The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen

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The ribonucleoside analog ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) shows antiviral activity against a variety of RNA viruses and is used in combination with interferon-α to treat hepatitis C virus infection. Here we show *in vitro* use of ribavirin triphosphate by a model viral RNA polymerase, poliovirus 3D^{pol}. Ribavirin incorporation is mutagenic, as it templates incorporation of cytidine and uridine with equal efficiency. Ribavirin reduces infectious poliovirus production to as little as 0.00001% in cell culture. The antiviral activity of ribavirin correlates directly with its mutagenic activity. These data indicate that ribavirin forces the virus into 'error catastrophe'. Thus, mutagenic ribonucleosides may represent an important class of anti-RNA virus agents.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) shows antiviral activity against a variety of RNA viruses[1–3] and is used in combination with interferon-α to treat hepatitis C virus infection[4,5] and as monotherapy for lassa fever virus infection[6] and severe respiratory syncytial virus infection[7]. Since the discovery of the broad-spectrum antiviral activity of ribavirin in 1972 (ref. 1), it has been suggested that the active form of ribavirin is the monophosphate, RMP (ref. 8). RMP inhibits inosine monophosphate dehydrogenase (IMPDH), causing a decrease in the intracellular concentration of GTP (refs. 2,8). This decrease potentially diminishes viral protein synthesis and limits replication of viral genomes. However, inhibition of IMPDH may not be sufficient for antiviral activity[2,7,9–11]. Other mechanisms of action have been proposed but not fully explored, including RMP inhibition of guanylyltransferase activity[12] and inhibition of viral transcription[13,14].

Ribavirin triphosphate (RTP) accumulates in cells after treatment with the nucleoside[15]. Therefore, we explored the possibility that ribavirin's antiviral effect requires direct incorporation into viral RNA. Here we demonstrate use of RTP by the poliovirus polymerase (3D^{pol}) *in vitro* and potent mutagenesis of poliovirus by means of ribavirin incorporation *in vivo*.

Ribavirin incorporation by the poliovirus RNA polymerase 3D^{pol}

A primer-extension assay for 3D^{pol} has been developed[16,17]. This assay uses a symmetrical primer/template substrate that we call 'sym/sub'. Stable, elongation-competent complexes are formed after a brief incubation of 3D^{pol} with sym/sub. This system permits evaluation of the kinetics and thermodynamics of 3D^{pol}-catalyzed nucleotide incorporation. Use of this substrate has shown that, in addition to correct ribonucleotides, 3D^{pol} uses incorrect ribonucleotides and deoxyribonucleotides[16].

Given that 3D^{pol} uses a variety of nucleotides as substrates, we sought to determine whether RTP could act as a substrate for this enzyme. First we used a sym/sub derivative containing cytidine as the first templating nucleotide (Fig. 1*a*, sym/sub-C). RTP was recognized by 3D^{pol} and incorporated into sym/sub-C (Fig. 1*b*). Prolonged reaction times permitted multiple cycles of ribavirin incorporation, indicating that ribavirin incorporation did not terminate elongation of nascent RNA (Fig. 1*b*, +2). We measured the rates of RMP incorporation at a variety of RTP concentrations (Fig. 1*c*) and used this information to determine the apparent dissociation constant (K_d) for RTP and the maximum rate of RMP incorporation (k_{pol}) (Fig. 1*d*). The K_d value was 430 μM and the k_{pol} value was 0.019/s (Table 1). Consistent with this, RTP competitively inhibited correct nucleotide incorporation with an inhibition constant (K_i) value in the range of 400 μM (data not shown). To prove that ribavirin was not a chain terminator, we did an experiment using a sym/sub-U derivative (Table 1). In the presence of RTP, 3D^{pol} extended sym/sub-U to create an 11-nucleotide product (Fig. 1*e*). When both RTP and UTP were present, 3D^{pol} extended this substrate to 12 nucleotides without accumulation of the 11-nucleotide product (Fig. 1*e*). The appearance of a 13-nucleotide product in this experiment was a reflection of misincorporation.

Modeling studies showed that the pseudo base (1,2,4-triazole-3-carboxamide) of ribavirin was capable of base-pairing equivalently with cytidine and uridine as long as rotation of the carboxamide moiety was not restricted (Fig. 2*a* and *b*). To test this experimentally, we used sym/sub-U instead of sym/sub-C. The efficiency of RMP incorporation opposite uridine was identical to that opposite cytidine (Table 1). Although the efficiency of RMP incorporation was low relative to incorporation of correct nucleotides (for example, GMP incorporation into sym/sub-C; Table 1), incorporation of RMP was equivalent to misincorporation of GMP (for example, GMP incorporation into sym/sub-U; Table 1).

To determine the effect of incorporated ribavirin on subsequent rounds of RNA synthesis, we evaluated the kinetics of CMP and UMP incorporation into sym/sub-R, a template containing ribavirin (Table 1). CMP and UMP were incorporated equivalently

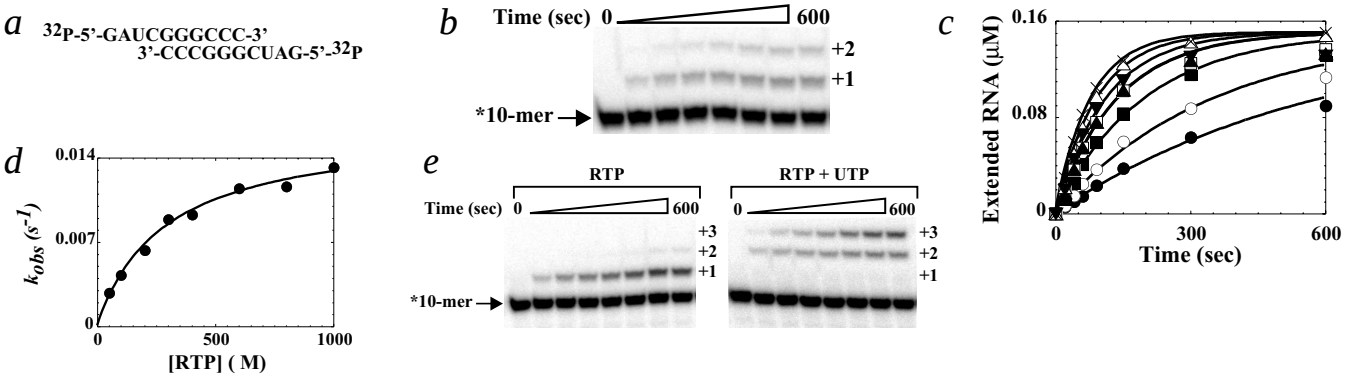

Fig. 1 RMP incorporation by 3D^{pol} *in vitro*. ***a***, Primer/template (sym/sub-C) sequences. ***b***, Denaturing PAGE of the ^{32}P-labeled products from poliovirus polymerase-catalyzed ribavirin incorporation into sym/sub-C; *, 10-nucleotide subtrate. ***c***, Kinetics of ribavirin incorporation with 50 μM RTP (●), 100 μM RTP (○), 200 μM RTP (■), 300 μM RTP (□), 400 μM RTP (▲), 600 μM RTP (▼), 800 μM RTP (△) and 1000 μM RTP (5). Solid lines, fit of the data to a single exponential. ***d***, Dependence of the observed rate of RMP incorporation on RTP concentration. Solid line, fit of the data to a hyperbola with a k_d value of 430 ± 79 μM and a k_{pol} value of 0.019 ± 0.002/s. ***e***, Complexes of 3D^{pol}–sym/sub-U were preassembled and then mixed with either 1 mM RTP or 1 mM RTP and 10 μM UTP. All '+1' product is 'extended' to '+2' and '+3' in the presence of UTP; *, 10-nucleotide subtrate.

opposite ribavirin (Table 1). CMP and UMP incorporation opposite ribavirin is, on average, 50,000% faster than incorporation of RMP opposite cytidine and uridine. The slow observed rate of RMP incorporation probably reflects isomerization of the 'pseudo base' from the 'syn' to the 'anti' conformation (Fig. 2*c*). Once ribavirin is in the RNA, it is presumably trapped in the 'anti' conformation and readily forms base pairs with incoming pyrimidines. These data demonstrate that RTP is a substrate for 3D^{pol} and indicate that incorporation of ribavirin into RNA should be mutagenic to viral RNA, promoting transitions of A to G and G to A.

Antiviral activity of ribavirin against poliovirus

Pharmacokinetic studies have shown that ribavirin collects in the livers of patients treated with ribavirin for chronic hepatitis C virus infection, and the ribavirin reaches steady-state levels of approximately 250 μM in hepatocytes (Z.H. and J.L., unpublished data). Therefore, we tested ribavirin's antiviral activity against poliovirus by treating cells with concentrations of ribavirin in the range of 100–1,000 μM. Cells treated with 100 μM ribavirin and then infected with poliovirus at a low multiplicity of infection showed a reduction in virus production to 50%; treatment with 1,000 μM ribavirin caused a reduction in virus production to 0.00001% (Table 2).

Ribavirin's effects on viral translation and replication

We used a poliovirus replicon (PolioLuc), in which the capsid-coding sequence was replaced by a luciferase gene[18] (Fig. 3*a*), to evaluate the effects of ribavirin on poliovirus translation and RNA synthesis in cell culture. Transfection of HeLa cells with replicon RNA results in the production of a polyprotein containing luciferase that is processed by the viral 2A protease to liberate active luciferase, and the replicon translates and replicates like wild-type poliovirus[19]. First, we did an experiment in the presence of 2 μg/ml brefeldin A, a drug that completely blocks poliovirus replication but not translation[20]. In cells treated with brefeldin A, PolioLuc translation was not substantially inhibited by ribavirin ($3.2\text{-}3.5 \times 10^4$ RLU (relative light units) at 100, 400 or 1,000 μM, compared to 6.5×10^4 RLU with no ribavirin) (Fig. 3*b*).

Ribavirin only moderately reduced RNA replication in PolioLuc-transfected cells (Fig. 3*c*, open bars, no brefeldin A). At a concentration of 1000 μM, ribavirin inhibited replication to 10% of wild-type levels at 4 hours after transfection (data not shown), with a recovery to 40% of wild-type levels by 6 hours after transfection (Fig. 3*c*, open bars). In parallel with the replicon transfections, cells were infected with a high multiplicity of infection of virus (10 plaque-forming units (PFU) per cell). Although it only modestly inhibited replicon RNA replication, ribavirin reduced viable virus production in a single round of infection to as low as 0.08% (Fig. 3*c*, filled bars).

To confirm that ribavirin inhibited virus production without substantially affecting RNA synthesis, we determined the levels of poliovirus RNA accumulated in ribavirin-treated, poliovirus-infected cells. Consistent with the data obtained using PolioLuc, peak viral replication in ribavirin-treated cells reached wild-type or nearly wild-type levels, while production of infectious virus from the cells was reduced to as low as 0.08% (Fig. 3*d*). These results are consistent with our hypothesis that ribavirin incorporation induces mutations in the viral genomes during multiple rounds of RNA replication in the cell, resulting in a substantial increase in the production of defective genomes.

Table 1 The pseudo base of ribavirin pairs equally with cytosine and uracil

Substrates		Kinetic parameters	
Nucleic acid:	Nucleotide	K_d	k_{pol}
sym/sub-C			
GAU**C**GGGCCC	RTP	430 ± 79	0.019 ± 0.002
CCCGGG**C**UAG	GTP	3.8 ± 0.7	56.7 ± 2.8
sym/sub-U:			
GCA**U**GGGCCC	RTP	496 ± 21	0.014 ± 0.001
CCCGGG**U**ACG	GTP	310 ± 30	0.013 ± 0.001
sym/sub-G:			
CAU**G**CCCGGG	CTP	19.2 ± 3.2	157 ± 8
GGGCCC**G**UAC			
sym/sub-R:			
CAU**R**CCCGGG	CTP	493 ± 41	8.5 ± 0.3
GGGCCC**R**UAC	UTP	551 ± 127	7.6 ± 0.6

K_d, in μM; k_{pol}, per second.

Table 2 Ribavirin is a mutagen of poliovirus, and the mutagenesis correlates directly with its antiviral activity

Mutagen	Viral titer produced	gua^r frequency	Increase in gua^r mutation frequency (%)
no mutagen	2×10^9	30	0
100 μM ribavirin	9×10^8	174	480
200 μM ribavirin	2×10^8	437	1,360
400 μM ribavirin	5×10^6	1243	4,040
1000 μM ribavirin	60	—	—
1 μM AZC	1×10^9	63	110
400 μM RIB4C	2×10^9	36	20
0.1 μg/ml BFA	5×10^7	23	0
75 μM 5FU	5×10^8	65	150
1900 μM 5FU	2×10^7	588	2,170
7500 μM 5FU	$< 1 \times 10^5$	—	—

Viral titer (in PFU/ml) was determined after 4 d, when 100% cytopathic effect is apparent in all conditions shown except for 400 μM ribavirin (titers obtained after 100% cytopathic effect at 6 d) and 1,000 μM ribavirin (which showed no cytopathic effect after 7 d). Input virus was 100 PFU. Gua^r frequencies (as gua^r/1×10^6 PFU) are the average of at least three experiments. Standard deviations are 10–20%. —, Mutation frequency could not be determined because viral titer was so extremely reduced. AZC, 5-azacytidine; 5FU, 5-fluorouracil; RIB4C, 1βD-ribofuranosyl-1,2,3-triazole-4-carboxamide; BFA, brefeldin A.

Ribavirin is an RNA virus mutagen

We measured the mutagenic potential of ribavirin on poliovirus using a gua^r assay. Poliovirus multiplication is inhibited by the presence of 2 mM guanidine in the culture medium (Fig. 3*e*). Guanidine inhibits the 2C^{ATPase} protein[21]; however, mutations in the 2C-coding sequence that confer resistance to guanidine have been identified (gua^r) (refs. 22, 23). A specific single-nucleotide mutation (C4605U) results in the gua^r phenotype (gua^r, see Methods) and this variant exists in the natural population of poliovirus at a frequency of approximately 1×10^{-5}. We quantified this variant by plaque assay in the presence of 2 mM guanidine (Fig. 3*e*). This assay provides a rapid method to evaluate the effect of different growth conditions on mutation frequencies, using gua^r as a genetic marker. The C-to-U mutation necessary for gua^r is consistent with incorporation of RTP as a GTP analog during negative-strand genome synthesis. There was a dose-dependent increase in the frequency of gua^r virus in poliovirus stocks grown in the presence of ribavirin (Fig. 3*e* and Table 2), thus confirming the mutagenic activity of ribavirin *in vivo*. Moreover, a direct correlation existed between the mutagenic activity of ribavirin and the antiviral activity of the compound (Table 2).

We used control experiments to demonstrate that known mutagens such as 5-azacytidine[24,25] and 5-fluorouracil[24,26,27] had dose-dependent antiviral activity at levels of mutagenesis similar to that of ribavirin (Table 2, AZC and 5FU). Furthermore, an IMPDH inhibitor that lacks antiviral activity[9] was not mutagenic (Table 2, RIB4C). Finally, a compound that inhibits poliovirus multiplication by a mechanism independent of both IMPDH and 3D^{pol} also lacked mutagenic activity (Table 2, BFA)[20,28].

To evaluate the spectrum of mutations induced by ribavirin, we analyzed sequences derived from independently cloned cDNAs of poliovirus capsid VP1, from virus grown in the presence or absence of 1000 μM ribavirin. Ribavirin-mutagenized genomes had a 600% increase in G-to-A and C-to-U transition mutations (Table 3), confirming the G-to-A mutagenic activity predicted by our *in vitro* ribavirin experiments. The C-to-U mutations seen are consistent with G-to-A transitions induced by incorporation of RTP as a GTP analog during negative-strand RNA synthesis. The increased frequency of G-to-A, C-to-U and total mutations were all highly significant ($P < 0.0004$, $P < 0.0001$ and $P < 2 \times 10^{-7}$, respectively).

Discussion

RNA viruses live as 'quasispecies', creating extraordinary genetic diversity through mutation. It has been proposed that, because of this high mutation rate, RNA viruses exist on the threshold of 'error catastrophe'[29,30], and a moderate increase in mutation rate can kill a RNA virus population by causing a 'genetic meltdown'[26,27,31]. An extrapolation of our sequencing data would indicate that, on average, each poliovirus genome (7,441 nucleotides long) synthesized after multiple rounds of replication inside an infected cell normally contains approximately two point mutations. In the presence of 1,000 μM ribavirin, each poliovirus genome synthesized contains approximately 15 points mutations. Thus, in our model RNA virus system, a suprisingly small increase (200-600%) in mutation rate produced a substantial antiviral effect (Table 2 and Table 3). Prolonged growth of human immunodeficiency virus (HIV) in the presence of mutagenic deoxyribonucleoside analogs inhibits multiplication of the virus[32].

In conclusion, the antiviral activity of ribavirin against poliovirus requires formation of RTP, this nucleotide is used by the viral RNA polymerase and the incorporated ribavirin is mutagenic. The ability of ribavirin monophosphate to inhibit IMPDH and thereby decrease cellular GTP pools probably serves to potentiate the mutagenic/antiviral effect by decreasing the concentration of 'competitor' nucleotide and thereby increasing the frequency of ribavirin incorporation. This unified model for the mechanism of action of ribavirin predicts that mutagenic ribonucleosides that

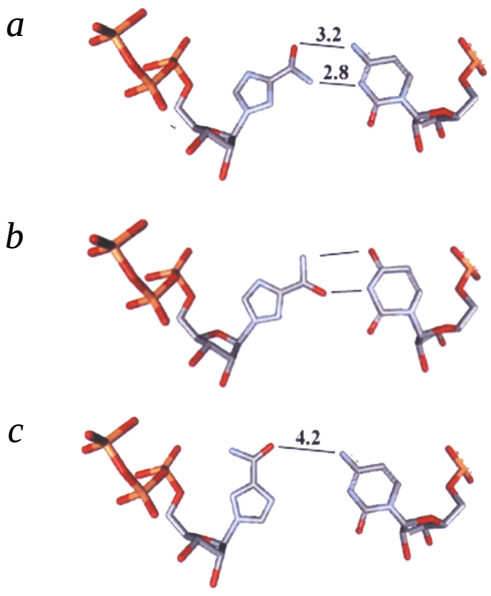

Fig. 2 Molecular modeling of ribavirin. ***a***, RTP with the pseudo base in the 'anti' conformation (left) forms base pairs with cytidine (right) in the template. Hydrogen-bond distances, Ångstrøms. ***b***, RTP also forms hydrogen bonds with uridine in the template after rotation of the carboxamide moiety. ***c***, RTP with the pseudo base in the 'syn' conformation is not within hydrogen-bonding distance to the template base. The pseudo base of ribavirin nucleoside exists mainly in the 'syn' conformation, as determined by x-ray crystallography[35] and solution nuclear magnetic resonance analysis of RTP (data not shown). Atoms: nitrogen, blue; oxygen, red; carbon, grey; phosphorous, orange. Hydrogen-bond distances, Ångstrøms.

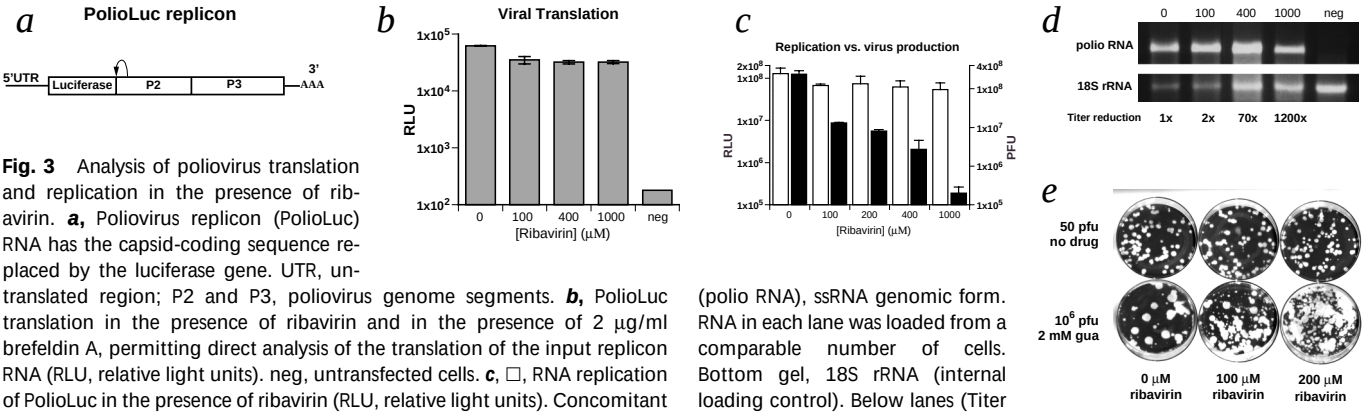

Fig. 3 Analysis of poliovirus translation and replication in the presence of ribavirin. ***a*,** Poliovirus replicon (PolioLuc) RNA has the capsid-coding sequence replaced by the luciferase gene. UTR, untranslated region; P2 and P3, poliovirus genome segments. ***b*,** PolioLuc translation in the presence of ribavirin and in the presence of 2 μg/ml brefeldin A, permitting direct analysis of the translation of the input replicon RNA (RLU, relative light units). neg, untransfected cells. ***c***, □, RNA replication of PolioLuc in the presence of ribavirin (RLU, relative light units). Concomitant poliovirus infections in the presence of ribavirin, ■. ***d***, Direct determination of poliovirus RNA replication in the presence of ribavirin. PolyA$^+$ RNA was isolated from infected cells treated with ribavirin (100, 400 or 1,000 μM) or untreated cells (0 μM) at the time of maximum virus replication and analyzed on a non-denaturing agarose gel. neg, RNA from uninfected cells. Poliovirus RNA (polio RNA), ssRNA genomic form. RNA in each lane was loaded from a comparable number of cells. Bottom gel, 18S rRNA (internal loading control). Below lanes (Titer reduction), Inhibition of infectious virus production in the ribavirin-treated cells. ***e***, Ribavirin is a mutagen to poliovirus. Virus stocks grown in the presence of increasing concentrations of ribavirin (below plates) were analyzed by plaque assay for the presence of the gua^r genetic marker. pfu, plaque-forming units; gua, guanidine.

can be incorporated by viral RNA-dependent RNA polymerases may represent an important class of antiviral agents for the treatment of RNA virus infections.

Methods

Analysis of 3D^{pol} *in vitro*. The polymerase 3D^{pol} was expressed and purified as described[33] and experiments using sym/sub derivatives were done as described[16]. Complexes of 3D^{pol}–sym/sub were preassembled and then mixed with the appropriate nucleoside triphosphate to initiate the reaction. Reactions were 'stopped' by the addition of EDTA. Product formation was monitored by phosphorimaging after denaturing PAGE. In kinetics experiments, when the value for k_{pol} was greater than 1/s, a rapid mixing/quenching device was used. All RNA was synthetic and was prepared by Dharmacon Research (Boulder, Colorado). Ribavirin triphosphate was obtained from Moravek Biochemicals (Brea, California). Structural models (Fig. 2) were constructed using WebLab Viewer.

Cells and viruses. HeLa S3 cells were propagated in OptiMEM (Life Technologies) supplemented with 2% dialyzed FCS (Life Technologies). In most experiments, 5×10^5 cells were plated in each well of a six-well dish 16–20 h before the experiment. A final volume of 2 ml was used, containing OptiMEM supplemented with 0.2% dialyzed FCS and mutagen or drug (ribavirin and 1-β–D-ribofuranosyl-1,2,3-triazole-4-carboxamide, provided by Schering-Plough; 5-azacytidine, 5-fluorouracil and brefeldin A, purchased from Sigma). In these conditions, 1 μM 5-azacytidine was moderately toxic to cells, killing approximately 10% over a 4-day period. All viral infections used a poliovirus stock grown from a plasmid-derived Mahoney strain poliovirus (pXpA) (ref. 34). Mutagenesis experiments (Table 2) used a viral inoculum of 100 PFU (± 3.5). Fig. 3*c* (infections with high multiplicity of infection) used 1×10^7–2×10^7 PFU. Mutagenesis experiments with 5-fluorouracil (Table 2) were done as described above except that mutagenized viral stocks were generated in cells grown in DMEM/F12 plus 10% FCS and were infected with an inoculum of 1×10^4 PFU. Replicon transfections and luciferase assays used PolioLuc RNA derived from pRLucRA (also called pRLuc rib+polyAlong), as described[17], and HeLa cells grown as described above. The translation experiment (Fig. 3*b*) used PolioLuc-transfected cells in the presence of 2 μg/ml brefeldin A, which completely abolishes poliovirus replication[20,28]. Translation was assayed by luciferase assay at 2 h after transfection. PolioLuc replication assays (Fig. 3*c*, open bars) were done in a similar way, in the absence of brefeldin A.

For direct quantification of viral RNA, 5×10^6 cells were infected with 5×10^7 PFU poliovirus in the growth and mutagen conditions described above. At 1, 6, 8 and 10 h after infection, viral RNA was isolated using oligo dT_{25} DynaBeads (Dynal, Oslo, Norway) and was separated by non-denaturing 1.5% agarose gel electrophoresis in 1× TAE in the presence of ethidium bromide. RNA from the time of maximum viral replication was used in Fig. 3*d* (8 h for 0 and 100 μM ribavirin, and 10 h for 400 and 1000 μM ribavirin). Samples of virus from each time point were obtained from cells before RNA isolation and quantified by plaque assay to determine the level of inhibition of infectious virus production.

Table 3 Sequence analysis of ribavirin treated poliovirus genomes

	mutations[a]		
	G-to-A	C-to-U	Total
no ribavirin	0.6	1.7	2.8
1000 μM ribavirin	6.5*	11.8*	20.3**

[a], per 104 nucleotides. Data for total mutations are greater than the sum of the two columns, as other transition (U to C, A to G) and transversion (G to U, G to C and U to A) mutations were also detected. *, $P < 0.0004$ and *, $P < 0.0000002$, compared with no ribavirin (two-tailed, unpaired Student's *t*-test). A total of 42,335 nucleotides was sequenced.

Gua^r and genome sequencing. Resistance to 2 mM guanidine was conferred by a single specific mutation: C to U at position 4,605 of the poliovirus genome (C4605U), in protein 2C (Pro to Ser at amino acid 161). The entire nonstructural genes of two independent gua^r mutants was sequenced to confirm that the C4605U mutation was the only change present. This mutation was additionally confirmed by sequencing the 2C gene of 20 independent gua^r virus isolates derived normally or in the presence of ribavirin or 5-fluorouracil mutagen, all of which had the C4605U mutation. gua^r virus was detected by plaque assay. HeLaS3 cells were plated 1 d before the experiment at 25% confluence in 10-cm dishes, and were infected with 50 PFU or 1×10^6 PFU poliovirus (Fig. 3*e*) from the appropriate viral stock (previously grown in the presence or absence of ribavirin). Cells were covered with a 20-ml overlay of 1% agar and DMEM/F12 plus 10% FCS supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (plus 2 mM guanidine hydrochloride (Sigma) in gua^r plaque assays). Plates were incubated at 37 °C for 72–80 h and then 'developed' using crystal violet staining. Two independent studies have identified several other specific gua^r mutations[22,23], all of which occur in the same region of 2C (amino acids 142–248). The specific mutation selected for in those studies[22,23] depended on the concentration of guanidine and the exact growth conditions used. Differences in the frequency of gua^r variants between our study and one published previously[26], in the presence of known RNA mutagens 5-fluorouracil and 5-azacytidine, probably depended on the fact that the studies quantified different gua^r mutations and used different growth conditions.

For sequence analysis, RNA was isolated using oligo dT_{25} DynaBeads from 1×10^6 cells (treated as described above) that had been infected at a multiplicity of infection of 10 and then collected at 10 h after infection. Random-

primed cDNA was synthesized from 1 μg RNA using Superscript II (Life Technologies), and VP1-coding sequence was amplified by PCR from 10% of the cDNA using high-fidelity PfuTurbo polymerase (Stratagene, La Jolla, California). An *Nhe*I–*Pst*I fragment encompassing the gene for VP1 was subcloned, and plasmid DNA was prepared from independent bacterial colonies and was sequenced from positions 2,625–3,400 of the poliovirus genome using BigDye terminator cycle sequencing, and was analyzed with DNASTAR. Virus capsid VP1 sequences were analyzed from 23 and 32 independent clones in conditions of no ribavirin and 1,000 μM ribavirin, respectively. The results in Table 3 were analyzed for statistical significance using a two-tailed Student's *t*-test with unassumed variance.

Acknowledgments

We thank D. Gohara for preparation of RTP molecular models; S. Scaringe for synthesis of ribavirin phosphoramidite; J. Lecomte for structural analysis of RTP; S. Firestine and J. Herold for discussions; J. Frydman and A. Frankel for comments; and G. Reyes for support. This work was supported in part by a National Cancer Institute National Institutes of Health Howard Temin Award CA75118 (to C.E.C.) and National Institute of Allergy and Infectious Diseases National Institutes of Health grants AI45818 (to C.E.C.) and AI40085 (to R.A.). S.C. is a Howard Hughes Medical Institute doctoral fellow.

RECEIVED 16 JUNE; ACCEPTED 19 OCTOBER 2000

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