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Adenovirus-Mediated Expression of Ribozymes in Mice

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Ribozymes are a new pharmaceutical class of reagents that offer potential in treating a number of different medical disorders, including infectious diseases and cancer. As a first step towards using ribozymes for the treatment of liver disorders such as viral hepatitis, adenovirus vectors that contain a ribozyme expression cassette under the control of different promoters directed against human growth hormone (hGH) were constructed and infused into transgenic mice that produce hGH from the gastrointestinal tract and liver. Adenovirus-mediated transfer of expressed ribozymes resulted in up to a 96% reduction of hepatic hGH mRNA over a period of several weeks in the transgenic mouse model. Furthermore, the concentration of ribozyme RNA correlated with the degree of hGH mRNA reduction. This study clearly demonstrates that ribozymes can function during the period of expression in an intact organ after somatic gene transfer.

Ribozymes are a powerful new therapeutic tool that offers a means to diminish and/or eradicate RNA molecules from cells (reviewed in reference 17). Although different ribozymes for catalytic self-cleavage exist in nature, the simplest and best characterized are the hammerhead ribozymes. Classically defined, hammerhead ribozymes are RNA molecules that hybridize to complementary RNA sequences in which the central part of the sequence forms a specific secondary structure where reactive groups are located close to each other and mediate specific cleavage of the target RNA at a consensus GUC target. The resulting RNA fragments are rapidly degraded, rendering the molecule nonfunctional. Thus, in theory, by altering the sequences that surround the catalytic site, it is possible to design ribozymes that cleave any specific target RNA molecule. A potential advantage of the ribozyme over other antisense technologies is related to the catalytic nature of the cleavage. Ribozymes can be either expressed in cells or synthesized and packaged for cellular uptake. Because ribozymes can be used to limit the synthesis of specific proteins or RNA viruses inside cells, a number of immediate therapeutic applications can be envisioned.

Currently, there are a few examples of ribozymes that are being developed for application to human diseases. Ribozymes directed against human immunodeficiency virus, when expressed from cells normally permissive to HIV, lead to a certain degree of protection from infection (reviewed in reference 13). A second application is cancer, for which a ribozyme directed against the *ras* oncogene or *bcl-abl* mRNA has been shown to alter the neoplastic phenotype of tumor cells in culture (reviewed in reference 13).

Even with these few examples that have been demonstrated to work in tissue culture systems, two major barriers to clinical application are the selection of ribozymes that function against RNA in cells and the efficient transfer and/or expression of active ribozymes in target tissues. There are a number of reasons why, in general, ribozymes designed to cleave RNA molecules function against naked RNA and not against RNA present inside cells (reviewed in reference 12). There are several criteria for the efficient functioning of ribozymes within a cell: (i) the target RNA sequence must be accessible in order to hybridize to the ribozyme; (ii) the ribozymes and target RNAs must be present in the same subcellular compartment; (iii) the ribozymes must be stable because it appears that the ratio of ribozyme RNA to target RNA should be relatively high; and (iv) the ribozyme should be expressed in all desired target cells within a particular tissue.

In order to attempt to obtain ribozymes for in vivo applications, two new improvements have recently been described (12). First, a new ribozyme library was used to isolate ribozymes that efficiently and selectively cleaved human growth hormone (hGH) RNA in cultured cells. Second, the ribozyme sequence was embedded in a stable loop region which, in turn, is part of an adenoviral vaI RNA, so that the catalytic secondary structure could form independently from the surrounding RNA structures of the ribozymes.

It is relatively simple to generate adenoviral vectors that can be used to transduce essentially 100% of hepatocytes after vasculature or biliary infusion in animals. In this study, hGH ribozyme expression cassettes were placed into recombinant adenovirus vectors and used to ablate hGH from tissue culture cells and to transiently ablate hGH mRNA from the livers of transgenic mice that produce hGH.

MATERIALS AND METHODS

Vector construction and generation of recombinant adenovirus. In order to produce recombinant adenoviruses that express ribozymes, the expression cassette containing the adenoviral vaI gene with RzE1 embedded in the loop structure was cut out from pGvaLRzE1 (12) as an *Xba*I-*Nhe*I fragment (578 nucleotides [nt]). This fragment was inserted in the *Xba*I sites of pXCJL.1 (16) and pAd.RSV-bPA (3) to generate pAd.polIIIRzE1 and pAd.RSVRzE1. pAd.T7RzE1 was produced by cloning the 717-nt *Pvu*II-*Nhe*I fragment from pGvaLRzE1 (with the T7 promoter) into the *Eco*RV site of pXCJL.1 A 3.2-kb fragment (*Bgl*II-*Pvu*II) containing the gene for the nucleus-localized T7 polymerase with a thymidine kinase polyadenylation signal (10) was cloned under the control of a PGK promoter into the *Eco*RV site of pAd.PGKbPA (3). A control adenovirus containing the vaL expression cassette without the ribozyme was generated by cloning the *Xba*I-*Nhe*I fragment of pGvaL into pAd.RSV-bPA.

The adenoviruses were prepared after cotransfection with pJM17 (16). Individual plaques were amplified in 293 cells, and adenoviral DNA was analyzed by *Hin*dIII digestion. Adenoviruses with the correct restriction pattern were pro-

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duced in large amounts and purified in a two-step CsCl gradient ultracentrifugation (5).

It is interesting that adenoviruses with the cloned vaL or vaLRzE1 expression cassette produced an additional band with lower density in the CsCl gradient. This new band contained an adenovirus with full-length DNA that was able to generate a cytopathic effect in 293 cells.

TNA preparation. Total nucleic acids (TNA) were extracted from mouse livers by homogenizing 100 mg of tissue (about 10^7 cells) in 4 ml of 10 mM Tris-Cl (pH 7.5)–1 mM EDTA–0.1 M NaCl–1% sodium dodecyl sulfate (SDS). The homogenates were incubated at 37°C overnight with 500-U/ml proteinase K, extracted with phenol-chloroform, and precipitated with ethanol. TNA pellets were dissolved in 500 μl of 0.2× SET (1× SET is 10 mM Tris-Cl [pH 7.5], 5 mM EDTA, and 1% SDS), and the concentration of nucleic acids was determined spectrophotometrically (an optical density at 260 nm of 1.0 is equivalent to 50 μg of nucleic acids per ml).

Solution hybridization. The procedure described by Townes et al. (18) was modified to quantitate hGH and ribozyme RNA. Ten picomoles of oligonucleotides (27- to 29-mer) were labelled with 20 pmol of [γ-^{32}P]ATP (6,000 Ci/mmol) and T4 kinase. Full-length, labelled oligonucleotides were separated on a urea–12% acrylamide gel, cut out, and eluted in 1 ml of 0.2× SET.

For hybridization, 10 μl of TNA (40 μg) was mixed with 20 μl of oligonucleotide-salt mix containing 6 volumes of H_2O, 3 volumes of 10× hybridization salts (3 M NaCl, 100 mM Tris-Cl [pH 7.5], 20 mM EDTA) and 1 volume of oligonucleotide (10,000 to 15,000 cpm) and incubated overnight at 45°C in an Ericomp thermocycler with cover heating. Hybridization samples were then diluted with 1 ml of S1 nuclease buffer (1 volume of 10× S1 buffer is 3 M NaCl, 0.3 M sodium acetate, 0.03 M zinc acetate [pH 4.5], 1 volume of herring sperm DNA [1 mg/ml], 8 volumes of H_2O) and incubated for 1 h at 37°C with 6 to 64 U of S1 nuclease (Gibco). The S1-resistant nucleic acids were precipitated with 100 μl of 6 M trichloroacetic acid for 1 h on ice and then collected on glass filters (Whatman GF/C) and washed three times with cold 3% trichloroacetic acid and 1% sodium PP_i and once with 95% ethanol before being counted in a scintillation cocktail for 5 min on a Packard scintillation counter.

The S1 nuclease concentration was optimized for each oligonucleotide used for hybridization according to the procedure described by Durnam and Palmiter (1). Each set of hybridizations included a standard curve with quantified amounts of a corresponding in vitro-transcribed RNA. For generating hGH and RzE1 in vitro, 2-μg transcripts of plasmid DNA containing the corresponding gene cloned behind a T7 promoter were linearized. For hGH, pGGH2.2 (10) was cut with *Hin*dIII, and for RzE1, pRzE1 was cut with *Nhe*I (12). After phenol extraction and ethanol precipitation, the template DNAs were incubated for 1 h at 37°C with 12.5 μl of TKB (20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 10 mM β-mercaptoethanol, 0.1 M KCl, 10 mM $MgCl_2$, 20% glycerol), 10 U of RNasin, 2.5 μl of 5 mM nucleoside triphosphates, 2.5 μl of 10 mM dithiothreitol and 100 U of T7 polymerase (Biolabs) in a total volume of 25 μl. After digestion with 23 U of DNase I, the transcripts were purified twice by phenol extraction and ethanol precipitated. The hGH and RzE1 transcripts were 2.1 and 0.25 kb in length, respectively. The RNA concentration was determined spectrophotometrically. Different amounts of RNA (1 to 160 pg) were diluted in 10 μl of TNA (40 μg) extracted from control liver tissue (C57BL/6 negative for hGH and RzE1 RNA) and hybridized overnight at 45°C with the corresponding oligonucleotides. The oligonucleotides are as follows: for hGH, 5′ GGCTGGTGGGCACTGGAGTG GCAACTT 3′ at nt 1607 to 1581 (against the 3′ untranslated region of hGH RNA), and for ribozyme RNA, 5′ TTTCGTCCTCACGGACTCATCAGTG 3′ (against the catalytic ribozyme region) and 5′ TCGTTGGGTCCACACGCTGC ACTGCAT 3′ (against the cloned loop region). The reaction mixtures for hGH RNA and ribozyme RNA were incubated with 40 U of S1 nuclease and 8 U of S1 nuclease, respectively.

To calculate the amount of RNA per cell in a tissue extract, we assumed that there are 10^8 hepatocytes per g of liver.

Cell culture. The 293 cells were cultured in Hg Dulbecco minimal essential medium and 10% fetal calf serum (Gibco BRL). CHO cells were grown in Dulbecco minimal essential medium (Gibco BRL) containing 200 mM asparagine, 200 mM proline, and 5% fetal calf serum. The plasmids for the transfection of CHO cells, pCMV hGH (12) and pAd/RSVhAAT (5), were purified by two rounds of CsCl gradient centrifugation. Transfection of 2.5×10^5 CHO cells in 6-cm dishes with 7 μg of pCMVhGH and 7 μg of pAd/RSVhAAT was carried out by calcium phosphate coprecipitation according to the protocol described earlier (12).

Animal studies. All animal studies were performed in accordance with the guidelines of the University of Washington animal care committee. Transgenic mice that express hGH from the gastrointestinal tract under the transcriptional control of the fatty acid-binding protein promoter (15) ($FABP^{-351\ to\ +21/hGH}$) were used. Specifically, line $FABP^{-351\ to\ +21/hGH}$ founder 39 heterozygous mice were mated to C57BL/6 mice. The positive offspring were identified by a sparse hair pattern detected at weaning and confirmed by serum hGH determinations. Purified recombinant adenovirus vectors were diluted in phosphate-buffered saline and injected in a volume of 100 to 200 μl into 5- to 7-week-old animals by tail vein injection. Serum samples were obtained from mice by retro-orbital bleeding.

hGH and hAAT analysis. hGH levels in CHO culture supernatant and mouse serum were estimated by enzyme-linked immunosorbent assay (ELISA) as described previously (11). The hGH ELISA has a linear range from 0.5 to 30 ng/ml and does not recognize mouse growth hormone in the serum. Human α1-antitrypsin (hAAT) concentrations were determined by an ELISA (5) with a sensitivity of 1 ng/ml.

RESULTS

Construction of recombinant adenovirus vectors that express the hGH ribozyme. The hammerhead ribozyme RzE1 (12) cleaves the hGH RNA within exon 4 at nt 1017. The homology of the regions flanking the catalytic ribozyme structure to the target RNA are 8 and 7 nt on the 5′ and 3′ ends, respectively. The ribozyme sequence was positioned in the center of a stable stem-loop structure, allowing for a correctly folded ribozyme region within the expressed RNA. Expression of RzE1 with polymerase III (Pol III) or T7 polymerase in transiently transfected cells repressed hGH synthesis almost completely (12). The effect of the expressed ribozyme was most likely the result of RNA cleavage and not an antisense phenomenon because a similar ribozyme with a single nucleotide mutation in the catalytic site of the molecule did not affect hGH production (12).

Relatively high intracellular concentrations of ribozyme RNAs would increase the likelihood of RNA cleavage in cells. Because recombinant adenovirus vectors are very efficient at gene transfer, the previously described ribozyme directed against the hGH mRNA (RzE1) was placed into recombinant E1-lacking adenoviral vectors flanked by adenovirus vaI DNA sequences with or without a Rous sarcoma virus long terminal repeat (RSV-LTR) or T7 promoter (Fig. 1). The vaI DNA sequence functions as an internal Pol III promoter. Alternatively, a T7 phage promoter placed upstream of the expression cassette can drive a high level of expression by coexpression of T7 RNA polymerase (10, 11). Thus, the gene from a nucleus-localized T7 RNA polymerase was cloned under the control of the PGK promoter, allowing constitutive expression of this RNA polymerase. Finally, to test expression from a more standard gene Pol II promoter, an adenovirus ribozyme expression cassette containing the RSV-LTR promoter was produced by cloning the promoter upstream of the vaI DNA promoter (Ad.RSVRzE1).

Function of recombinant adenovirus vectors that express ribozymes. In order to determine whether ribozymes expressed from these vectors could eradicate hGH production from transduced cells, CHO cells were transiently transfected with plasmids that expressed hGH and hAAT under control of the cytomegalovirus promoter previously shown to produce about 1,000 mRNA molecules per cell (12). The hAAT serves as a control for any general effects the adenovirus or ribozyme may have on overall cell function. There was a modest, general decline in both hGH and hAAT secretion into the culture medium when a control adenovirus vector containing the vaL cassette without the ribozyme was used. It is important to note that cells transduced with any of the three RzE1-containing adenovirus vectors (Fig. 1) had much reduced or no detectable hGH protein in the culture medium (Fig. 2). The Ad.RSVRzE1 vector and the combination of the Ad.T7RzE1 and Ad.T7pol vectors appeared to be the most efficient at eliminating hGH mRNA. This suggested that the transcription of ribozyme RNA was greater when either a Pol II or T7 promoter was placed upstream of the Pol III promoter than when the Pol III promoter was used alone. The effect of the expressed hGH ribozyme was specific because even though there are 60 potential NUH ribozyme cleavage sites in the hAAT cDNA, there was no specific decline in hAAT secretion into the culture medium (Fig. 2).

Adenovirus-mediated ribozyme transfer into mice. To dem-

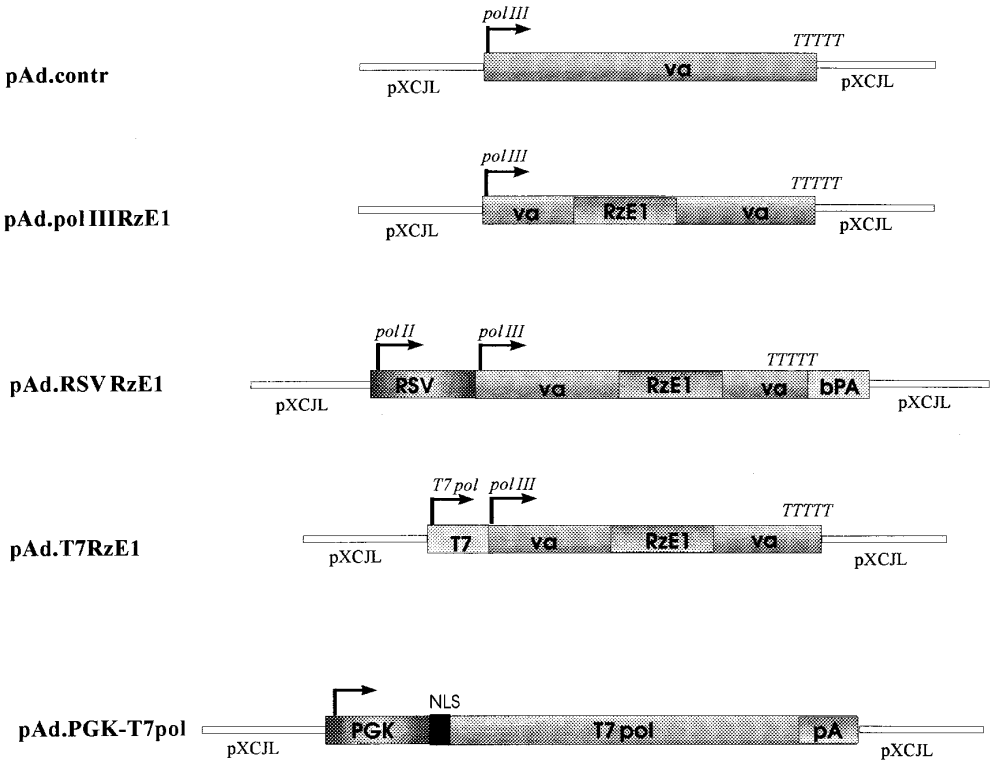

FIG. 1. Adenovirus ribozyme expression cassettes. The RzE1 against exon 4 of the hGH RNA embedded in a vaI RNA expression cassette was cloned into pXCJL.1 in order to produce recombinant adenovirus. In Ad.polIIIRzE1 a vaI internal Pol III promoter drives the ribozyme expression. The Pol III transcription stops at an oligo(dT) tract at the 3′ end of the expression cassette. In Ad.RSVRzE1 an RSV-LTR promoter can drive a Pol II transcription of the ribozyme gene. The Ad.T7pol vector produces T7 polymerase, which allows transcription from the T7 promoter. The site of initiation of transcription for the respective promoters is indicated. NLS, nuclear localization signal.

[1]onstrate that expression of ribozymes would function in the hepatocytes of animals, the different adenoviruses were infused into transgenic mice that express hGH from the gastrointestinal tract and liver. A large fraction of the hGH mRNA was found in nonhepatic tissues in these animals. For the initial studies, 5×10^9 PFU of Ad.polIIIRzE1 or of AdT7.RzE1-Ad.T7pol (1:1 ratio) was infused into transgenic animals. This dose of vector has been previously shown to transduce 80 to 90% of hepatocytes (9) with an average copy number of 15 to 30 genomes per cell (19). After gene transfer, serum samples

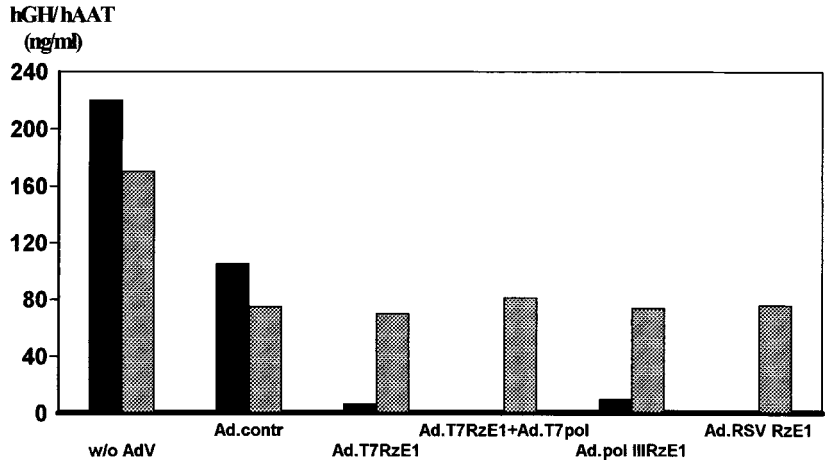

FIG. 2. Effects of different adenoviruses expressing hGH RzE1 on hGH synthesis in a cellular assay. CHO cells were transfected with pCMV hGH and pAd/RSVhAAT. The calcium phosphate-DNA precipitate was added together with 1,000 PFU of recombinant adenoviruses per cell, which was sufficient to transduce 100% of CHO cells. w/o AdV, no adenovirus added; Ad.T7RzE1+AdT7pol, a multiplicity of infection of 500 of each adenovirus added. The DNA and adenovirus mixture was left on CHO cells for 6 h, and then the medium was changed. Forty-eight hours later the supernatants were analyzed for hGH and hAAT levels by ELISA (performed in duplicate). Data are means of two independent experiments. The variation was less than 10% at each point. The infection of CHO cells by adenoviruses had a nonspecific effect which resulted in a general reduction of transgene expression. Ad.T7pol (multiplicity of infection, 1,000) had the same nonspecific effect on hGH and hAAT expression as that observed with the control adenovirus, Ad.contr (not shown). Solid bar, hGH; stippled bar, hAAT.

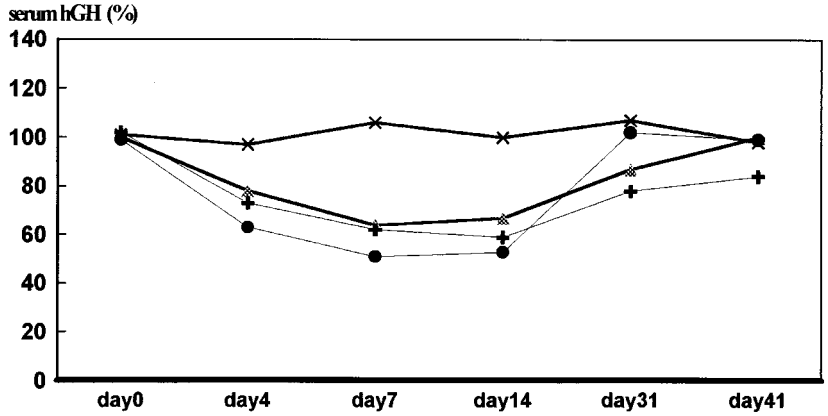

FIG. 3. hGH levels in mice treated with recombinant adenovirus vectors. The hGH transgenic mice were infused with different adenovirus vectors at a dose of 5×10^9 PFU on day 0. Serum samples were analyzed for growth hormone by ELISA at various points after adenovirus administration. Each point represents at least two animals. The variation between samples at each point was ≤15%. A serum hGH concentration of 100% corresponds to 22 ng of hGH per ml of serum, which was the mean value found in untreated transgenic animals. Symbols: ×, control adenovirus; △, Ad.polIII.RzE1; ✚, Ad.RSVRzE1; ●, Ad.T7RzE1-Ad.T7pol.

were analyzed for serum hGH (Fig. 3). Animals injected with a control vector had no change in serum hGH levels, whereas infusion of a vector containing hGH ribozyme decreased serum hGH by up to 50% over a 2-week period. Because of the nonhepatic sources of hGH production, this method could not be used to estimate the relative production of hGH from the livers of recipients.

To more accurately estimate the ability of expressed ribozymes to decrease or eliminate hGH mRNA from the livers of treated mice, RNA solution hybridization (Fig. 4A) was used to quantitate hepatic hGH mRNA. Untreated transgenic animals contained about 300 hGH mRNA copies per cell. In the first experiments, animals were infused with 5×10^9 PFU of different recombinant vectors that express the hGH ribozyme. The relative amount of hGH mRNA was expressed as a percentage of the amount in normal untreated transgenic animals. While animals infused with a control vector had no detectable decline in hGH RNA levels, animals infused with Ad.T7RzE1-Ad.T7pol had up to a 90% decline in hGH RNA levels (Fig. 4B). The decrease in expression was more modest with the Ad.polIIIRzE1 vector. The hGH mRNA trough occurred between days 4 and 14 and returned to pretreatment levels by 1 month postinfusion.

To determine if the quantity of ribozyme present correlated with the decline in hGH mRNA, ribozyme levels were quantitated for the animals infused with the vectors described above (Fig. 4A and C) with an oligonucleotide specific for the 3′ untranslated region of hGH mRNA. The level of ribozyme did correlate with the amount of hGH reduction. The estimated number of ribozyme copies per hepatocyte (cell) was relatively high. For every nanogram of RzE1 RNA per 10^6 cells present, there were about 17,500 RNA molecules per cell. Thus, during maximal ribozyme expression (2.2 ng/10^6 cells), the molecular ratio of ribozyme to target mRNA was about 50 to 1. Ribozyme expression declined to low or undetectable levels over a period of about 1 month. This is not unexpected because recombinant adenovirus vectors are eventually cleared in immunocompetent animals, whereas gene expression is indefinite in *scid* mice, which are deficient in B and T cell immunity (14).

In order to determine whether an infusion of a higher dose of adenovirus could eradicate the hGH mRNA, two groups of hGH transgenic mice (three per group) were infused with 10^{10} PFU of either Ad.polIIIRzE1 or Ad.T7RzE1-Ad.T7pol (1:1). Infusion of this dose of adenovirus into mice is known to transduce most of the hepatocytes. Seven days after the ribozyme-containing adenoviruses were administered, the amount of hGH mRNA that remained was determined as a percentage of the hGH mRNA concentration in animals that received a control adenovirus. The hGH mRNA amounts in mice that received Ad.polIIIRzE1 and those that received Ad.T7RzE1-Ad.T7pol were 14.5% ± 6.3% and 3.5% ± 2.5%, respectively.

DISCUSSION

Functional ribozymes expressed from hepatocytes offer a new potential therapy for a number of serious infectious diseases, including viral hepatitis. For DNA viruses, ribozymes can be designed against key RNA molecules that will interfere with viral replication. For RNA viruses such as hepatitis C virus, ribozymes can, in theory, be designed to directly cleave the genome. There are two major obstacles to developing such therapies for clinical use. The first is the inability to predict ribozymes that will function against native RNA in cells. This problem has been overcome in part by the recent development of a ribozyme library that can be used to obtain sequences that have a high probability of functioning against native RNA in vivo (3). This reduces the hit-and-miss approach of testing potential ribozyme cleavage sites present in most RNA molecules. The second problem is the inability to express high enough levels of ribozymes in all cells of a particular target tissue. By surrounding potential ribozymes with sequences known to stabilize RNA, greater concentrations of ribozymes can be achieved. Furthermore, the development of better promoters may also be useful for achieving higher levels of expressed ribozymes in cells.

With the modest advances in somatic gene transfer, sequences that allow for expression of ribozymes can now be delivered by a number of vector systems (7). Recombinant adenovirus vectors offer efficient gene transfer to the liver and other tissues, but their use has been limited because of an associated immune response directed against transduced cells. Nevertheless, there is cautious optimism that these problems can be solved (2, 6, 14) or that new vectors (7) can be developed in the near future.

For efficient elimination of RNA molecules by ribozymes, expression should be optimized. The studies presented here demonstrate that a Pol III promoter, while adequate, may not be the best. The presence of a T7 promoter and concomitant

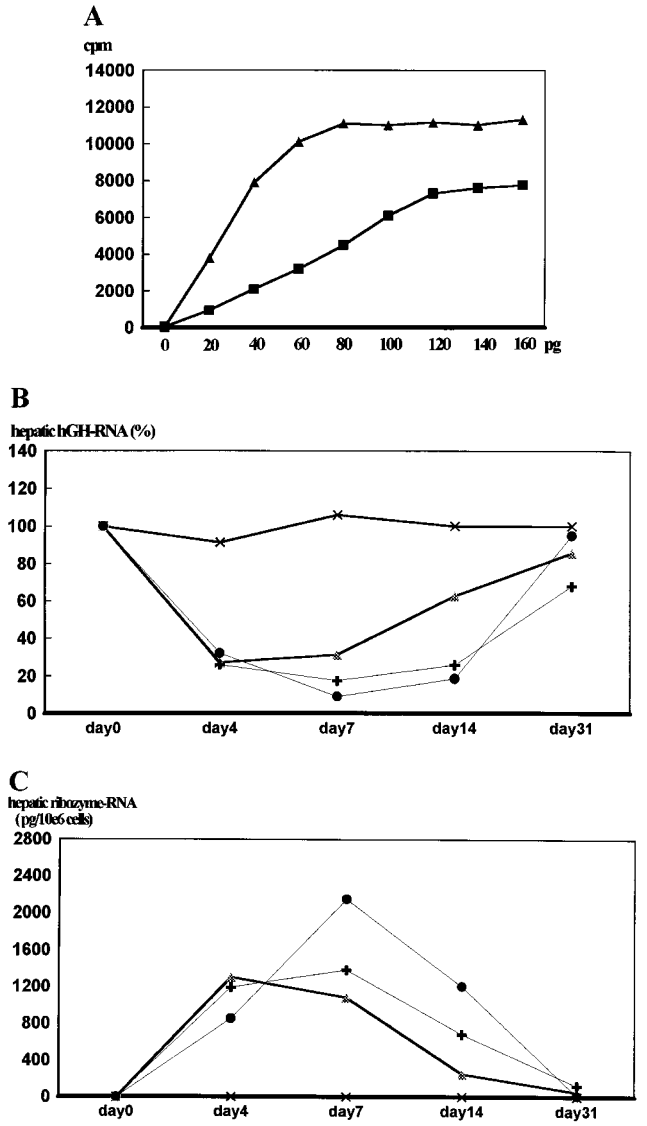

FIG. 4. hGH and ribozyme RNA levels in adenovirus-treated mice. Transgenic mice were infused with 5×10^9 PFU of recombinant adenovirus. (A) Standard curves for quantifying hGH and ribozyme-va RNA by solution hybridization with oligonucleotide probes. Data are expressed as S1-resistant counts per minute as a function of standard RNA concentration for hGH (▲) or RzE1 (■) (see Materials and Methods for details). Forty micrograms of TNA from the livers of hGH recipients were analyzed for hGH mRNA (B) and ribozyme RNA (C) by solution hybridization. Data are presented as amounts of RNA relative to that in untreated animals. An hGH concentration at 100% corresponds to 350 pg of hGH RNA per 10^6 cells. The mean concentration for two animals analyzed for each time point are given. The variation between points was less than 15%. Assuming that there are 10^8 cells per g of liver, there are approximately 300 copies of hGH mRNA and 17,500 copies of ribozyme RNA per cell (1 ng of ribozyme RNA is about 8×10^9 molecules). Symbols: ×, control adenovirus; △, Ad.polIII.RzE1; ✚, Ad.RSVRzE1; ●, Ad.T7RzE1-Ad.T7pol.

production of T7 polymerase appeared to result in greater diminution of hGH RNA. The reduction of hGH mRNA correlated well with ribozyme RNA levels. Because the Pol III promoter was downstream of the T7 or RSV-LTR promoter, these studies cannot distinguish between the relative proportion of transcripts produced from each of the respective promoters. Further studies are required to address the relative amount of ribozymes expressed from each of the promoters.

Although reasonably high concentrations of ribozyme RNA were achieved in our model system, the ratio of ribozyme RNA to target RNA required to eliminate the majority of RNA was in the range of 50:1 to 100:1. It is unclear whether the low level of hGH mRNA that was detected after adenovirus administration was the result of hepatocytes that escaped transduction, RNA molecules that escaped ribozyme cleavage, cleavage products detected by solution hybridization, or low-level transgene expression in nonparenchymal liver cells. Nevertheless, the absolute quantity of ribozyme needed to correct a clinical disorder will more than likely be highly variable. An adenovirus containing a functional ribozyme under the transcriptional control of the cytomegalovirus promoter directed against the *ras* oncogene has been shown to revert the phenotype of cultured cancer cells to that of normal cells (4). It is important to note that because the expression sequences are relatively short, higher concentrations of ribozymes could be produced in a vector containing multiple ribozyme expression sequences. Additionally, two different ribozymes directed against different portions of a specific RNA molecule could be expressed from a single vector. This may allow for more efficient cleavage and decrease the likelihood of selecting ribozyme-resistant mutant sequences.

It is assumed that the fall-off of ribozyme-mediated gene expression in animals was related to an immune response directed against adenovirus vectors. Further studies are required to establish whether the Pol III promoter and/or the T7 promoter will function long-term in tissues. Additionally, it is not possible to exclude long-term toxicity from constitutive high-level ribozyme expression. Recent studies demonstrate that a transgenic mouse expressing a ribozyme directed against the beta 2 microglobulin mRNA was able to decrease as much as 90% of the corresponding RNA in some tissues (8). Importantly, expression in these animals is stable, making general toxicity unlikely.

The study described here clearly demonstrates that delivery of an adenovirus that expresses a ribozyme to a somatic tissue such as the liver results in almost complete eradication of the targeted RNA. It is not possible to exclude the possibility that some of the decrease in hGH mRNA was due to an antisense rather than a ribozyme cleavage mechanism. There are several factors that make an antisense mechanism unlikely. First, when a mutation was placed into the catalytic region of the ribozyme and expressed from cells producing hGH, there was no effect on hGH production (12). Second, optimal diminution of RNAs with antisense molecules generally requires consecutive sequences of homology that are greater than 17 nt. The hGH ribozyme used here contains 8 and 7 nt of homology which are interrupted by the catalytic domain. Although the transgenic model used to demonstrate efficacy is a nonclinical one, it demonstrates proof of principle that (i) somatic gene transfer resulting in the expression of ribozymes is possible and (ii) the level of ribozyme expression correlates with the ability to specifically eradicate a specific mRNA from a target cell in animals.

The initial success of nearly eliminating a specific RNA from liver tissue is important for the eventual use of these types of strategy in clinical medicine. Furthermore, this study clearly demonstrates that the use of expressed ribozymes delivered to animals via gene therapy vectors will need to be studied in greater detail to obtain the desired absolute quantity or the desired length of expression. Notably, not all disorders potentially treatable with ribozymes may require long-term ribozyme expression.

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