with the 231-bp Eco RV–Hind III fragment from TRP1ARS1. Chromatin structure was mapped for at least two independent clones for all experiments described.
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15. A Pst I fragment encompassing the *lacZ* gene and *CYC1* TATA box from the plasmid pLG669-Z [L. Guarente and M. Ptashne, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2199 (1981)] was ligated into the Pst I site of pRS104 (*11*), at 823 map units of TRP1ARS1; the Kpn I–Hind III fragment of the resulting plasmid containing the *lacZ* gene and TRP1ARS1 sequences was then inserted into the polylinker of the CEN-containing shuttle vector pRS314 [R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)], creating pRS314-17Δ80, and introduced into yeast cells. The GAL4 binding site is 300 bp upstream of the transcription start site for *lacZ* in this plasmid. Cells were grown in the presence of 2% glucose or 2% galactose, and assayed for β-galactosidase activity as described [J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972); M. D. Rose, F. Winston, P. Hieter, *Methods In Yeast Genetics: A Laboratory Course Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990)]. Samples for chromatin mapping were prepared as described (*11*) and analyzed by the indirect end-label technique (*13*). Digestion was with Pvu II, and the blot shown in Fig. 2 was hybridized with the 252-bp Pvu II–Cla I fragment from pRS314.
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18. The vector for constitutive expression of GAL4 is pCL1, a derivative of YCp50 bearing a *LEU2* marker and transcribing the GAL4 coding sequence from the *ADH1* promoter [S. Fields and O. Song, *Nature* **340**, 145 (1989)].
19. The expression vector for GAL4(1–147)H is derived from pAS1, a 2-μm-based plasmid in which the *ADH1* promoter drives expression of a fusion of the first 147 amino acids of GAL4 with the hemagglutinin epitope. The *TRP1* gene present on pAS1 was replaced by the *LEU2* gene from pRS425 (*13*) before transformation of *leu*$^-$ yeast cells.
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18 May 1993; accepted 19 October 1993

Tethering Ribozymes to a Retroviral Packaging Signal for Destruction of Viral RNA

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Cellular compartmentalization of RNAs is thought to influence their susceptibility to ribozyme cleavage. As a test of this idea, two retroviral vectors—one encoding a hammerhead ribozyme designed to cleave *lacZ* transcripts and another encoding the *lacZ* messenger RNA—were coexpressed inside retroviral packaging cells. Because of the retroviral packaging signal, the ribozyme would be expected to colocalize with the *lacZ*-containing viral genomic RNA but not with the *lacZ* messenger RNA. The ribozyme was found to reduce the titer of infectious virus containing *lacZ* by 90 percent, but had no effect on translation of *lacZ* messenger RNA. These results indicate that sorting gene inhibitors to appropriate intracellular sites may increase their effectiveness.

The ability to target ribozymes to cleave viral RNAs in vitro has led to speculation about their potential therapeutic value as antiviral agents in vivo (*1*). To develop ribozymes for this purpose, however, one must consider the characteristics that distinguish these two settings. In test tubes, ribozymes and their substrates diffuse freely, and trans-cleavage can proceed as fast as RNA duplex formation (*2*). In cells, RNAs do not appear to diffuse freely but rather are sorted to specific cellular locations (*3*). Such compartmentalization of viral RNAs in vivo may reduce their availability to ribozymes. We propose a strategy that takes advantage of the cell's propensity to compartmentalize biological molecules. We show that delivery of a ribozyme to the same cellular location as its target can substantially increase the effectiveness of the ribozyme.

Our experimental system exploits properties of retroviral replication as well as retroviral vector–mediated gene transfer. We used two types of Moloney murine leukemia virus

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(MoMLV) vectors (Fig. 1A). The retroviral vector B2A contains the *lacZ* gene (*4*). The *lacZ*-encoding transcripts were targeted for cleavage by two hammerhead ribozymes (*5, 6*) and were thus used to report ribozyme-mediated inhibition. The retroviral vector N2A:Hamβ1G encodes the selectable marker neo^r (a gene for neomycin resistance) and a hammerhead ribozyme. The vector N2A:Hamβ2G is identical to N2A:Hamβ1G except for sequence changes in the flanking arms of the hammerhead that make it recognize a different region of the *lacZ* coding sequence (Fig. 1B) (*7*). The N2A:Hamβ vectors were used to transfer and express ribozyme-containing RNAs in an ecotropic packaging cell line (E86/B2A) containing the B2A retroviral vector (*4, 8*). An inactive variant of the hammerhead sequence, Hamβ1D (Fig. 1B), was inserted into N2A to serve as a control for the importance of ribozyme activity.

We confirmed the trans-cleavage activity of these ribozymes in vitro by using short RNA oligonucleotides corresponding to ribozyme and substrate sequences. This analysis also demonstrated that the Hamβ1D mutation eliminates hammerhead ribozyme activity (*9*).

In E86/B2A cells, nearly identical *lacZ*-encoding transcripts have two distinct fates (Fig. 2A). Some of the transcripts serve as mRNAs and are transported to the cytoplasm for translation. One can assess the abundance of these mRNAs by measuring the activity of the *lacZ* gene product β-galactosidase (β-gal) within the cells. The remaining transcripts serve as genomic RNAs for the replication of the retroviral vector and are sorted to sites of viral budding on the surface of the packaging cells. The abundance of these genomic RNAs can be assessed by determination of the β-gal viral titer released from the packaging cells.

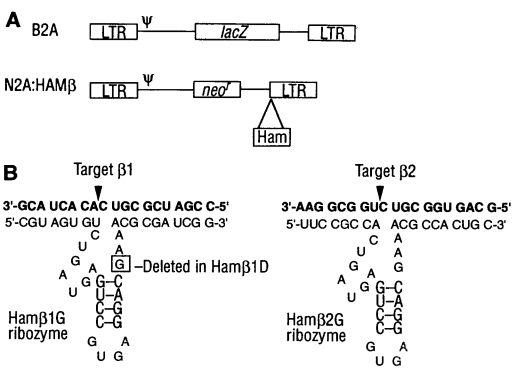
We hypothesized that the MoMLV encapsidation machinery would colocalize ribozyme-encoding genomic RNAs with B2A genomic RNAs (*10, 11*) (Fig. 2B). In contrast, the N2A:Hamβ and B2A mRNAs would probably be localized in different nuclear tracts because they would be transcribed from proviruses integrated at distant sites on the cellular chromosomes (*3, 11, 12*). Thus, if colocalization of ribozyme and substrate RNAs enhances trans-cleavage of the substrate, one would predict the reduction in titer of β-gal virus to be greater than the reduction in β-gal activity in these cells.

The ribozyme-containing templates were introduced into E86/B2A cells by retroviral vector–mediated gene transfer instead of transfection to avoid problems with variable *lacZ* expression associated with clonal isolation of E86/B2A cell lines (*13, 14*). We infected the E86/B2A cells at a multiplicity of infection (MOI) of 10 with the various ribozyme and control vectors, allowed the cells to expand, and then quantified the β-gal activity within the cells and the neo^r and β-gal virus released by the cells (Fig. 3A) (*15, 16*). No substantial reduction of β-gal activity was observed inside cells containing a functional hammerhead vector (N2A:Hamβ1G or N2A:Hamβ2G) as compared with cells containing a control vector (N2A or N2A:Hamβ1D). Similarly, no difference was seen in titers of neo^r virus released from the various vector-containing cells. However, the titers of β-gal virus released from cells containing an active hammerhead ribozyme were reduced by 89.5 ± 3.4% (average ± SD, n = 10, N2A:Hamβ1G and N2A:Hamβ2G in five experiments) as compared with that of cells containing the inactive ribozyme N2A:Hamβ1D (Fig. 3, A and B).

Internal controls provided evidence that the reduction in virus titer was due to the ribozyme and not to an unrelated cellular change. The fact that β-gal activity was comparable in E86/B2A cells containing a ribozyme or a control vector suggests that B2A transcription was unchanged in these cells. Also, the observation that the neo^r viral titers were similar for all cells indicates that the packaging machinery was operating correctly and had not become limiting.

To determine whether the ratio of ribozyme to substrate RNA affects the reduction in β-gal viral titer, we infected 10^4 E86/B2A cells at various MOIs. With N2A:Hamβ1G and N2A:Hamβ2G, the reduction in β-gal viral titer decreased as the MOI was decreased from 10 to 2 to 0.4. In contrast, no substantial change in β-gal viral titer occurred when control vectors were used to infect at these MOIs (Fig. 3C). In another experiment, we infected E86/B2A cells at MOIs of 10, 1.0, and 0.1 and again found that reduction in β-gal viral titer was directly related to the MOI (*14*).

To confirm that the decrease of β-gal viral titer from E86/B2A cells infected with N2A:Hamβ1G (MOI 10) was due to destruction of B2A RNA, we quantified the viral RNAs in the packaging cells, in the viral particles released from these cells and in NIH 3T3 cells infected with the viral supernatants. RNase protection analysis (Fig. 4) (*17*) demonstrated that B2A RNA concentrations were substantially reduced in both viral particles and infected cells only when the active hammerhead retrovirus was present (Fig. 4A). The ratio of B2A to N2A:Hamβ RNAs in packaging cells



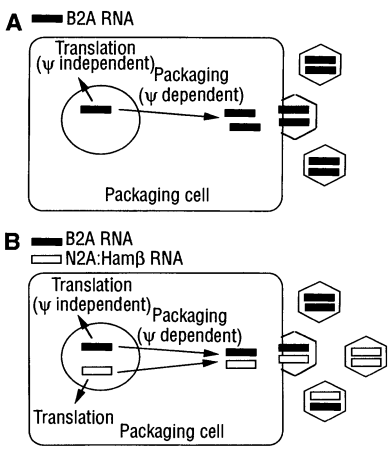


Fig. 1 (left). Structures of the retroviral vectors and ribozymes. (**A**) The B2A vector contains the *lacZ* gene under the control of the MoMLV long terminal repeat (LTR) promoter. The N2A:Hamβ vectors contain hammerhead ribozyme sequences in their 3′ LTRs, which are expressed as part of the full-length vector transcript. Both B2A and N2A:Hamβ contain the MoMLV packaging signal, ψ, which is also present in full length vector-derived transcripts. Oligonucleotides corresponding to the Hamβ1G, Hamβ2G, and Hamβ1D ribozymes were cloned into the Apa I and Bgl II restriction sites in the U3 region of the 3′ LTR of the retroviral vector N2A (*25*) to generate the N2A:Hamβ1G, N2A:Hamβ2G, and N2A:Hamβ1D vectors. (**B**) RNA sequences of the Hamβ1G and Hamβ2G hammerhead ribozymes are shown base-paired to their *lacZ* target sequences. A single nucleotide, the boxed G, was deleted from the catalytic core of Hamβ1G to create the inactive control ribozyme Hamβ1D. The target β1 and target β2 sequences reside in the middle of the *lacZ* coding sequence in the B2A-derived transcripts and are shown in bold (*7*). Substrate cleavage sites are indicated by arrows. **Fig. 2 (right).** Fates of transcribed retroviral vector RNAs inside packaging cells and proposed colocalization of N2A:Hamβ and B2A genomic RNAs in such cells. (**A**) Nearly identical B2A-derived RNAs serve as mRNAs and genomic RNAs in a packaging cell. The viral proteins gag, pol, and env are constitutively expressed inside packaging cells, which allows packaging of ψ-containing transcripts into viral particles (*8, 10*). (**B**) Coexpression of B2A and N2A:Hamβ RNAs in a packaging cell should result in both RNAs being used for translation and packaging. The B2A and N2A:Hamβ genomic RNAs should be colocalized by the encapsidation machinery, whereas the corresponding mRNAs are unlikely to be colocalized.

containing the active ribozyme (B/N_G) was 0.12 ± 0.03 (*18*), whereas in packaging cells containing the inactive ribozyme the corresponding ratio (B/N_D) was 0.10 ± 0.01. The similarity of these ratios is consistent with the observation that β-gal activity was similar in these cells. The low ratios of *B/N* indicate that there was a substantial excess of hammerhead-containing RNA over target RNA in the packaging cells. In RNAs isolated from viral particles, B/N_D was essentially unchanged (0.11 ± 0.02), whereas B/N_G was reduced to 0.02. The substrate escape ratio, calculated as $(B/N_G)/(B/N_D)$ and representing the residual fraction of substrate-containing transcripts in N2A:Hamβ1G samples relative to N2A:Hamβ1D samples, was 0.18 ± 0.03. In RNAs from infected NIH 3T3 cells, B/N_D was again 0.10 ± 0.01, B/N_G was 0.02, and the substrate escape ratio was 0.20 ± 0.02. The fact that the substrate escape ratio changes from 1.2 in packaging cells to ~0.19 in viral particles and infected cells (an 84% reduction) correlates well with the reduction of β-gal viral titer in these cells (Fig. 3, A and B). No cleavage products were detected by RNase protection analysis; one possible explanation is that cleavage occurred just prior to RNA encapsidation, and cellular RNases degraded the products.

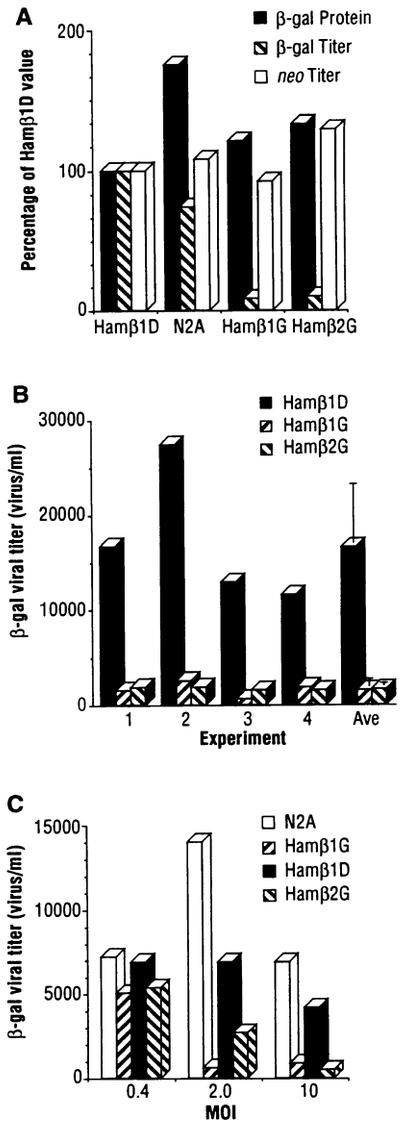


Fig. 3. Inhibition of β-gal viral titer from E86/B2A cells containing active ribozymes. (**A**) Amphotropic viral supernatants containing the N2A:Hamβ1G, N2A:Hamβ2G, N2A:Hamβ1D, or parental N2A retroviral vector (*13*) were used to infect E86/B2A cells (10^4) at an MOI of 10 in the presence of polybrene (hexamethrine bromide, 8 μg/ml) for 2 hours. The cells were expanded to 5 × 10^6 in DMEM with 10% fetal calf serum, and the media were changed. Twelve hours later, the internal β-gal activity and the titers of released β-gal and *neo*^{r} viruses were determined. The β-gal activities were determined by a standard ONPG (*O*-nitrophenyl-β-D-galactopyranoside) assay (*4*), and the results are plotted relative to the β-gal activity found in E86/B2A cells infected with N2A:Hamβ1D (*16*). We determined the β-gal and *neo*^{r} viral titers by infecting NIH 3T3 cells (10^5) with serial dilutions of the supernatants removed from the various pools of E86/B2A cells and assaying for B2A infection by a standard X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)–agarose staining procedure (*26*) at 36 hours after infection. Cells were assayed for N2A:Hamβ infection by selection with G418 (0.7 mg/ml). These values are also plotted relative to N2A:Hamβ1D values (*16*). (**B**) Four sets of E86/B2A cells were infected with N2A:Hamβ1D, N2A:Hamβ1G, and N2A:Hamβ2G in parallel as described above (A), and the titers of β-gal virus released from the cells were determined. The average titers from this experiment are shown with standard deviations. (**C**) The E86/B2A cells (10^4) were infected with the various N2A:Hamβ or N2A retroviral vectors at MOIs of 0.4, 2, and 10. Cells were expanded, and β-gal viral titers were determined as in (A).

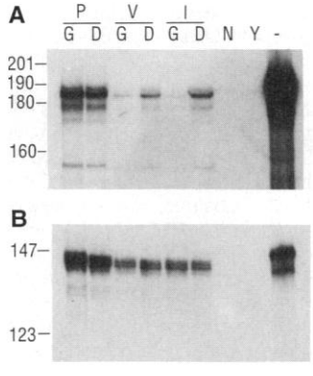


Fig. 4. Quantitation of B2A and N2A:Hamβ1 RNAs from packaging cells, virions, and infected cells. RNase protection analysis with (**A**) a 190-nt *lacZ*–specific RNA probe to quantitate B2A RNA or (**B**) a 150-nt *neo*^{r}-specific probe to quantitate N2A:Hamβ1 RNA (*17*). Probe RNA was protected from digestion by hybridization to the following RNA samples: P, RNA from E86/B2A packaging cells infected with N2A:Hamβ1G (G) or N2A:Hamβ1D (D); V, RNA from virions released from the same cells; and I, RNA from NIH 3T3 cells infected with B2A/N2A:Hamβ1 virus. Lane N contains uninfected NIH 3T3 cell RNA; lane Y contains yeast RNA; and lane (−) contains 10% of input RNA probe without RNase treatment. DNA fragments from a restriction digest of pBR322 with Msp I were ^{32}P end-labeled and denatured to produce molecular size markers (indicated on the left in nucleotides). Protected RNAs were analyzed on a 6% polyacrylamide gel with 8 M urea and exposed to x-ray film for 5 days (A) or 12 hours (B).

Our results support the hypothesis that colocalization of ribozymes with their substrates is essential for efficient cleavage (*19*). Because such colocalization could be rate limiting, it may be possible to increase the efficacy of ribozymes as antiviral agents by increasing the rate at which the ribozyme finds its substrate in vivo. For example, tethering a human immunodeficiency virus (HIV)–specific ribozyme to the HIV packaging signal or to the *rev* response element (*20*) may enhance the ribozyme's activity by colocalizing it with HIV transcripts inside cells. This strategy may also reduce the amount of nonspecific RNA cleavage by limiting the number of cellular RNAs exposed to the ribozyme.

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separately at 50°C for 10 min in reaction buffer [50 mM Hepes (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$], equilibrated to 37°C for 2 min, and then mixed at 37°C to start the cleavage reactions. Portions of the reaction mixtures were removed at various time points, added to a stop solution (10 mM EDTA), and the products separated on a 15% polyacrylamide gel in 8 M urea. With 500 nM ribozyme and 25 nM substrate, the half-time ($t_{1/2}$) for the reactions with either Hamβ1G or Hamβ2G was ~30 min. This $t_{1/2}$ value is approximately that expected for the conditions used (*5, 21*). No cleavage was observed with the Hamβ1D ribozyme.

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13. Plasmids encoding the N2A:Hamβ1G, N2A:Hamβ2G, N2A:Hamβ1D, and N2A retroviral vectors were introduced into the amphotropic packaging cell line AM12 (*22*) by electroporation with a Bio-Rad Gene Pulser. Stably transduced cells were selected with G418 (0.7 mg/ml), and the G418-resistant colonies were pooled. We determined the titer of *neo*^{r} virus in the supernatants from these cells by infecting NIH 3T3 cells with serial dilutions of these supernatants and then selecting with G418.
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15. The E86/B2A cells (10^4) infected with the various retroviral vectors were expanded in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum without G418 selection. In a separate experiment, selection with G418 further reduced the titer of β-gal virus released from active hammerhead-containing cells by a few percent, but had no effect on β-gal activity within the cells or on the titer of *neo*^{r} virus released.
16. The β-gal assay values obtained from infected E86/B2A cells were as follows: 160 U (1000 × A_{420}/hour) (N2A:Hamβ1D), 290 U (N2A), 205 U (N2A:Hamβ1G), and 220 U (N2A:Hamβ2G), where A_{420} is the absorbance at 420 nm. Untransduced E86 cells (*8*) were assayed simultaneously and were used to zero the A_{420} readings. The *neo*^{r} viral titers (per milliliter) were as follows: 6.5×10^4 (N2A:Hamβ1D), 7.0×10^4 (N2A), 6.0×10^4 (N2A:Hamβ1G), and 9.0×10^4 (N2A:Hamβ2G). The β-gal viral titers (per milliliter) were as follows: 8.1×10^3 (N2A:Hamβ1D), 6.0×10^3 (N2A), 6.5×10^2 (N2A:Hamβ1G), and 8.0×10^2 (N2A:Hamβ2G). The β-gal viral titer of the parental E86/B2A cell line was 9.0×10^3 per milliliter.
17. E86/B2A cells (10^4) were infected with N2A:Hamβ1G and N2A:Hamβ1D viruses at an MOI of 10 and expanded to 10^8. The cell medium was changed and was removed 24 hours later. Virion RNA was isolated as in (*23*). Total RNA was isolated from the cells by the guanidinium isothiocyanate method (*24*). Viral supernatants (1 ml) were used to infect NIH 3T3 cells (10^4) for 3 hours in the presence of polybrene (8 μg/ml). These cells were expanded to 10^8 and total RNA was isolated. RNase protection analysis was performed on 50 μg of packaging cell and infected cell RNAs and on 20% of the isolated virion RNAs with an RPA II ribonuclease protection assay kit (Ambion). Body-labeled RNA probes were transcribed by T7 RNA polymerase from templates generated by the polymerase chain reaction (PCR). The B2A and N2A:Hamβ1G plasmids were amplified with PCR primers specific for the *lacZ* and *neo*^{r} genes as described in the RPA II kit. The *lacZ* probe (190 nt) was complementary to 181 nt of RNA flanking the cleavage site for Hamβ1G on B2A transcripts. The *neo*^{r} probe (150 nt) was complementary to 140 nt of N2A:Hamβ1 RNA.
18. To determine the ratio of B2A and N2A:Hamβ1 RNAs, we added *lacZ* and *neo*^{r} RNA probes (6×10^4 cpm of each) to the samples and performed RNase protection analysis as above (*17*). Mixing the probes controlled for any differences in RNA loading. Products were quantified with an Image Acquisition and Analysis System (AMBIS, Inc., San Diego, CA) and with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). An internally controlled PCR approach confirmed the decrease in B2A retroviral RNA in infected NIH 3T3 cells; the substrate escape ratio was reproducibly between 0.09 and 0.14.
19. In this experimental system, we cannot directly test whether the presence of the packaging signal on the ribozyme-encoding transcripts is essential for inhibition of B2A titer, because it cannot be deleted from the N2A:Hamβ vectors if one is using retroviral vector–mediated gene transfer for introduction of N2A:Hamβ vectors into E86/B2A cells. Therefore, we cannot rule out the possibility that the packaging pathway is intrinsically more susceptible to inhibition than the mRNA pathway. For example, differences in ionic conditions or RNA-binding proteins associated with these two pathways may influence ribozyme cleavage activity or target accessibility.
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27. We thank E. Gilboa and C. Smith for E86/B2A cells; J. Doudna, C. Rusconi, and G. Fang for critical reading of the manuscript; A. Gooding, C. Grosshans, and M. Kissinger for technical support; T. McConnell, D. Herschlag, and R. Rempel for useful discussions; and the W. M. Keck Foundation. B.A.S. was a Damon Runyon–Walter Winchell Cancer Research Fund Postdoctoral Fellow (DRG 1146), and T.R.C. is an Investigator of the Howard Hughes Medical Institute and an American Cancer Society Professor.

4 June 1993; accepted 27 September 1993

Activation of Exocytosis by the Heterotrimeric G Protein G_{i3}

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Secretagogues of rat peritoneal mast cells, such as mastoparan and compound 48/80, induce mast cell exocytosis by activating directly the guanosine triphosphate–binding proteins that are required for exocytosis. The introduction of a synthetic peptide that corresponds to the carboxyl-terminal end sequence of $G\alpha_{i3}$ into the cells specifically blocked this secretion. Similar results were obtained when antibodies to this peptide were introduced. The $G\alpha_{i3}$ was located in both the Golgi and the plasma membrane, but only the latter source of $G\alpha_{i3}$ appeared to be essential for secretion. These results indicate that $G\alpha_{i3}$ functions to control regulated exocytosis in mast cells.

The nonhydrolyzable analog of guanosine triphosphate (GTP), guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), when introduced into patch-clamped (*1*) or streptolysin O (SLO)–permeabilized mast cells (*2, 3*), stimulates exocytosis independently of phospholipase C (PLC). This suggests that a GTP-binding protein, designated G_E, may act downstream of PLC in the control of regulated secretion (*4*). However, whereas both small GTP-binding proteins of the Ras (*5*) and Rab (*6*) families as well as heterotrimeric G proteins (*7*) have been implicated in exocytosis, the identity of G_E has remained obscure. Certain positively charged secretagogues of rat peritoneal mast cells, including mastoparan (*8*), substance P (*9*), compound 48/80 (*7*), neomycin (*10*), and a variety of kinins (*11*), induce exocytosis in a receptor-independent manner by interacting directly with heterotrimeric G proteins. Although they activate phosphoinositide metabolism, these secretagogues can also induce exocytosis independently of PLC, presumably by directly activating G_E (*10*). The finding that treatment with pertussis toxin (Ptx) inhibits exocytosis under these conditions indicated that G_E is a Ptx-sensitive heterotrimeric G protein (*10*). Therefore, we analyzed the Ptx-sensitive G proteins present in mast cells to identify the G protein that fulfills the functional role of G_E in these cells.

Mast cell proteins were adenosine diphosphate (ADP)–ribosylated by Ptx and separated by both two-dimensional gel electrophoresis and SDS–polyacrylamide gel electrophoresis (PAGE) in the presence of

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