Ribozyme-Mediated Repair of Sickle β-Globin mRNAs in Erythrocyte Precursors

Ning Lan, Richard P. Howrey, Seong-Wook Lee,*
Clayton A. Smith, Bruce A. Sullenger†

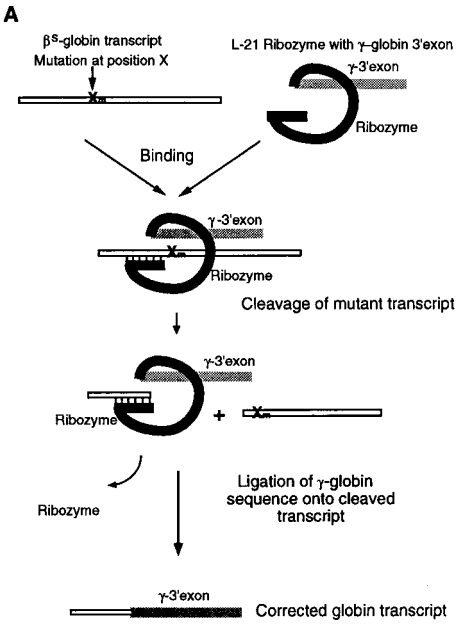
Sickle cell anemia is the most common heritable hematological disease, yet no curative treatment exists for this disorder. Moreover, the intricacies of globin gene expression have made the development of treatments for hemoglobinopathies based on gene therapy difficult. An alternative genetic approach to sickle cell therapy is based on RNA repair. A trans-splicing group I ribozyme was used to alter mutant β-globin transcripts in erythrocyte precursors derived from peripheral blood from individuals with sickle cell disease. Sickle β-globin transcripts were converted into messenger RNAs encoding the anti-sickling protein γ-globin. These results suggest that RNA repair may become a useful approach in the treatment of genetic disorders.

The genetic basis of sickle cell disease is an A to T transversion in the sixth codon of the β-globin gene (*1*). Individuals who are homozygous for this mutation accumulate long polymers of sickle hemoglobin (HbS) in their erythrocytes, which leads to a chronic hemolytic anemia and cumulative tissue damage (*2*). Even though the genetic basis of this disease has been well established, it has been difficult to develop gene therapy-based treatment for this hematological malady because globin gene expression is highly regulated and has been difficult to recapitulate after gene transfer (*3*). Here we describe an alternative method of genetic therapy based on RNA repair that should be able to restore the regulated expression of corrected globin RNAs in erythrocyte precursors (*4*).

The self-splicing group I intron from *Tetrahymena thermophila* (*5*) was previously shown to mediate trans-splicing of oligonucleotides in vitro (*6*). More recently, we demonstrated that a slightly shortened version of this ribozyme, called L-21 (*7*), could repair truncated *lacZ* transcripts in *Escherichia coli* (*8*) and in the cytoplasm of mammalian cells (*9*). To determine if trans-splicing could be used to repair mutant transcripts associated with a common genetic disorder, we tested whether such splicing ribozymes could amend mutant β-globin transcripts in erythroid lineage cells derived from the peripheral blood of patients with sickle cell disease (Fig. 1). More specifically, we wanted to determine whether splicing ribozymes could convert sickle β-globin (β^S-globin) transcripts into RNAs encoding γ-globin because fetal hemoglobin, which contains γ-globin, has been shown to greatly impede polymerization of HbS (*10*). In this splicing reaction, the ribozyme must recognize the sickle β-globin transcript by base pairing to an accessible region of the RNA upstream of the mutant nucleotide via an internal guide sequence (IGS), cleave the β^S-globin RNA, release the mutation containing cleavage product, and splice on the revised sequence for the globin transcript (Fig. 1A).

We generated erythrocyte precursors from normal umbilical cord blood (UCB) and from peripheral blood from patients with sickle cell disease by culturing the blood cells in medium without serum that was supplemented with erythropoietin, Flt-3 ligand, and interleukin-3 (IL-3) (*11*). Nucleated red blood cells (RBCs) appear by day 7 under these culture conditions and by 3 weeks they constitute 70 to 90% of the total number of cells in the culture, as evidenced by Wright–Giemsa and immunofluorescent staining (Fig. 1B). Moreover, because most of these erythroid lineage cells are late RBC precursors (Fig. 1B), they are rich in globin transcripts (*12*).

To ascertain which regions of the β-globin transcript are accessible to ribozymes, we developed an RNA mapping strategy that is based on a trans-splicing ribozyme library and RNA tagging (*9*). To generate the mapping library, we randomized the guide sequence of the *Tetrahymena* group I trans-splicing ribozyme so that the 5′ end of the RNAs in the library began with 5′-GNNNNN-3′, where G represents guanine and N represents equal amounts of the four nucleotides (nt) A, G, C, or U (*13*). To map the β-globin transcript in vitro, we incubated the mapping library with total RNA isolated from erythrocyte precursors under splicing conditions (*14*). To identify accessible uridine residues, the trans-splicing reaction products were reverse transcribed (RT) and amplified by the polymerase chain reaction (PCR) with primers specific for the ribozyme's 3′ exon tag (*9*) and for the β-globin target RNA. The uridine at position 61 of β-globin RNA appeared to be particularly accessible because five of nine clones sequenced contained splice junctions at this nucleotide (Fig. 2A). To determine which nucleotides were accessible on β-globin transcripts inside cells, we transfected the mapping library into erythrocyte precursors (*15*). We isolated total



B

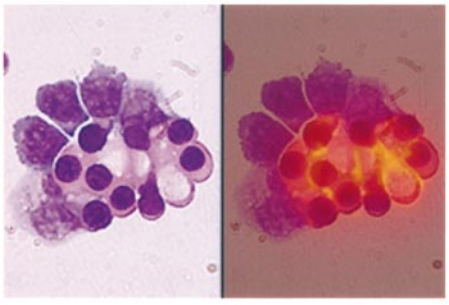


Fig. 1. Ribozyme-mediated repair of sickle β-globin transcripts in human erythrocyte precursors. (**A**) Scheme for the conversion of β^S-globin mRNAs into transcripts encoding γ-globin. X_m, sickle β-globin point mutation; γ-3′ exon, restorative globin sequence. (**B**) Erythrocyte precursors generated from the peripheral blood of sickle cell patients. Cells stained with Wright-Giemsa stain alone (left) and with a fluorescently labeled antibody (E6) specific for the erythroid lineage cell surface marker, band 3 (right) (*25*).

N. Lan, S.-W. Lee, B. A. Sullenger, Center for Genetic and Cellular Therapies, Departments of Surgery and Genetics, Duke University Medical Center, Durham, NC 27710, USA.
R. P. Howery, Center for Genetic and Cellular Therapies, Department of Pediatrics, Duke University Medical Center, Durham, NC 27710, USA.
C. A. Smith, Center for Genetic and Cellular Therapies, Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA.

*Current Address: Department of Molecular Biology, Dankook University, Seoul, Korea.
†To whom correspondence should be addressed: E-mail: sulle001@mc.duke.edu

RNA from these cells and identified reactive uridines by RT-PCR amplification and sequence analysis. In five of the nine clones examined, the 3′ exon tag had been spliced onto the uridine at position 61 (Fig. 2A). These mapping results, taken together with the fact that sickle β-globin transcripts contain a point mutation at position 70 (*1*), encouraged us to focus on developing ribozymes that recognize the uridine present at position 61 on the β-globin mRNA. Thus, the IGS on the L-21 trans-splicing ribozyme was changed to 5′-GGGUGC-3′ to generate a ribozyme, called Rib61, specific for site 61. In addition, an inactive version of this ribozyme, called Rib61d, which lacks part of the catalytic core of the enzyme, was generated as a control (*8*).

Rib61 can trans-splice a 3′ exon tag onto β-globin transcripts in vitro. We incubated the trans-splicing ribozymes Rib61-3′tag and Rib61d-3′tag under splicing conditions with β^S-globin RNA generated by in vitro transcription or total RNA isolated from erythrocyte precursors. To determine whether trans-splicing had occurred in any of the RNA samples, we performed RT-PCR analyses with one primer specific for the β-globin target RNA and the other primer specific for the 3′ exon tag sequence (Fig. 2B) (*16*). An amplified fragment of the expected size [93 base pairs (bp)] was generated from samples containing Rib61-3′tag and either in vitro transcribed β^S-globin RNA or total RNA isolated from sickle cell patient and UCB-derived RBC precursors. No such RT-PCR product was generated from samples that lacked a ribozyme or that contained the inactive version of the ribozyme. Sequence analysis of the spliced products demonstrated that the ribozyme had reacted with the intended uridine in each of five junctions analyzed (*17*). To evaluate splicing efficiency, we changed the 3′ exon attached to Rib61 to allow for coamplification of trans-spliced products and unreacted β^S-globin substrate RNAs by quantitative-competitive RT-PCR (*18*, *19*). This ribozyme, called Rib61-3′eff, converted 8% of the globin RNA to product (Fig. 2C).

To determine whether Rib61-3′tag could react with β-globin transcripts inside primary human cells, we transfected the ribozyme into erythrocyte precursors derived from UCB and sickle cell patients (*15*). RT-PCR amplification generated a fragment of the expected size (93 bp) from the total RNA isolated from a sickle cell patient and UCB-derived RBC precursors that had been transfected with the active ribozyme (Fig. 3). By contrast no such product was generated from RNA samples isolated from cells that were not transfected or that were transfected with the inactive ribozyme. When Rib61-3′tag was added to the RNA extraction buffer used to isolate total RNA from a sample of mock-transfected erythrocyte precursors, no amplification product was generated (Fig. 3), which suggests that the observed trans-splicing products were generated inside the RBC

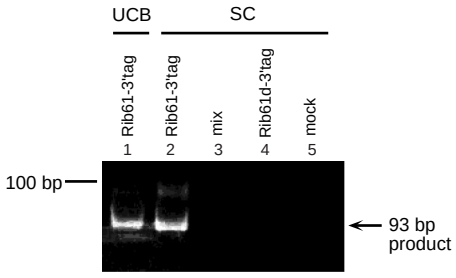


Fig. 3. Trans-splicing a 3′ exon onto β-globin transcripts in RBC precursors. Erythrocyte precursors derived from normal UCB or peripheral blood of sickle cell patients (SC) were mock-transfected (mock and mix) or transfected with the active (Rib61-3′tag) or inactive (Rib61d-3′tag) ribozymes (*15*). RNA was harvested from these cells and trans-spliced products were analyzed as described (Fig. 2B). In the "mix" sample, Rib61-3′tag was added to the RNA extraction buffer before RNA isolation. The migration of a 100-bp DNA molecular mass marker is indicated.

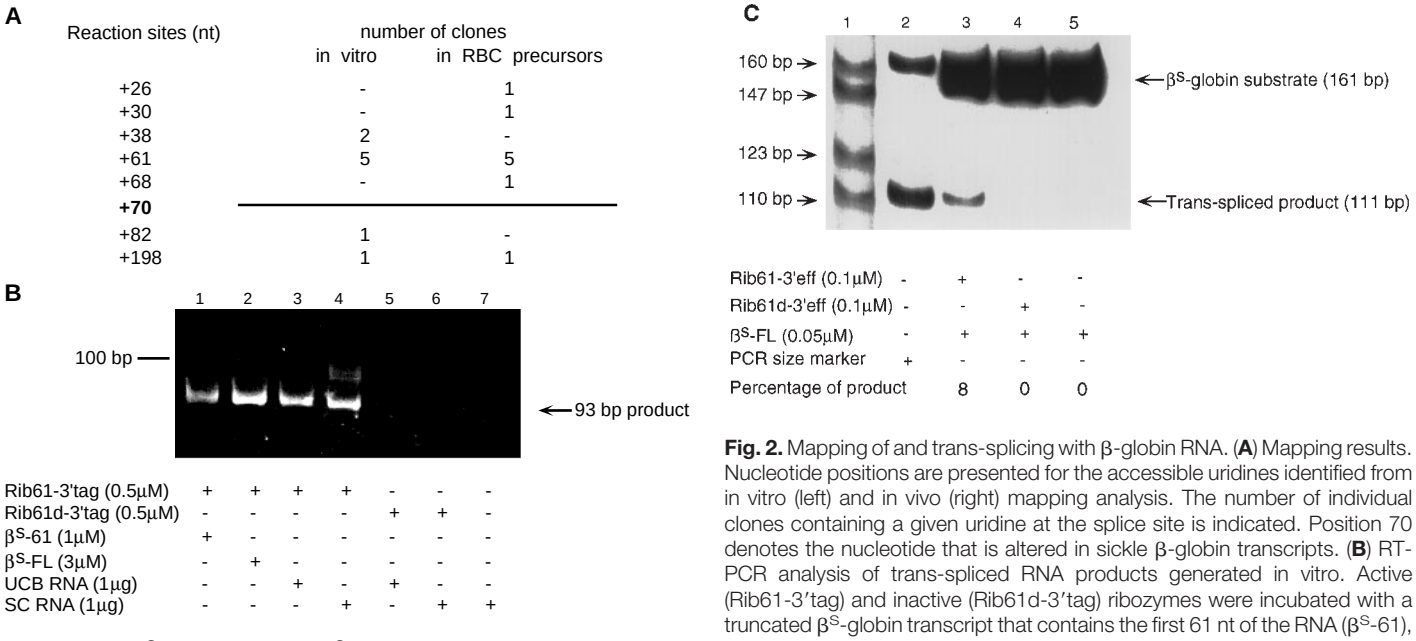


Fig. 2. Mapping of and trans-splicing with β-globin RNA. (**A**) Mapping results. Nucleotide positions are presented for the accessible uridines identified from in vitro (left) and in vivo (right) mapping analysis. The number of individual clones containing a given uridine at the splice site is indicated. Position 70 denotes the nucleotide that is altered in sickle β-globin transcripts. (**B**) RT-PCR analysis of trans-spliced RNA products generated in vitro. Active (Rib61-3′tag) and inactive (Rib61d-3′tag) ribozymes were incubated with a truncated β^S-globin transcript that contains the first 61 nt of the RNA (β^S-61), the full-length β^S-globin transcript (β^S-FL), or total RNA isolated from erythrocyte precursors derived from normal UCB (UCB RNA) or from peripheral blood of sickle cell patients (SC RNA). Amplification of trans-splicing reaction products is expected to yield a 93-bp product (*16*). The migration of a 100-bp DNA molecular mass marker analyzed in parallel is indicated. (**C**) Rib61 can convert a significant fraction of sickle β-globin RNA to product. Full-length sickle β-globin RNA (50 nM) was incubated with the active ribozyme (Rib61-3′eff, 100 nM; lane 3), with the inactive version of the ribozyme (Rib61d-3′eff, 100nM; lane 4), or without ribozyme (lane 5) for 180 min at 37°C. Reaction products were amplified by quantitative competitive RT-PCR analysis in the presence of radiolabeled deoxynucleotide triphosphates and products were separated on an 8% acrylamide gel. The abundance of the unreacted β-globin substrate RNA and trans-spliced products was determined by Phosphorimager analysis. A DNA fragment containing an artificially generated trans-spliced product and unreacted substrate was amplified to provide 111- and 161-bp markers (lane 2). Molecular mass markers were provided by digesting pBR322 DNA with Msp I (lane 1).

precursors and not during RNA analysis.

To create a trans-splicing ribozyme that could convert β^S-globin transcripts into RNAs encoding γ-globin, we changed the 3′ exon on Rib61-3′tag to contain the human γ-globin cDNA sequence (nt 29 to 545) (*20*). This trans-splicing ribozyme, called Rib61-3′γ, can quickly and accurately trans-splice its 3′ γ-globin exon onto β^S-globin RNAs in vitro. Radioactively labeled Rib61-3′γ was allowed to react with an excess of unlabeled full-length (β^S-FL) or truncated (β^S-61) β^S-globin substrate RNA (Fig. 4A). In both reactions, Rib61-3′γ was quickly converted to free ribozyme (Rib) plus ligated globin exons (β^S-61-3′γ) with an approximate half-time ($t_{1/2}$) of 60 min. Rib61-3′γ reacted even faster ($t_{1/2} \sim$ 25 min) with a short 13-nt substrate (5′SA_5). The ligated globin exons (β^S-61-3′γ) did not accumulate as well as the shorter 5′S-3′γ product in these reactions, however, because β^S-FL and β^S-61 did not suppress the cleavage activity of the liberated ribozyme as effectively as 5′SA_5 (*17*, *21*). The inactive version of the ribozyme (Rib61d-3′γ) was unable to mediate this splicing reaction (*17*).

When Rib61-3′γ was transfected into erythrocyte precursors derived from sickle cell patients and UCB (*15*), trans-spliced products were detected by RT-PCR amplification of a fragment of the expected size (62 bp) (Fig. 4B). No such product was generated from RNA samples isolated from cells that were not transfected or that were transfected with the inactive ribozyme. Moreover, no amplification product was generated from RNA samples in which Rib61-3′γ was added to the RNA extraction buffer before the mock-transfected cells were lysed (Fig. 4B).

Sequence analysis of eight different subclones derived from sickle cell patient samples demonstrated that in each case the ribozyme had correctly spliced its γ-globin 3′ exon onto nt 61 of the β-globin target transcript and in the process maintained the open reading frame for translation of the mRNA (Fig. 4C). Thus, trans-splicing ribozymes are able to correct mutant globin transcripts in primary human RBC precursors with high fidelity.

RNA repair may be a particularly appropriate genetic approach with which to treat sickle cell disease because the process should restore the regulated expression of anti-sickling versions of β-globin and simultaneously reduce the production of β^S-globin. Moreover, the efficiency of β-globin RNA repair probably does not have to be 100% to benefit patients. Sickle cell trait is a benign condition that is not associated with increased morbidity or mortality (*22*) and sickle cell patients that express γ-globin at 10 to 20% the level of β^S-globin in most of their RBCs have greatly improved clinical prognoses (*23*). Whether this level of β-globin conversion can be achieved is currently unclear. However, previously we demonstrated that a trans-splicing ribozyme could revise up to 50% of a *lacZ* substrate RNA in mammalian cells (*18*) and Rib61 could react with almost 10% of β^S-globin RNAs in vitro (Fig. 2C). If enhanced repair efficiency in vivo is required, ribozymes that recognize other accessible sites (Fig. 2A) in the β^S-globin RNA could be evaluated individually or in combination with Rib61, and strategies that colocalize ribozymes with β^S-globin RNAs inside cells could be evaluated (*24*). Alternatively, mutant ribozymes with enhanced activities could be developed by in vitro selection experiments and tested in vivo. With development of efficient ribozymes and gene transfer systems, RNA repair may prove to be a useful approach to treat sickle cell disease and other inherited disorders.

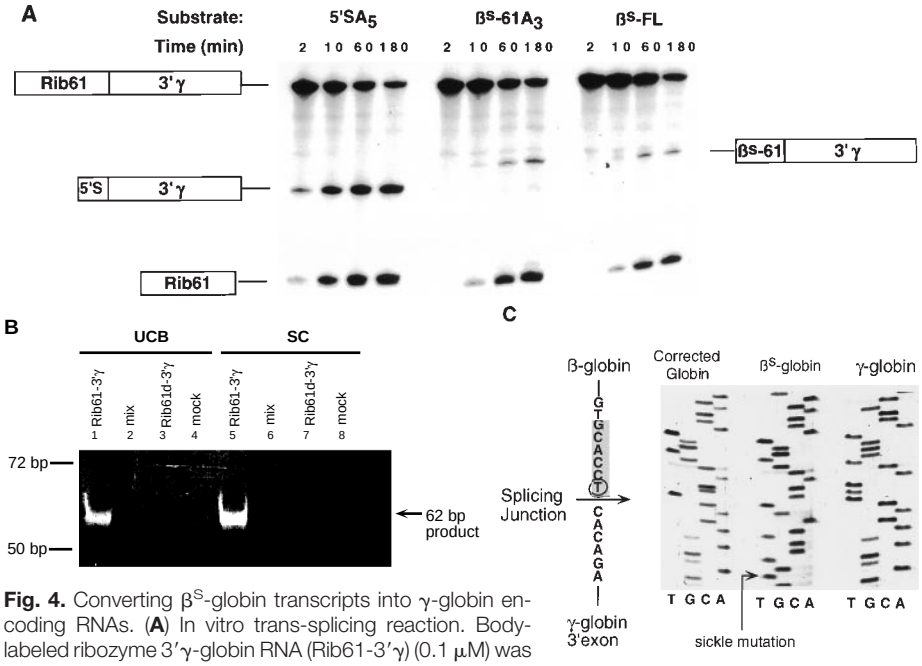


Fig. 4. Converting β^S-globin transcripts into γ-globin encoding RNAs. (**A**) In vitro trans-splicing reaction. Body-labeled ribozyme 3′γ-globin RNA (Rib61-3′γ) (0.1 μM) was incubated under splicing conditions with an excess of full-length β^S-globin RNA (β^S-FL) (2.5 μM), truncated β^S-globin RNA (β^S-$61A_3$: 61 nt of β^S-globin RNA plus 3 adenosine residues) (2.5 μM) and a 13-nt substrate (5′SA_5:GGGCACCUAAA) (2.5 μM) for the indicated times (*16*). Trans-spliced products (5′S-3′γ and β^S-61-3′γ) and free ribozyme (Rib61) are indicated. (**B**) RT-PCR analysis of amended RNAs generated in erythrocyte precursors from normal UCB or peripheral blood of sickle cell patients (SC). Cells were mock-transfected (mock and mix) or transfected with the active (Rib61-3′γ) or inactive (Rib61d-3′γ) ribozymes. Amended RNAs were amplified by RT-PCR and yield a DNA fragment of 62 bp (*16*). In the mix samples, Rib61-3′γ was added to the RNA extraction buffer before RNA isolation. The migration of size markers of 72 and 50 bp is indicated. (**C**) Sequence of amended β^S-globin transcripts. The expected sequence for a corrected transcript around the splicing junction is shown, with the complement to the IGS shaded and the uridine at position 61 circled. β^S-globin and γ-globin sequences are provided for comparison and the mutant nucleotide in the sickle β-globin transcript is indicated. Translation of the amended transcript would yield γ-globin with three amino acids derived from β-globin.

REFERENCES AND NOTES

1. V. M. Ingram, *Nature* **180**, 326 (1957).
2. O. S. Platt and G. J. Dover, in *Hematology of Infancy and Childhood*, D. G. Nathan and F. A. Oski, Eds. (Saunders, Philadelphia, 1993), pp. 732–782.
3. S. H. Orkin, *Cell* **63**, 665 (1990); C. Andrin and C. Spencer, *Biochem. Cell Biol.* **72**, 377 (1994); M. Sadelain *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6728 (1995).
4. B. A. Sullenger, *Chem. Biol.* **2**, 249 (1995); T. R. Cech, *Biotechnology* **13**, 323 (1995).
5. T. R. Cech, *Annu. Rev. Biochem.* **59**, 543 (1990).
6. T. Inoue, F. Sullivan, T. R. Cech, *J. Mol. Biol.* **189**, 143 (1986); M. Been and T. R. Cech, *Cell* **47**, 207 (1986).
7. A. J. Zaug, C. A. Grosshans, T. R. Cech, *Biochemistry* **27**, 8924 (1988).
8. B. A. Sullenger and T. R. Cech, *Nature* **371**, 619 (1994).
9. J. T. Jones, S.-W. Lee, B. A. Sullenger, *Nature Med.* **2**, 643 (1996).
10. H. R. Sunshine, J. Hofrichter, W. A. Eaton, *Nature* **275**, 238 (1978); M. J. Behe and S. W. Englander, *J. Mol. Biol.* **133**, 137 (1979).
11. UCB samples were obtained from labor and delivery samples, and peripheral blood samples were obtained from sickle cell patients with hemoglobin SC disease who were undergoing scheduled phlebotomy, in accordance with IRB policy on discarded materials. Mononuclear cells were isolated by Ficoll/Hypaque gradient separation and resuspended at 1×10^6 cells per milliliter in BIT 9500 serum-free medium (Stem Cell Technology) supplemented with Flt-3 ligand (25 ng/ml) (Immunex), IL-3 (2.5 ng/ml) (R&D), and erythropoietin (1 unit/ml) (R&D). These

cells were then cultured at 37°C overnight and transferred to fresh plates to eliminate adherent cells.
12. J. A. Grasso, N. C. Chromey, C. F. Moxey, *J. Cell Biol.* **73**, 206 (1977); F. Ramirez *et al*., *J. Biol. Chem.* **250**, 6054 (1975).
13. T. B. Cambell and T. R. Cech, *RNA* **1**, 598 (1995).
14. The mapping library was generated by PCR amplification of plasmid pT7L-21 (*7*) with a 5′ primer containing a randomized sequence at positions corresponding to the ribozyme's IGS (5′-GGGGGGATCCTAATACGACTCACTATAGNNNNNAAAAGTTATCAGGCATGCACC) and a 3′ primer specific for 3′ exon tag sequences present in the pT7L-21 plasmid (5′-AGTAGTCTTACTGCAGGGGCCTCTTCGCTATTACG). The resulting cDNA library was in vitro transcribed using T7 RNA polymerase to generate the RNA mapping library.
15. RBC precursors (1×10^6 cells) were resuspended in 200 μl of Opti-MEM (Gibco- BRL), and ribozymes (2.5 to 5 μg generated by in vitro transcription) were transfected into these cells using 20 μl of DMRIE-C (Gibco-BRL) in 1 ml of Opti-MEM for 4 hours. Then, DMEM (Gibco-BRL) with 10% fetal calf serum (1 ml) and erythropoietin (2 units/ml) were added to the cells. Total RNA was isolated by using TRI Reagent (Molecular Research Center) containing EDTA (10 mM) 16 to 24 hours after transfection. Transfection of these cells with a reporter RNA demonstrated that 1 to 2% of the erythrocyte precursors take up RNA (*17*).
16. Ribozyme 3′ exon (100 to 500 nM) and substrate RNAs (1 to 5 μM or 1 μg of cellular RNA) were denatured at 95°C for 1 min in reaction buffer [50 mM Hepes (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$] and then equilibrated at 37°C for 3 min. The substrates were then added to the ribozymes along with guanosine (100 μM) to start the reactions, which proceeded at 37°C for 3 hours. For reaction mixtures that contained radiolabeled ribozyme, we removed samples at the times indicated and added them to an equal volume of EDTA (10 mM) to stop the reaction. Reaction products were analyzed on a 4% polyacrylamide gel containing 8 M urea. For RT-PCR analysis, trans-splicing products were reverse transcribed at 37°C for 20 min in the presence of L-argininamide (10 mM) from a primer specific for the 3′ exon sequence as described (*8, 9*). The resulting cDNAs were amplified for 30 cycles (*in vitro* ribozyme reactions) or 30 to 90 cycles (*in vivo* ribozyme reactions) using a 3′ exon primer (3′tag primer, 5′-ATGCCTGCAGGTCGACTC; 3′γ-globin primer, 5′-CCGGAATTCCCTTGTCCTCCTCTGTGA) (*9*, *20*) and a 5′ primer specific for the β-globin mRNA (5′-GGGGATCCCTGTGTTCACTAGCAACC). The amplified products were separated on a 10% acrylamide gel and visualized by ethidium bromide staining.
17. N. Lan, unpublished results.
18. J. T. Jones and B. A. Sullenger, *Nature Biotech.* **15**, 902 (1997).
19. Quantitative-competitive RT-PCR analysis of trans-splicing reaction efficiency was performed by incubating β^S-globin RNA with Rib61- 3′eff. The 3′ exon attached to Rib61-3′eff contains a priming sequence for a downstream RT-PCR primer called down-1 that is also found on the unreacted β^S-globin transcript. After the distance between the down-1 site and an upstream priming site on β^S-globin is spliced, RNA is reduced from 161 to 111 bp. Unreacted globin substrate RNAs as well as revised β-globin products were coamplified by using a single set of PCR primers to yield different-sized products that were separated on an acrylamide gel, and PhosphorImager analysis was used to quantify the efficiency with which the ribozyme had converted the globin RNA to product in the reaction (*18*).
20. C. Cavallesco *et al*., *Gene* **12**, 215 (1980).
21. In the reactions with the two longer β-globin substrates (β^S-$61A_3$ and β^S-FL) more free ribozyme is generated than spliced exons. The spliced products have accumulated to about 25% of the level of the free ribozyme at 60 and 180 min. Moreover, 15% and 28% of the 3′ exon originally attached to the ribozyme is present in the trans-spliced product band at 60 and 180 min, respectively. This reduced accumulation does not appear to result from 3′ exon hydrolysis, however, because no free 3′ exon is detected (Fig. 4A). Rather, this reduction results from cleavage of the trans-spliced products at other sites in these long RNAs by the free ribozyme generated in the reaction. These shorter cleavage products run off the bottom of the gel (*17*). We did not observe such miscleavage in the case of the short 13-nt substrate $5'SA_5$, however, because the activity of the free ribozymes appears to be suppressed by the excess of unreacted substrate present in the sample (*17*). The observation that the longer substrates do not appear to suppress this miscleavage as well as their shorter counterpart suggests that not all the longer RNAs are folded into a conformation that allows for ribozyme binding. In the long run, ribozymes with increased substrate specificity will be developed to solve this miscleavage problem.
22. M. T. Ashcroft and P. Desai, *Lancet* **2**, 784 (1976); D. A. Sears, *Am. J. Med.* **64**, 1021 (1978); M. S. Kramer, Y. Rooks, H. A. Pearson, *N. Engl. J. Med.* **299**, 686 (1978).
23. D. R. Powars *et al*., *Blood* **63**, 921 (1984); D. L. Rucknagel *et al*., in *Developmental Control of Globin Gene Expression*, G. Stamatoyannopoulos and A. W. Nienhuis, Eds. (Liss, New York, 1987), pp. 487–496.
24. B. A. Sullenger and T. R. Cech, *Science* **262**, 1566 (1993); E. Bertrand *et al*., *RNA* **3**, 75 (1997).
25. M. J. Telen, R. M. Scearce, B. F. Haynes, *Vox Sang.* **52**, 236 (1987).
26. We thank C. Rusconi, P. Zarrinkar, L. Milich, J. Jones, K. Phillips, M. Barman, R. Kaufman, M. Telen, E. Gilboa, H. Lyerly, and R. Rempel for useful discussions, M. Telen for her generous gift of the E6 antibody, and the nurses in labor and delivery and the medical oncology treatment center for their assistance in collecting blood samples. Supported in part by the Korean Academic Research Fund of the Ministry of Education (S.-W.L.) and by a grant (HL57606) from the National Heart Lung and Blood Institute (B.A.S.).

8 December 1997; accepted 9 April 1998

The Tetrameric Structure of a Glutamate Receptor Channel

Christian Rosenmund, Yael Stern-Bach, Charles F. Stevens*

The subunit stoichiometry of several ligand-gated ion channel receptors is still unknown. A counting method was developed to determine the number of subunits in one family of brain glutamate receptors. Successful application of this method in an HEK cell line provides evidence that ionotropic glutamate receptors share a tetrameric structure with the voltage-gated potassium channels. The average conductance of these channels depends on how many subunits are occupied by an agonist.

Voltage-gated potassium channels are tetramers, and nicotinic acetylcholine receptors are pentamers (*1*). Brain glutamate receptors are often assumed to be pentamers (*2*) because they, like the acetylcholine receptor, are ligand-gated. We developed a method to count the number of subunits in the brain glutamate receptor. The key observation upon which our counting method is based is that the mean single-channel current depends on how many of a receptor's binding sites have an agonist bound. The number of binding sites can then be counted by observing the distinct electrophysiological states that a receptor passes through as successively more binding sites become occupied. Successful application of this counting method requires solving three problems. First, because our method counts binding sites, the number of binding sites must equal the number of subunits; in addition, the binding sites must be equivalent so that sites are not missed. We therefore used the α-amino-3-hydroxy-5-methyl-4-isoxazol propionate (AMPA)–receptor $GluR3_{flip}$ (and mutant versions) expressed in a mammalian (HEK) cell line (*3*), because this receptor forms homomultimers.

Second, at the saturating concentrations of agonist that are needed to ensure full binding-site occupancy, the lifetime of each occupancy state is too brief to resolve. To prolong the lifetime of each state, we slowed down the agonist binding rate by interposing a very slow step, the dissociation of a high-affinity competitive antagonist; thus, we started the receptor with all of its binding sites occupied by a competitive antagonist and then made the sites available for agonist binding, one by one, as the bound antagonist molecules slowly dissociated. We used a rapid superfusion system to change an outside-out patch's environment from a saturating concentration of the high-affinity antagonist 6-nitro-7-sulphamoyl-benzo(F)quinoxalinedion (NBQX) to a saturating concentration of agonist (*4*).

Finally, if the receptor's normal desensitization mechanisms were intact, the receptor would desensitize long before this progression through the various occupancy states is complete (*5*). We therefore used single channels of a GluR6/GluR3 chimera

C. Rosenmund, Workgroup Cellular Neurobiology, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany.
Y. Stern-Bach, The Hebrew University–Hadassah Dental School, Department of Anatomy and Embryology, Post Office Box 12272, Jerusalem, Israel.
C. F. Stevens, Howard Hughes Medical Institute, The Salk Institute, La Jolla, CA 92037, USA.

*To whom correspondence should be addressed. E-mail: cfs@salk.edu