ioplan microscope was modified by removing the illumination optics to allow entry of the laser. The typical laser power used to form the trap (exiting the microscope objective) was 50 mW.
16. The fluorescence was detected with an image-intensified charge-coupled device camera (PAULTEK), the signal was recorded on an SVHS VCR, and an Argus Image Processor (Hamamatsu) and a frame grabber (National Instruments PCI 1402) were used to perform background subtraction. The lengths of the DNA molecules, measured between the center of the 1-μm bead and the free end of the molecule, were obtained from frame-grabbed images. The length of stained DNA (48.5 kb) extended by flow was measured to be 19.2 ± 0.82 μm at a flow rate of 72 μm/s in 50% sucrose. Under these conditions, DNA is 93% extended (*24*).
17. The salmon protamine used in these experiments contains 21 positively charged arginines distributed throughout the length of the protein (32 amino acids).
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19. Stokes' law, $f = 6\pi\eta rv$, where η is the buffer viscosity (15.4 cp), r is the sphere radius, and v is the buffer velocity (50 μm/s), was used to calculate the frictional force on the fully formed toroid. The toroid used to calculate the frictional force had a 90-nm outer diameter and a 30-nm inner diameter and was 20 nm thick (*1*).
20. Because we cannot confirm that every protamine has dissociated from the decondensed DNA molecule, these experiments provide only a maximum value for the off-rate.
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25. The flow cell contains two channels, each pumped at the same speed with a single syringe pump. The depth of the flow cell was 40 μm and the molecule was typically held 20 μm beneath the coverslip. Flow velocities were maintained at ~50 μm/s. Using a computer-controlled stage with 0.1-μm resolution to manipulate the position of the flow cell relative to the optical trap, we moved the DNA molecule to the protein side of the flow cell to initiate condensation.
26. Work was performed at Lawrence Livermore National Laboratory (LLNL) under the auspices of the U.S. Department of Energy under contract W-7405-ENG-48. Funding was provided by a LLNL Labwide Laboratory Directed Research Development Award. We thank J. Holzrichter, M. Colvin, and M. Cosman for their suggestions, support, and encouragement; J. W. Cosman and C. Barry for help during the early stages of the study; and J. T. Cosman for generating the computer graphics image of DNA.

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Imidazole Rescue of a Cytosine Mutation in a Self-Cleaving Ribozyme

Anne T. Perrotta, I-hung Shih, Michael D. Been*

Ribozymes use a number of the same catalytic strategies as protein enzymes. However, general base catalysis by a ribozyme has not been demonstrated. In the hepatitis delta virus antigenomic ribozyme, imidazole buffer rescued activity of a mutant with a cytosine-76 (C76) to uracil substitution. In addition, a C76 to adenine substitution reduced the apparent pK_a (where K_a is the acid constant) of the self-cleavage reaction by an amount consistent with differences in the pK_a values of these two side chains. These results suggest that, in the wild-type ribozyme, C76 acts as a general base. This finding has implications for potential catalytic functions of conserved cytosines and adenines in other ribozymes and in ribonuclear proteins with enzymatic activity.

Transphosphoesterification reactions catalyzed by self-cleaving and self-splicing RNAs (ribozymes) require loss of a proton from the participating 2′- or 3′-hydroxyl group to promote its nucleophilic attack on the cleavage-site or splice-site phosphate (*1*, *2*). Metal ions can assist in this reaction, and metal-ion catalysis is one of several strategies that ribozymes share with protein enzymes (*1*, *2*). Enhanced nucleophilicity of the hydroxyl group could also result from base-catalyzed deprotonation (*1*–*3*). The pK_a values of the nucleoside side chains (pK_a ~ 3.5 to 4.5), however, appear to be too low to provide efficient general acid-base catalysis at physiologic pH (*4*). Although pK_a values can be shifted closer to neutrality in particular RNA structures (*2*, *5*), it has not been demonstrated that an RNA side chain can act as a general base in catalysis (*1*, *2*).

Department of Biochemistry, Box 3711, Duke University Medical Center, Durham, NC 27710 USA.

*To whom correspondence should be addressed. E-mail: been@biochem.duke.edu

The two hepatitis delta virus (HDV) ribozymes are structurally related self-cleaving RNAs (*6*, *7*) that require a 2′-hydroxyl group on the ribose located immediately 5′ of the cleavage site phosphate (*8*) and that generate products containing a 2′,3′-cyclic phosphate and a 5′-hydroxyl group (*9*). Thus, implied is a cleavage mechanism that involves nucleophilic attack of the 2′-hydroxyl or 2′-alkoxide on the cleavage-site phosphorus (Fig. 1). In the HDV ribozymes, a specific cytosine (C75 in the genomic ribozyme, designated γC75, and its counterpart C76 in the antigenomic ribozyme) has been hypothesized to accept the proton from the attacking 2′-hydroxyl group (*10*, *11*).

To establish that the cytosine base at position 76 was essential for cleavage in the antigenomic ribozyme, we tested whether exogenous cytosine could rescue activity of C76 mutants. We introduced mutations at C76 into the PEX1 antigenomic ribozyme sequence (*12*); consistent with previous findings (*13*), self-cleavage activity of C76u and C76g was undetectable under standard conditions (Fig. 2A). At 37°C, the rate constants were down by a factor of 10^6. Cleavage activity of the C76u ribozyme was partially restored when cytosine was added to the reaction mixture (*14*). Rescue of activity by exogenous bases and base analogs has previously been demonstrated in hammerhead ribozymes containing abasic residues (*15*). In those studies, rescue occurred through compensation of structural changes introduced by the abasic residue. To test whether cytosine rescue of the C76u mutant might reflect a catalytic role for the base, we substituted imidazole for cytosine. For C76u and C76g, addition of 200 mM imidazole (pH 7.4) to the reaction mixtures enhanced cleavage activity at least 250- and 25-fold, respectively (Fig. 2A and Table 1). The 3′ product band in these reactions was the same size as the normal 3′ product, which suggests that imidazole-dependent cleavage occurred at the wild-type cleavage site in the 101-nucleotide (nt) precursor. We tested several other buffers, but only imidazole and 4(5)-methylimidazole enhanced cleavage activity (*14*). A divalent cation (Mg^{2+}, Ca^{2+}, or Mn^{2+}) was required for cleavage of all constructs (Fig. 2A) (*16*).

Imidazole would most likely be acting as either a general base (pK_a ~ 7.0) or a nucleophile in the cleavage reaction. If imidazole acted as a nucleophile in a single-displacement reaction, it should show up in one of the products. However, a 2′,3′-cyclic phosphate would be generated if the adjacent 2′-hydroxyl was the nucleophile. Therefore, we characterized the 5′ cleavage product. For this analysis, the sequence 5′ to the cleavage site in both PEX1 and C76u was shortened from 8 to 3 nt. Wild-type and mutant precursor RNAs were 5′-end-labeled and allowed to cleave in the absence and presence of imidazole, respectively. The 5′ cleavage products for both ribozymes comigrated on polyacrylamide gels under denaturing conditions in which short fragments containing 3′(2′)-terminal phosphates were resolved from frag-

ments containing 2′,3′-cyclic phosphates (Fig. 2B). To confirm generation of a cyclic phosphate, we isolated 5′ products from both reactions and hydrolyzed them with acid. We observed a shift in mobility after hydrolysis when the products were fractionated by gel electrophoresis (Fig. 2B) or thin-layer chromatography (*16*). This shift is consistent with the presence of an additional negative charge carried by a terminal phosphate. These results demonstrate that the mutant ribozyme cleaved at precisely the wild-type position and suggest that cleavage most likely occurred by attack of the adjacent 2′-hydroxyl group on the phosphorus.

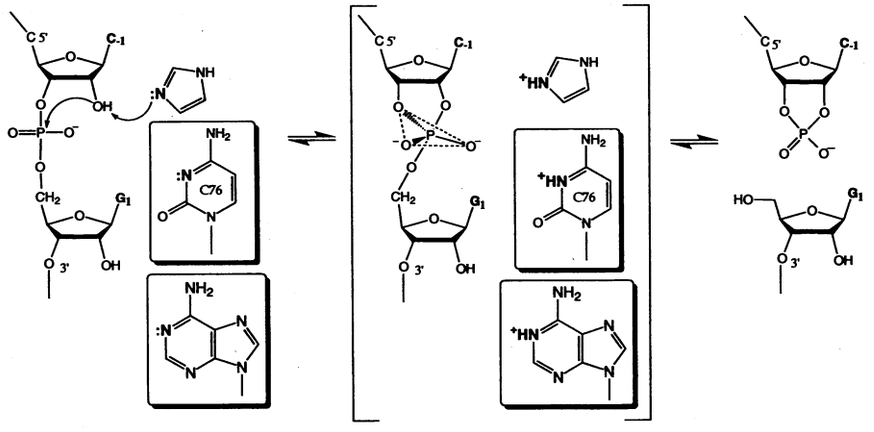

Fig. 1. Proposed mechanism for general base catalysis by imidazole and RNA side chains. In the C76u mutant, the ring nitrogen of imidazole is the potential proton acceptor. In the wild-type (top inset) and C76a mutant (bottom inset), cytosine and adenine, respectively, could act as general bases. Other likely players in catalysis for which there is no specific evidence in the HDV ribozymes, including metal ions and the proton donor for the 5′ leaving group, are not shown.

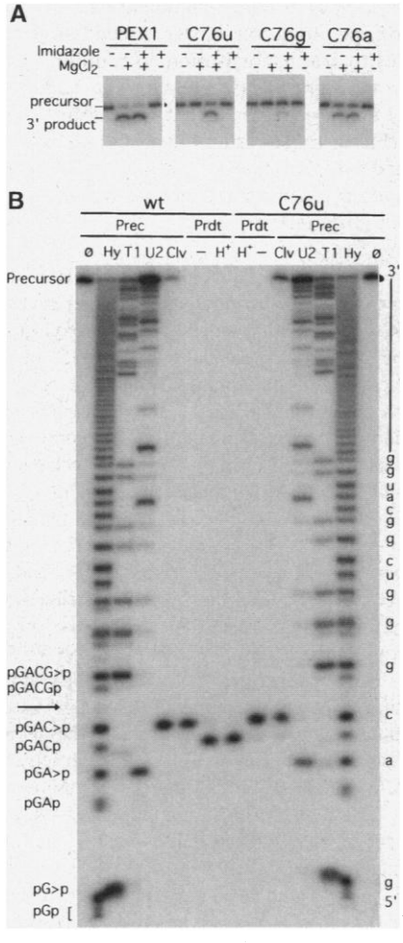

Fig. 2 (A) Dependence of ribozyme cleavage activity on imidazole and Mg^{2+}. Radiolabeled precursor RNA was heated for 1 min to 95°C in 0.1 mM EDTA (pH 8.0) and then preincubated at 37°C for 10 min in 0.5 mM spermidine containing 1 mM EDTA buffered with either 40 mM tris-HCl (pH 7.5) or 200 mM imidazole (pH 7.4). All pH values given in this paper were measured under final reaction conditions. Reactions (37°C) were initiated by adding $MgCl_2$ (11 mM final concentration). Samples were quenched with formamide containing 50 mM EDTA and fractionated on a 6% polyacrylamide gel containing 7 M urea. Reaction time for PEX1 was 5 s; reaction time for all mutants was 4 hours. **(B)** Cleavage of wild-type and C76u in imidazole generates the same 5′ product. Cleavage products of wild-type (wt) and C76u precursors (5′-end-labeled with ^{32}P) were fractionated on a sequencing gel under conditions in which small fragments containing 2′,3′-cyclic and 2′(3′)-terminal phosphates were well resolved (19% acrylamide, 1% bisacrylamide). The shortened 5′ sequence (3 nt, 5′-GAC-) facilitated resolution of the 5′ product on the basis of charge. Precursor (Prec) lanes are untreated (lanes ø), alkaline hydrolysis ladder (lanes Hy), T1 G ladder (lanes T1), U2 A ladder (lanes U2), or allowed to self-cleave (lanes Clv). The wild type (wt) cleaved for 10 s in $MgCl_2$, and the C76u cleaved for 4 hours in $MgCl_2$ with imidazole. Product lanes (Prdt) are the isolated 5′ product receiving no further treatment (lanes −) or the same fragment treated with 0.1 M HCl for 4 hours at room temperature (lanes H^+). Positions of the bands corresponding to mono-, di-, tri-, and tetranucleotides containing either a terminal (p) or cyclic (>p) phosphate are indicated on the left. Arrow is the position corresponding to the ribozyme cleavage site in the ladder of fragments.

The magnitude of imidazole rescue was greatest for the C76u mutant (Fig. 3A and Table 1); therefore, we focused on the mechanism of rescue of this ribozyme. The cleavage rate constant for the C76u ribozyme was linearly dependent on imidazole concentration (25 to 200 mM), and the slope of the line increased with increasing pH (Fig. 3B). Extrapolation to zero imidazole gave an imidazole-independent rate constant of 2.1×10^{-3} $hour^{-1}$ at pH 7.4. At higher imidazole concentrations, the increase in the rate constant showed only slight curvature (k_{obs} = 0.71 $hour^{-1}$ at 500 mM imidazole, pH 8.3); however, degradation of RNA became more pronounced at these higher concentrations. If there is a saturable imidazole binding site, the equilibrium dissociation constant (K_d) is estimated to be >1.2 M. The pH dependence of the apparent second-order rate constant indicated an apparent pK_a of 7.0 ± 0.1 (Fig. 3C). The concentration and pH dependence data for the C76u mutant suggest that imidazole base is the active species, and, although other mechanisms dependent on the concentration of imidazole base are plausible (see below), these data are consistent with imidazole-enhanced cleavage by a general base mechanism.

There are, however, at least two additional mechanisms that could explain imidazole-dependent cleavage in the C76u mutant. First, imidazole may structurally compensate for the mutation but not participate in the chemistry. Pyrazole (1,2-diazole; pK_a ~ 2.5), a close structural analog of imidazole but a much weaker base, did not rescue activity (*16*). Competition experiments indicated that pyrazole inhibited the imidazole-dependent reaction [inhibition constant (K_i) ~ 300 mM], so it appeared to bind but not to facilitate cleavage. The second possibility is that imidazole could coordinate a catalytic metal ion, presumably replacing a ligand lost with the mutation of C76. For example, the N-3 of C^{60} in yeast $tRNA^{Phe}$ is a Mg^{2+} ligand. That interaction is through a water molecule (*17*), and mutating the cytosine affected the concentration and specificity of Mg^{2+}-catalyzed hydrolysis of the tRNA (*18*). Thus, catalytic metal ion coordination appears to be a potentially viable mechanism. However, if imidazole substituted for C76 as a metal ligand in the ribozyme reaction, pyrazole should have fulfilled a similar function. Additionally, increasing the $MgCl_2$ concentration from 10 to 250 mM or adding Mn^{2+} (2 and 10 mM, with and without Mg^{2+}) did not restore site-specific self-cleavage activity in the C76u mutant in the absence of imidazole. In the presence of imidazole, addition of 2 mM Mn^{2+}

did not further stimulate the reaction (*16*). Finally, metal coordination to γC75 in the crystal structure of the HDV genomic ribozyme cleavage product was not observed (*10*), although, if metal binding requires coordination to both γC75 and the scissile phosphate, the metal ion may not remain strongly bound in the cleavage product (*10*).

The simplest explanation for imidazole-dependent cleavage of the C76u ribozyme is that imidazole acts as a general base to accept a proton from the 2′-hydroxyl group of the nucleotide 5′ to the cleavage site (Fig. 1). This interpretation could mean that, in the wild-type antigenomic sequence, the N-3 of C76 accepts the proton, as proposed for γC75 in the genomic ribozyme (*10*). If C76 is acting as a general base, replacing it with a base of a different pK_a would be expected to shift the apparent pK_a of the cleavage reaction in a predictable direction and amount. Because the C76a mutant was marginally active, we examined it in this regard. If it is assumed that there are structural similarities for the two HDV ribozymes, the adenine N-6 amino group could potentially form the same hydrogen bond network as the cytosine N-4 amino group, thus positioning the N-1 of adenine near the N-3 of the cytosine it replaced. However, the N-1 of adenosine ($pK_a \sim 3.5$) is a poorer proton acceptor than the N-3 of cytidine ($pK_a \sim 4.2$) (*4*). The pH dependence of the cleavage rate constants for the two ribozymes revealed a decrease in the apparent pK_a from 6.1 for wild type to 5.6 for the C76a mutant (Fig. 3D). Finding that the apparent pK_a had decreased by an amount roughly equivalent to the pK_a difference of the two nucleosides is consistent with both cytosine and adenine functioning as general bases at position 76.

The general base mechanism for imidazole in these studies is supported by a strong precedent for the role of imidazole, or an imidazole side chain, as a general base in RNA cleavage reactions (*3*, *19*). There is also precedent for exogenous imidazole acting as a general base—for example, to rescue the activity of histidine mutations of bacterial luciferase (H44A) (*20*) and horseradish peroxidase (H42A) (*21*). Imidazole rescue of a side-chain mutant of a naturally occurring RNA enzyme expands the application of this

Table 1. Rate constants for cleavage of PEX1 (wild type) and C76 mutants in the absence and presence of 200 mM imidazole. In reactions without imidazole, the buffer was 40 mM tris-HCl (pH 7.5). All reactions in imidazole were done at pH 7.4. The reaction temperature for PEX1 was 25°C to allow for manual measurements; the other reactions were done at 37°C. Otherwise, the conditions were the same as those described in Figs. 2 and 3. Maximal rate constants for cleavage of C76g and C76u in the absence of imidazole were estimated from the average extent of cleavage after 20 hours; there was a twofold variation in these numbers between experiments. Rate constants for the other reactions were obtained from a fit to a mechanism for a single exponential reaction. The values reported are the averages of three determinations, and, other than for PEX1, variation between rate constants in individual experiments was within 10%. Enhancement (fold) is defined as [k_{obs} (imidazole)]/[k_{obs} (no imidazole)].

Ribozyme	k_{obs} (min^{-1})		Enhancement (fold)
	No imidazole	200 mM imidazole	
PEX1	12 ± 2	14 ± 2	1.2
C76a	3.0×10^{-3}	3.1×10^{-3}	1.0
C76g	($\leq 1.5 \times 10^{-5}$)	3.8×10^{-4}	≥ 25
C76u	($\leq 1.7 \times 10^{-5}$)	4.2×10^{-3}	≥ 250

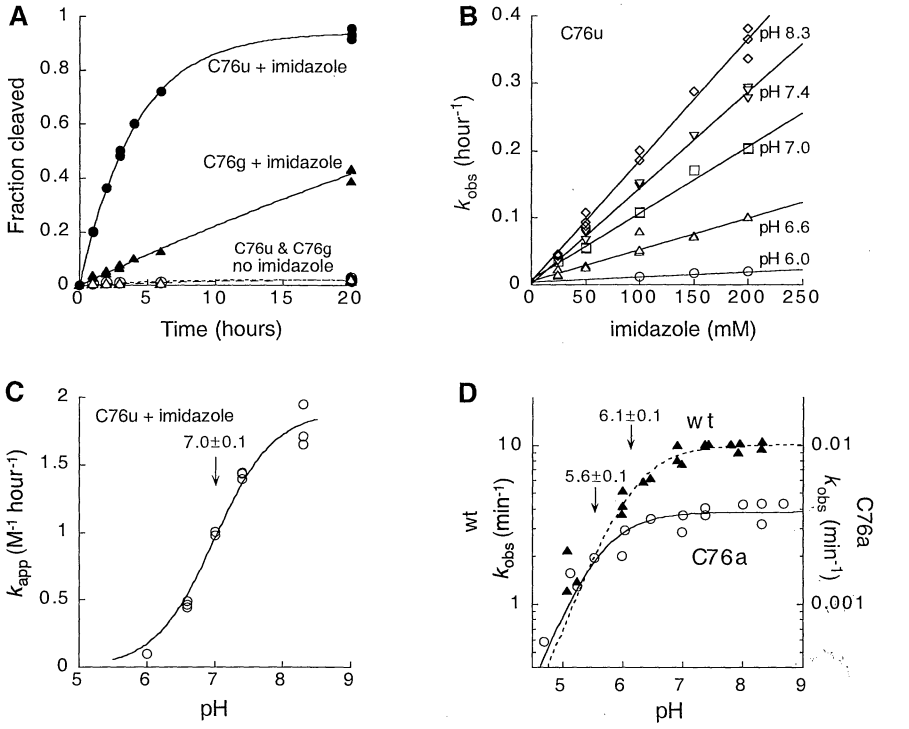

Fig. 3. (**A**) Kinetics of cleavage of C76u (circles) and C76g (triangles) with (closed symbols) and without (open symbols) imidazole. RNA was preincubated, as described in Fig. 2, in either 200 mM imidazole (pH 7.4) or 40 mM tris-HCl (pH 7.5). $MgCl_2$ was added to start the reactions, and aliquots were quenched and fractionated on polyacrylamide gels. Results were quantified by PhosphorImager analysis, and the fraction cleaved was calculated from the amount of radioactivity in the precursor and 3′ product after correcting for the label in the 5′ product. Rate constants are reported in Table 1. (**B**) Concentration and pH dependence of the cleavage rate constant for C76u in imidazole. Pseudo-first-order rate constants in increasing imidazole concentrations (25 to 200 mM) at the pH indicated were obtained as described above. In the pH range 6.0 to 8.3, the fraction of unprotonated imidazole ranges from 10% to 95% (assuming a pK_a of 6.95). k_{obs} was linearly dependent (R values ≥ 0.95) on imidazole concentration in this range with apparent second-order rate constants (M^{-1} $hour^{-1}$) of 7.8×10^{-2} (pH 6.0), 4.7×10^{-1} (pH 6.6), 9.9×10^{-1} (pH 7.0), 1.4 (pH 7.4), and 1.8 (pH 8.3). (**C**) The pH dependence of C76u cleavage rate constants in imidazole. The apparent second-order rate constant for cleavage in imidazole is plotted as a function of pH. Fitting the data to the expression $k_{app} = k_{max}/(1 + 10^{pK_a - pH})$ gives an apparent pK_a of 7.0 ± 0.1 (arrow). (**D**) Comparison of pH dependence of wild-type (PEX1) and the C76a ribozymes. Conditions for cleavage reactions were the same as for Fig. 2 except for the buffer system, which was 25 mM acetic acid, 25 mM MES, 50 mM tris (pH 4.0 to pH 8.0) or 50 mM MES, 25 mM tris, 25 mM 2-amino-2-methyl-1-propanol (pH 7.0 to 9.0) (*24*) instead of 40 mM tris-HCl. The triple buffer systems were titrated with HCl or NaOH, and the ionic strength was maintained within 10% of 0.5. The observed rate constants (min^{-1}) are plotted as a function of pH. Apparent pK_a values (arrows) for the two ribozymes were obtained by fitting the data to $k_{obs} = k_{max}/(1 + 10^{pK_a - pH})$. Data analysis and graph preparation were done with KaleidaGraph (Synergy Software, Reading, PA).

method and suggests that general acid-base catalysis by nucleotide side chains is a mechanism that can be used in RNA-catalyzed reactions.

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11. In both ribozymes, numbering is from the cleavage site so that cleavage occurs between positions −1 and 1. Because of differences in the sequences of the two ribozymes, we use a γ prefix to denote genomic nucleotide positions. γC75 and C76 were first identified by mutagenesis as essential for cleavage activity (*13, 22, 23*) . Changing γC75 to U, G, or A resulted in no detectable self-cleavage activity in the genomic ribozyme; these mutations were reported to decrease activity by a factor of at least 5×10^5 (*23*). In the antigenomic ribozyme, changing C76 to U or G results in little or no activity (decreased by a factor of $>10^4$), whereas the C76 to A mutant is marginally active (decreased by a factor of 2×10^3) (*13*). Because the published data for the C-to-A mutations in the two ribozymes conflicted, we tested the genomic γC75a mutation. It cleaved with a rate constant of 3.7×10^{-3} min^{-1}, down a factor of 5×10^3 relative to the wild-type sequence—essentially the same effect as in the antigenomic ribozyme (T. S. Wadkins and M. D. Been, unpublished data). Cross-linking data indicate that C76 is within 10 Å of the cleavage-site phosphate [C. Bravo, F. Lescure, P. Laugâa, J.-L. Fourrey, A. Favre, *Nucleic Acids Res.* **24**, 1351 (1996); S. P. Rosenstein and M. D. Been, *Biochemistry* **35**, 11403 (1996)]. Solid evidence for γC75 being part of the active site of the genomic ribozyme was seen with the crystal structure of the 3′ cleavage product: the O-2 of γC75 could form a hydrogen bond to the 5′-hydroxyl leaving group (*10*) . Ferré-D'Amaré *et al.* (*10*) noted that the base of γC75 is within a region of negative electrostatic potential, with its N-4 amino group participating in a network of hydrogen bonds. They proposed that this environment may perturb the pK_a of the base and that, in the precursor, the N-3 of γC75 may be positioned to accept the proton from the 2′-hydroxyl group of the ribose at position −1.
12. The antigenomic ribozymes used in this study are derivatives of PEX1 [A. T. Perrotta and M. D. Been, *J. Mol. Biol.* **279**, 361 (1998)] and were prepared by transcription from restriction endonuclease–cut plasmid DNA with T7 RNA polymerase. Precursor RNA was purified by electrophoresis on polyacrylamide gels and eluted. The precursor RNAs were either labeled internally by including guanosine [α-^{32}P]triphosphate in the transcription mixture or 5′-end-labeled with adenosine [γ-^{32}P]triphosphate and polynucleotide kinase after dephosphorylating with calf intestinal phosphatase (*6*). The mutations at position 76 and modifications to the sequence 5′ to the cleavage site were generated by oligonucleotide-directed mutagenesis on single-stranded uracil-containing templates of the plasmid (*6*).
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Male Attractiveness and Differential Testosterone Investment in Zebra Finch Eggs

Diego Gil,*† Jeff Graves, Neil Hazon, Alan Wells

Good-genes hypotheses of sexual selection predict that offspring fathered by preferred males should have increased viability resulting from superior genetic quality. Several studies of birds have reported findings consistent with this prediction, but maternal effects are an important confounding variable. Those studies that have attempted to control for maternal effects have only considered differential maternal investment after egg laying. However, female birds differentially deposit testosterone in the eggs, and this influences the development of the chick. This study shows that female birds deposit higher amounts of testosterone and 5α-dihydrotestosterone in their eggs when mated to more attractive males.

Female preferences for ornaments that indicate male genetic quality would allow females to enhance the viability of their offspring (*1*). Evidence of female preference for traits indicative of "good genes" has been found in some species (*2*). In birds, several studies have found evidence for enhanced survival of offspring fathered by highly ornamented males (*3*, *4*), but it remains possible that this effect is a result of differential female investment in the offspring of these males. Differential investment by females in chicks fathered by attractive males has been experimentally demonstrated in lab and field studies (*5*).

Although some of the studies reporting good-genes effects in birds have attempted to control for maternal effects, these studies have only considered investment after laying (*4*). However, it is possible that differential investment may occur before laying. Females of several bird species deposit varying amounts of testosterone in their eggs (*6–9*), and this variation influences the development of the chick. Chicks that hatch from eggs with high amounts of testosterone beg for food more intensively, grow faster than other chicks, and are more likely to become dominant once they fledge (*6*, *10*).

We predicted that female birds would deposit higher amounts of testosterone when mated to attractive males than when mated to less attractive males. In the zebra finch (*Taeniopygia guttata*), the attractiveness of the male can easily be manipulated by using leg bands of different colors. Females pair preferentially with red-banded males and avoid green-banded ones. This manipulation has a more substantial effect on male attractiveness than any other

School of Environmental and Evolutionary Biology, University of St. Andrews, St. Andrews, Fife KY16 9TS, UK.

*Present address: Laboratoire de Psychophysiologie et d'Ethologie, UPRESA-CNRS 7025 Université de Paris X, 92001 Nanterre, France.

†To whom correspondence should be addressed: E-mail: Diego.Gil@u-paris10.fr.