

Efficient expression of ribozyme and reduction of stromelysin mRNA in cultured cells and tissue from rabbit knee via Adeno-associated Virus (AAV)

Research Article

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Summary

The potential use of Adeno-associated virus (AAV) as an efficient gene delivery vector is increasingly being recognized in human gene therapy. We have investigated the utility of recombinant AAV (rAAV) vectors for the delivery and expression of hammerhead ribozymes targeted against a cellular mRNA encoding a matrix metalloproteinase, stromelysin. Stromelysin expression has been linked to the pathogenesis of human osteoarthritis. We have constructed several rAAV backbone plasmids containing single or multiple hammerhead ribozymes expression cassettes under the control of either the tRNA, U1, or U6 promoter, and have used these plasmids to generate rAAV. These rAAV vectors also contain a selectable marker, the truncated nerve growth factor receptor (NGFR) driven by the cytomegalovirus immediate early gene promoter. rAAV expressing stromelysin-specific ribozyme transduced *ex vivo* cultured rabbit synovial fibroblasts (RSFs) with a greater than 95% efficiency. Stable ribozyme expression can readily be detected throughout the life span of RSFs in culture. Furthermore, ribozyme mediated knockdown of stromelysin mRNA was detected in RSFs infected by a rAAV containing the tRNA-based transcription unit.

I. Introduction

Human arthritic disorders are the major cause of chronic disability among adults (Vincenti, Clark, and Brinckerhoff, 1994). Several etiological factors have been reported to play roles in the initiation and progression of degenerative cartilage disorders. For example, a group of proteolytic enzymes, including several members of the matrix metalloproteinase (MMP) family, are strongly implicated in the pathology of arthritis (Vincenti, Clark, and Brinckerhoff, 1994) (Cawston, 1996). MMPs degrade extracellular matrix components such as collagens, gelatins, proteoglycan, and fibronectin (Woessner, 1991).

MMPs normally play an important role in embryogenesis, wound healing, and tissue remodeling

(Werb, Alexander, and Adler, 1992); however, abnormal expression has been implicated in a wide range of human diseases such as atherosclerosis, arthritis (Murphy and Hembry, 1992), glomerulonephritis, corneal ulceration, periodontitis, encephalomyelitis, and tumor metastasis (Stetler-Stevenson, Aznavoorian, and Liotta, 1993).

Most MMPs (except for MMP-11 and MT-MMP) are secreted into the extracellular matrix as proenzymes. Stromelysin (MMP-3) may be a key mediator in arthritic diseases. It degrades proteoglycans and a wide range of other matrix components (Woessner, 1991) and activates the proenzyme forms of collagenase (Suzuki *et al.*, 1990), gelatinases (Miyazaki *et al.*, 1992) (Ogata, Engchild, and Nagase, 1992), and other MMPs, leading to initiation of a proteolytic cascade. Synovial fibroblasts derived from

osteoarthritic or rheumatoid synovium produce high levels of stromelysin upon stimulation (Brinckerhoff and Auble, 1990). In addition, there is a significant up-regulation of stromelysin and other MMPs in articular tissues from patients with osteo- or rheumatoid arthritis (Hembry *et al.*, 1995) (Okada *et al.*, 1992). Thus targeted inhibition of one or more of these proteolytic activities may be a valid therapeutic approach for arthritis.

Ribozymes are RNA-based enzymes that have the ability to cleave RNA molecules in a sequence-specific manner. Sequence specificity comes from the base-pairing of ribozyme sequences with nucleotides spanning the cleavage site of the target RNA. Cleaved RNA is rapidly degraded in the cell, resulting in a decrease in expression of the encoded protein. Because of their sequence-specificity, ribozymes can be utilized as a therapeutic agent to down-regulate a specific RNA in the background of other cellular RNAs (Rossi, 1999). We have previously reported that a chemically stabilized synthetic hammerhead ribozyme targeting nucleotide position 1049 in the human stromelysin mRNA significantly reduced the target RNA levels upon intra-articular injection (Flory *et al.*, 1996). In this study, we have used recombinant AAV (rAAV) for the expression of hammerhead ribozymes targeting stromelysin mRNA at the same nucleotide position.

Because ribozymes can function as RNA molecules they can be synthesized in a variety of transcription units besides RNA polymerase II mRNAs. However, the success of ribozyme efficacy is dependent upon at least three parameters: ribozyme intracellular localization, ribozyme cleavage activity, and ribozyme RNA levels. Ribozyme localization is somewhat dependent upon the inherent characteristics of the particular transcription unit used and of the RNA elements contained within the transcript (Bertrand *et al.*, 1997; Thompson *et al.*, 1995b). Ribozyme cleavage activity is effected by flanking sequences contained within the transcript (Chowrira, Pavco, and McSwiggen, 1994; Thompson *et al.*, 1995a) and ribozyme RNA levels are limited by promoter strength and ribozyme RNA stability (Thompson *et al.*, 1995a) (Thompson *et al.*, 1995b) (Rossi, 1999). Thus, we have designed and constructed a variety of ribozyme transcription units that maintain catalytic activity and have the potential to accumulate to high levels in the nucleus or to utilize endogenous splicing machinery to promote ribozyme/target RNA hybridization. We have previously reported the construction of a modified tRNA promoter for the expression of an HIV specific hammerhead ribozyme (Thompson *et al.*, 1995a). In this report, we compare the efficacy of ribozymes expressed from modified tRNA, U6 snRNA, and U1 snRNA transcription units.

Ribozyme expression *via* retroviral and adenoviral vectors has been reported in several studies (Macejak *et al.*, 1999) (Thompson *et al.*, 1995a), however the utility of AAV for ribozyme expression has not been explored

extensively. Retroviruses and adeno-associated virus (AAV), by virtue of integrating into chromosomal DNA, are attractive gene delivery vehicles for the treatment of human disorders in which a long-term therapy is essential for an effective treatment. The attractive features of AAV as a vector are non-pathogenicity, low immunogenicity, stable and efficient expression of transgenes from the integrated or episomal form, infection of non-dividing cells, broad host range, generation of high titer (8×10^8 IU/ml), and physically stable virions. Recently, AAV-mediated expression of ribozymes targeting a mutated rhodopsin mRNA was shown to slow the rate of photoreceptor degeneration in a transgenic rat model (Lewin *et al.*, 1998).

AAV is a small non-pathogenic human parvovirus whose genome (4681 bases) is a single-stranded DNA of either polarity, flanked by inverted terminal repeats (ITRs). AAV-ITRs are 145 bases in length and function as the sole cis-acting elements essential for chromosomal excision (rescue), integration, replication, and encapsidation of nascent viral DNA (Muzyczka, 1992). AAV infects a variety of mammalian cells with a broad host range; however, some human megakaryocytic cell lines are refractory to AAV infection, presumably because they lack the putative AAV receptor (Ponnazhagan *et al.*, 1996). AAV can establish a lytic or latent infection in mammalian cells in the presence or absence of a helper virus, respectively. Productive AAV infection is dependent on a helper DNA virus, which is usually adenovirus; however, herpes virus and vaccinia virus can substitute for the helper functions of adenovirus (Schlehofer, Ehrbar, and zur Hausen, 1986) (Thomson *et al.*, 1994). In the absence of a helper virus, the AAV genome preferentially integrates into a defined region of human chromosome 19 (q13.3-qter), and establishes a stable latent infection (Kotin *et al.*, 1990) (Samulski *et al.*, 1991). In this study, we demonstrate the utility of rAAV as a vector for ribozyme expression in primary synovial cells of the rabbit knee, and report the feasibility of rAAV-mediated gene therapy for arthritic disorders.

II. Results

A. rAAV-LacZ transduction of synoviocytes and chondrocytes.

Although AAV is known to infect a variety of mammalian cell lines, some cells, such as megakaryocytic MB-O2 and MO7e cell lines, are refractory to AAV infection (Ponnazhagan *et al.*, 1996). Therefore, we first tested whether AAV can infect primary cells from the intra-articular lining of rabbit knees. Primary synovial fibroblasts from rabbit knee tissue (RSFs) were cultured *ex vivo* and were infected with rAAV containing a β -galactosidase gene driven by the cytomegalovirus immediate early promoter. rAAV-mediated LacZ expression was readily detected in RSF cells (**Figure 1**). The LacZ expression was detectable 30 hrs post-infection and remained stable during the entire life span of primary RSF cells in culture (5 – 7 passages, data not shown).

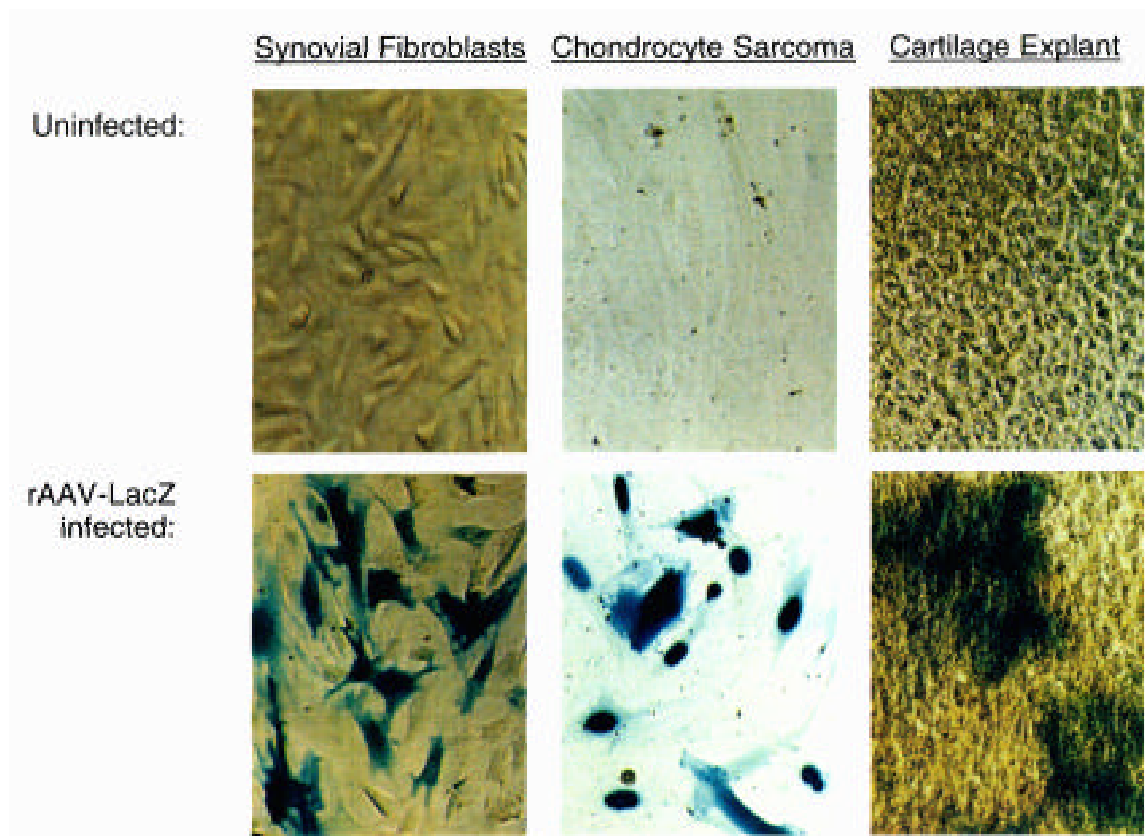


Figure 1: rAAV Transduction of Synoviocytes and Chondrocytes. -galactosidase expression in uninfected or rAAV-LacZ infected cells. Synovial fibroblasts (primary culture isolated from rabbit knee), chondrocyte sarcoma (human cell line SW1353), and cartilage explants (rabbit femoral groove tissue) are shown, rAAV-LacZ contains the LacZ gene encoding -galactosidase.

Rabbit cartilage explants infected with rAAV also demonstrated LacZ expression (**Figure 1**). It is not clear, however, whether the infected cells in the cartilage explants were chondrocytes or another cell type(s). In addition, rAAV-mediated LacZ expression can readily be detected in the chondrocyte sarcoma cell line SW1353 (**Figure 1**). Together, these observations indicate that rAAV-LacZ can infect the *ex vivo* cultured RSFs and cells of rabbit cartilage explants.

B. rAAV vectors and ribozyme transcription units

The backbone plasmid, in which all ribozyme transcription units were inserted, contains a modified nerve growth factor receptor (NGFR) gene, driven by the cytomegalovirus early promoter, as a selection marker. Because of a deletion in the c-terminus of the cDNA, NGFR is biologically inactive when expressed from rAAV-infected cells. However, rAAV-mediated expression of this altered NGFR can be readily detected on the membrane of transduced cells by Fluorescence Activated Cell Scanning (FACS; see **Figure 5A**).

Several ribozyme transcription units were cloned into the backbone rAAV plasmid, and the resulting plasmids were used to generate rAAV particles. The rAAVs used in this study are shown in **Figure 2** and transcription units are shown in **Figure 3A**. The modified tRNA transcription unit was originally developed to increase the copy number and maintain catalytic activity of ribozyme expressed inside cells (Thompson *et al.*, 1995a). To generate ribozyme with minimal flanking sequence we chose the U6 snRNA promoter since the U6 promoter is extragenic except for the "G" at +1. We have developed a U6+1-Rz (called "U6C", see **Figure 3A**) that contains a 5'/3' stem interaction for stability, analogous to that in the improved "TRZ-motif" (Thompson *et al.*, 1995a). Certain splicosomal RNAs interact with pre-mRNA molecules during normal pre-mRNA processing and the sequences which mediate these interactions in the context of a snRNP are known. The first snRNP to contact a pre-mRNA contains U1 RNA which hybridizes to the 5' splice site. We reasoned that replacing the U1 RNA sequences that hybridize to the 5' splice site with a ribozyme (**Figure 3A**) could promote hybridization of a U1-ribozyme snRNP to a target cleavage site in the nucleus.

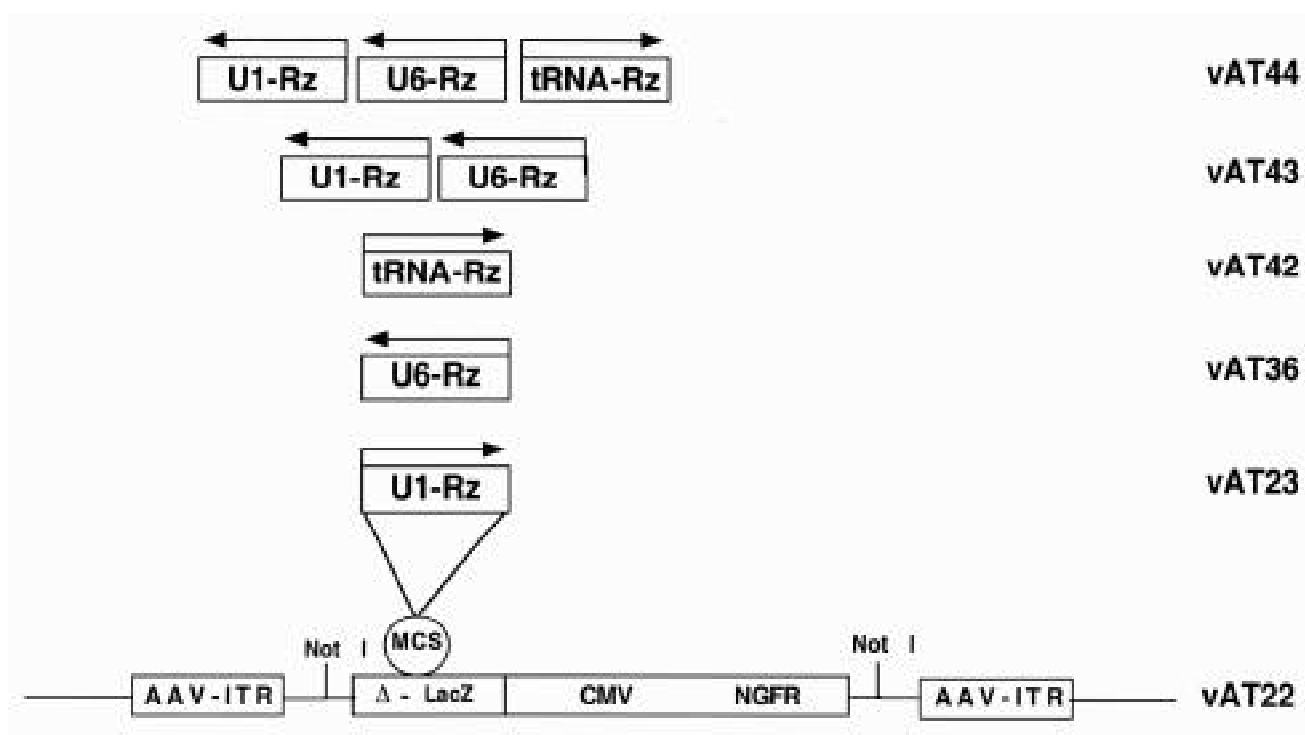


Figure 2: Schematic Representation of rAAV Vectors. Recombinant viruses contain the ribozyme (Rz) transcription units as noted. vAT22 is the "empty" (contains no ribozyme transcription unit) control virus derived from the backbone plasmid pAT22. AAV-ITR, AAV inverted terminal repeat sequence; Δ -LacZ, sequence derived from the LacZ gene to increase genome size for efficient packaging; MCS, multiple cloning site; CMV, cytomegalovirus immediate early gene promoter; NGFR, truncated nerve growth factor receptor cDNA.

To confirm that cleavage activity was maintained by each ribozyme within the U1-, U6-, or tRNA-derived RNA transcripts, ribozyme RNAs were synthesized *in vitro* to contain the flanking sequences predicted for each transcription unit. The chimeric ribozyme RNAs were then tested for cleavage activity against a RNA containing the target site. All ribozyme transcripts contained comparable cleavage activity *in vitro* that was partially reduced compared to a chemically synthesized ribozyme with no extraneous sequence (**Figure 3B**). To further characterize these ribozyme transcripts within cells, we analyzed their stability in 293 cells following Actinomycin D treatment (**Figure 3C**). The U6- and tRNA-derived ribozyme RNAs had a similar stability with a half-life of 1-1.5 h. On the other hand, no reduction in the level of U1-derived ribozyme RNA was detected over 4h. In addition, we observed that the U1-ribozyme was immunoprecipitable with anti-trimethyl-G or anti-SM antibodies (our unpublished results), indicating that the U1-ribozyme transcript goes through a maturation/modification process analogous to authentic U1 RNA.

C. Efficient transduction and expression of a stromelysin-specific hammerhead ribozyme in RSFs

Since rAAV could infect RSFs and the ribozyme chimeric transcripts retained cleavage activity, a series of rAAV containing one or more expression cassettes encoding ribozymes targeting stromelysin mRNA were prepared (**Figure 2**). Although ribozyme expression was detected in 293 cells, the promoter activity of the ribozyme transcription units in primary RSFs was uncertain. Thus, we investigated ribozyme expression in rAAV-infected RSFs. RSF cells were infected (moi = 20) with rAAV containing either single or multiple transcription units (see **Figure 2**). Northern analyses demonstrate that each transcript was expressed in RSFs (data from initial experiments not shown, but see **Figure 5B**). We then investigated the duration of ribozyme expression. RSF cells were infected with a subset of rAAV (containing either U6 or both U1 and U6 transcription units). Cells were harvested and total RNA purified for Northern analysis at different cell passages post-infection. Cells were infected at passage 3, and analyzed for RNA 48 hours after infection, prior to passaging (**Figure 4**, P3 lane).

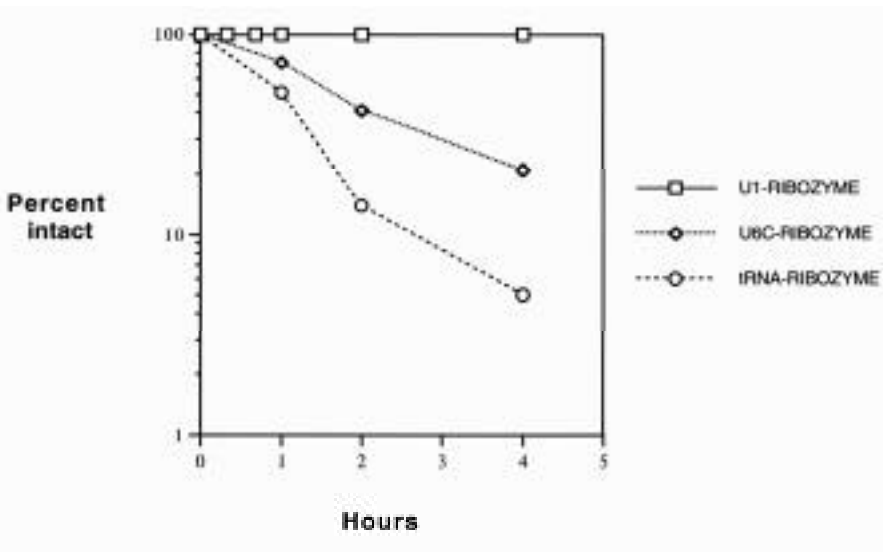
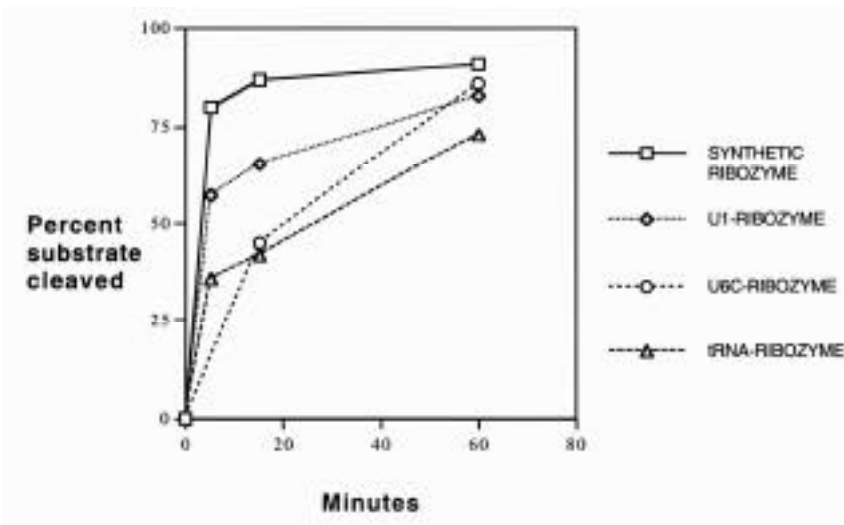
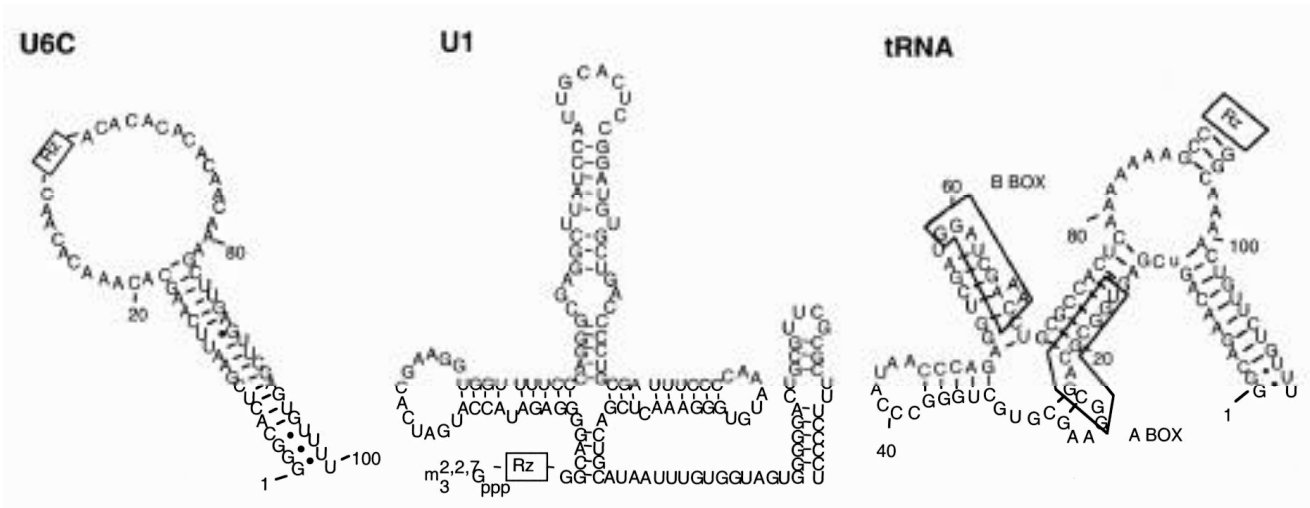


Figure 3: Ribozyme Transcription Units. A) Predicted RNA secondary structures of ribozyme transcripts used. Rz, site of inserted ribozyme. B) *In Vitro* cleavage activity of chimeric ribozyme transcripts shown in A. C) Stability of ribozyme transcripts in 293 cells, following treatment with Actinomycin D.

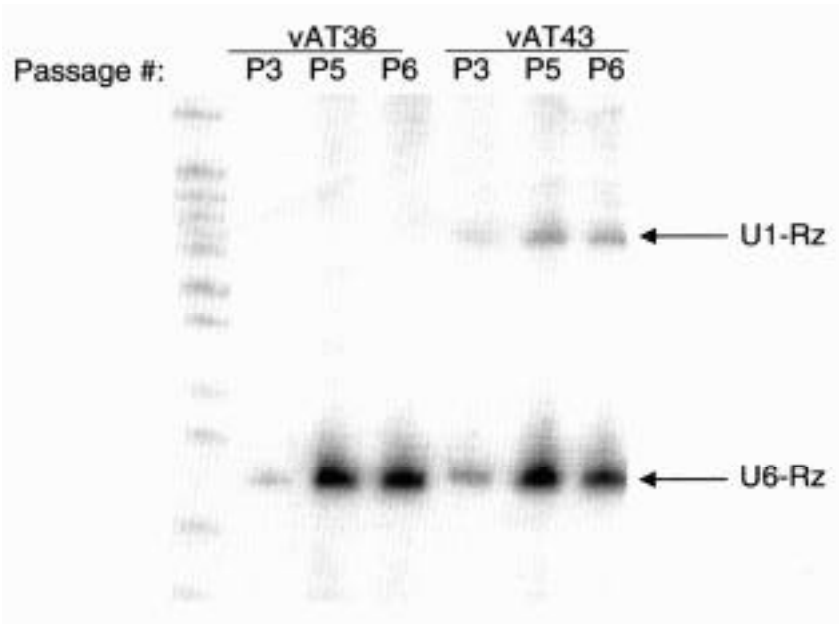


Figure 4: Expression of Ribozyme Throughout the Life Span of rAAV-Infected RSF Cells. Northern analysis of total RNA from RSFs infected with rAAVs as noted. P3, P5, P6, passage 3, 5, 6, respectively. Arrows denote U1-derived (U1-Rz) or U6-derived (U6-Rz) ribozyme transcripts.

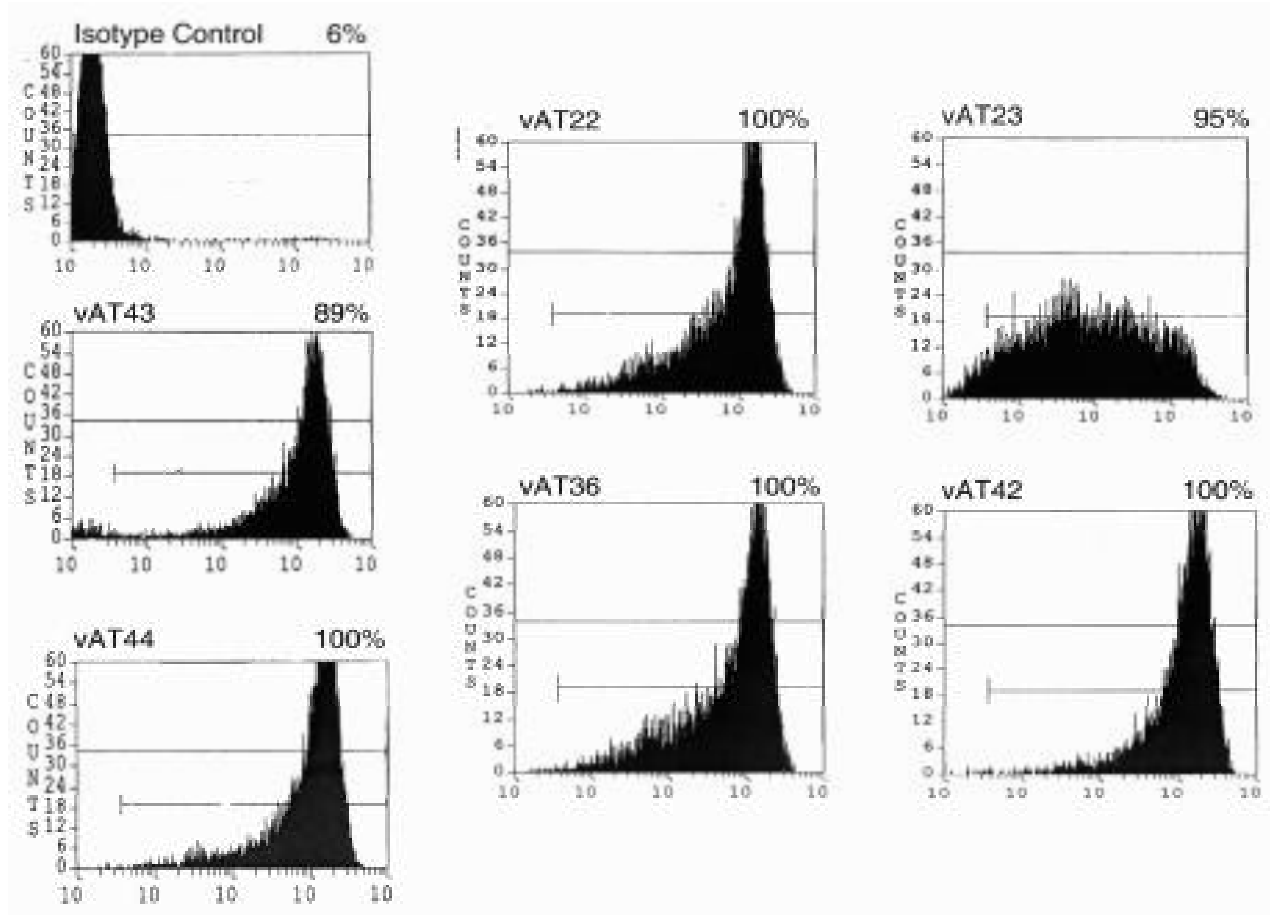
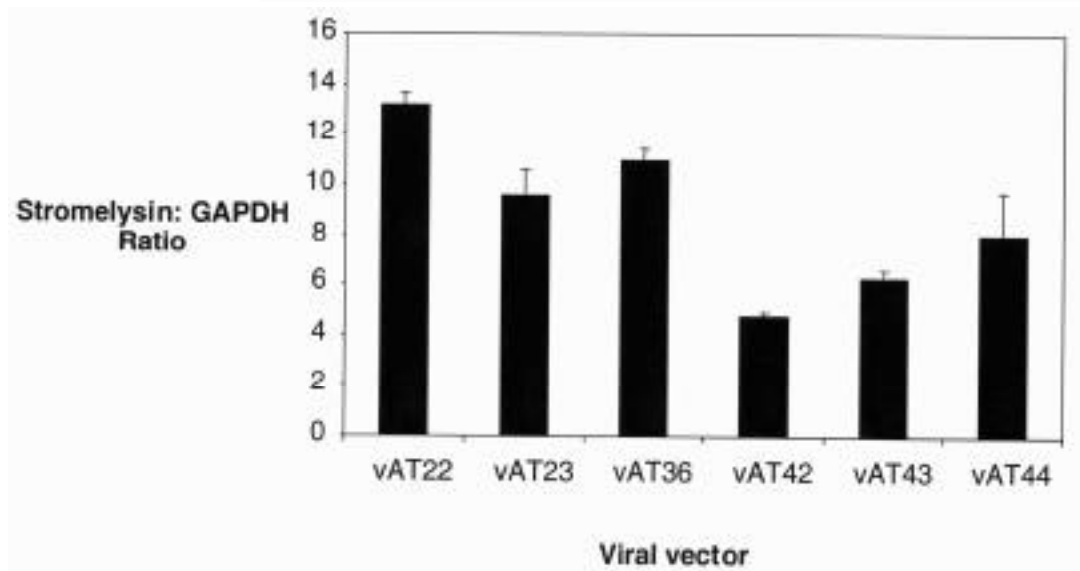
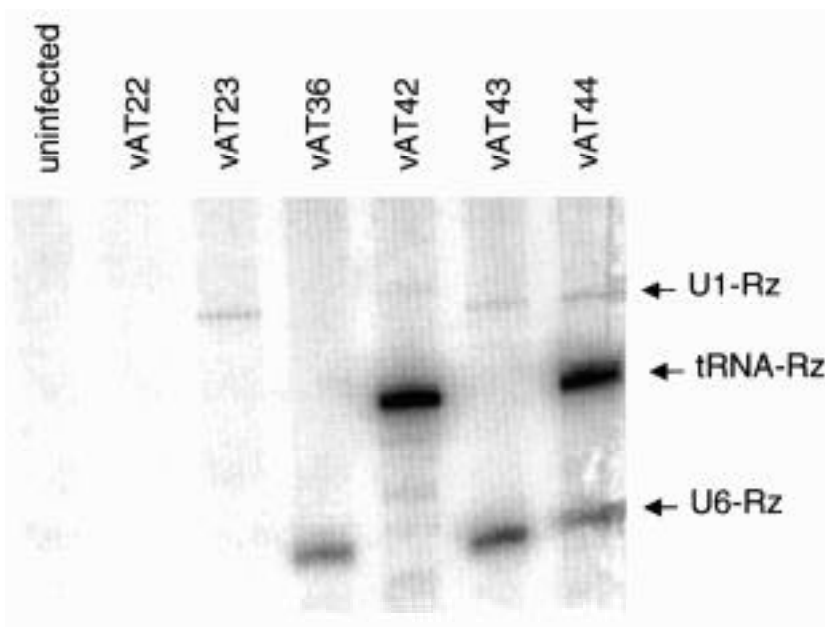


Figure 5: Transduction, rAAV-Mediated Ribozyme Expression and Reduction in Stromelysin RNA Levels in RSF Cells. A) FACS analysis of NGFR expression in cells infected with rAAVs as noted. Proportion of cells transduced is noted for each rAAV infection.

**Figure 5 (Continued):
Transduction, rAAV-
Mediated Ribozyme
Expression and
Reduction in
Stromelysin RNA**

Levels in RSF Cells. B) Northern analysis of total RNA from RSFs infected with rAAVs as indicated. Arrows denote ribozyme (Rz) transcripts. **C)** RNase Protection analysis of stromelysin RNA in RSFs infected with rAAVs as indicated. A, B, and C were all performed with same populations of rAAV-infected cells.



Ribozyme expression from U6 and U1 transcription units is readily detectable during the entire life span of RSF cells in culture (6 passages; **Figure 4**). Ribozyme expression from the U1 and U6 promoters significantly increased upon passaging. Transduction efficiencies as determined by NGFR expression using FACS analysis were similar (>90%) with each of the rAAVs tested.

D. Effect of stromelysin specific ribozyme expression on target RNA

The inflammatory cytokine IL-1 is implicated in the initiation and progression of arthritic disorders in human and animal models (Cawston, 1996). IL-1 induced stromelysin mRNA expression peaks at 8-12 hours post-

treatment in RSFs (our unpublished results). RSFs were infected with rAAV, induced with IL-1 for 10 hours beginning 36 hours post-infection, and harvested 46 hours post-infection. Transduction efficiency with each virus was greater than 95%, with the exception that the vAT43 infection gave 89% transduction efficiency (**Figure 5A**). The cell surface expression of NGFR was heterogenous over the RSF population, particularly with vAT23, as evidenced by the broad shifted peak. Northern analysis demonstrated ribozyme expression with each of the infections (**Figure 5B**). The steady state levels of ribozyme were greatest in the tRNA-ribozyme chimera, followed by the U6- and U1-derived RNAs. The tRNA-ribozyme levels were reduced in the trimeric expression construct (vAT44) compared to the monomer cassette (vAT42), indicating possible promoter interference.

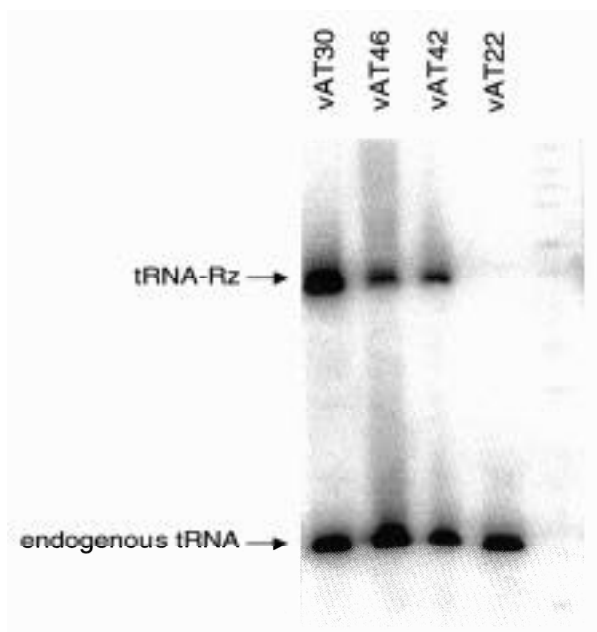
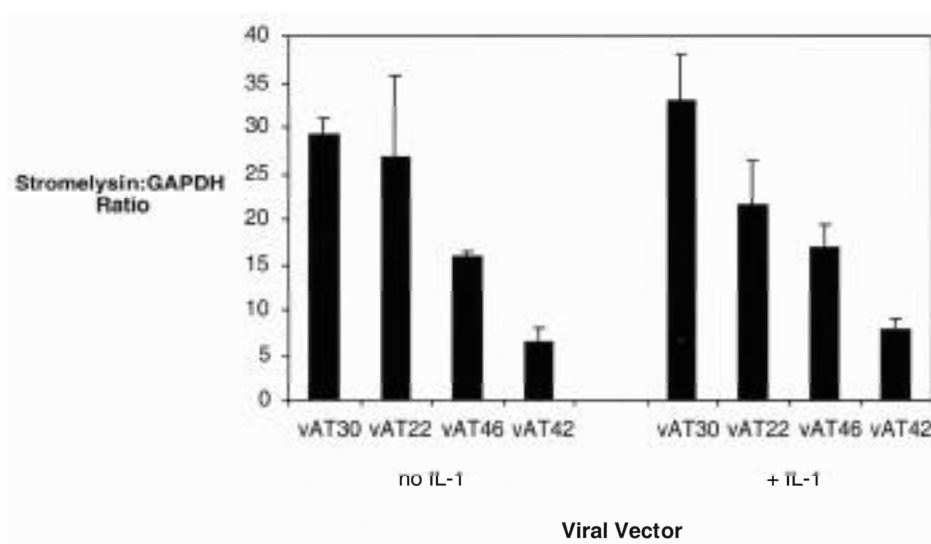


Figure 6: rAAV-Mediated Ribozyme Expression and Effect on Stromelysin RNA Levels with Active, Attenuated, or Irrelevant Ribozyme. **A)** Northern analysis of total RNA from RSFs infected with rAAVs as indicated. **B)** RNase Protection analysis of stromelysin RNA in RSFs infected with rAAVs as noted. Sets of cells not treated with IL-1 (no IL-1) or pretreated for 10h (+IL-1) prior to infection are indicated.



The level of stromelysin RNA was induced upon rAAV infection alone 3-4 fold over that of IL-1 treatment alone (data not shown). Thus, in terms of ribozyme efficacy, cells infected with the empty vector, vAT22, is the appropriate control for comparison. The level of stromelysin target RNA was slightly reduced in RSFs expressing U1- or U6-derived ribozymes compared to RSFs infected with the control vector (**Figure 5C**, vAT23 or vAT36 versus vAT22; $p < 0.05$). The greatest reduction (>60%) in stromelysin RNA was observed in RSF expressing tRNA-ribozyme (vAT42 versus vAT22; $p < 0.05$). Interestingly, the U1- and U6-derived ribozyme appeared to have an additive effect in vAT43 infected

cells, but this effect was lost in cells infected with virus containing the trimeric ribozyme cassette (vAT44).

E. Reduction in target RNA is due to a ribozyme mechanism.

To confirm that the reduction in stromelysin RNA observed was due to ribozyme cleavage activity and not due to over-expression of the tRNA motif itself, we compared target RNA levels in RSFs infected with rAAV encoding either a ribozyme targeting an irrelevant sequence (vAT30), the ribozyme targeting site 1049 of stromelysin (vAT42), or an attenuated version of the stromelysin ribozyme (vAT46); all genes were driven by the tRNA promoter within an otherwise

identical rAAV vector. The attenuated control contains two base substitutions in the ribozyme catalytic core domain. This attenuated analog is still capable of binding the target site, but has reduced cleavage activity. Previous work demonstrated the greatly reduced catalytic effects of base substitutions in the ribozyme core on ribozyme activity *in vitro* (Ruffner, Stormo, and Uhlenbeck, 1990) as well as in cell culture and *in vivo* (Jarvis *et al.*, 1996) (Flory *et al.*, 1996).

The active and attenuated versions of the stromelysin-specific ribozyme and the irrelevant HIV-specific ribozyme were each readily detectable in these cells by Northern analysis (**Figure 6A**). The probe used in this Northern blot was designed to hybridize to tRNA sequences, such that the same probe will detect all three ribozyme transcripts as well as endogenous tRNA. After normalization to endogenous tRNA, it appears that the irrelevant (HIV-specific) ribozyme (vAT30) level is the greatest, yet no reduction in the level of stromelysin RNA is observed with rAAV encoding irrelevant ribozyme (**Figure 6B**). As observed previously, RSF infected with rAAV containing active ribozyme (vAT42) significantly reduced stromelysin mRNA levels as compared to either control virus (**Figure 6B**; $p < 0.05$). This reduction was not dependent upon IL-1 treatment since vAT42-infected RSFs without IL-1 also displayed reduced target RNA levels compared to RSFs infected with either vector control vAT22 or vAT30 ($p < 0.05$). The attenuated version of the stromelysin-specific ribozyme (vAT46) did not show a statistically significant decrease in stromelysin mRNA levels relative to the control vector vAT22 in the presence or absence of IL-1. Statistical analysis was done with Kruskal-Wallis One Way Analysis of Variance on Ranks to test for differences between groups. When significant, post-hoc analysis was done using the Dunnett's test and p values < 0.05 were considered significant. These results confirm that the decrease in stromelysin mRNA observed in vAT42 infected cells is largely due to a ribozyme cleavage-dependent mechanism.

III. Discussion

Arthritis is a chronic debilitating disease affecting 16-40 million people in the USA (Lawrence *et al.*, 1989). There is a strong correlation between the levels of matrix metalloproteinase (MMP) expression and secretion from the intra-articular cell lining of the joint, and the initiation and progression of the extracellular matrix degeneration seen in human arthritic disorders, such as osteoarthritis and rheumatoid arthritis. In addition, MMPs are directly implicated in tissue remodeling and tumor metastasis in the angiogenic phase of tumor growth. For example, MMP2 is shown to trigger the migration of breast epithelial cells *via* specific cleavage of laminin-5 (Ln-5) (Brooks *et al.*, 1996) (Giannelli *et al.*, 1997). The cleaved form of Ln-5 is commonly discovered in tumors and in tissues undergoing remodeling, but not in quiescent

tissues. Therefore, MMPs are very attractive therapeutic targets whose abnormal levels of expression can potentially be controlled *via* genetic and non-genetic interventions. Besides T cells and macrophages, synovial fibroblasts and chondrocytes are directly involved in initiating the arthritic disease, primarily by overexpression and secretion of proteases into the intra-articular region of the joint. Cytokines secreted by T cells and macrophages are known to induce synovial fibroblasts to express and secrete augmented levels of MMPs into the joint cavity (Burger *et al.*, 1998). Natural inhibitors of MMPs, such as tissue inhibitor of metalloproteases (TIMPs), normally function to keep the activities of these proteases within physiological homeostasis during the course of wound healing, tissue remodeling, and embryogenesis (Nagase, 1996) (Brown, 1997). However, the perturbation of this balance in favor of increased MMPs expression and secretion would eventually lead to extracellular matrix destruction associated with arthritis and tumor metastasis. Because arthritic disorders, for the most part, are chronic in nature, a long-term therapeutic genetic intervention may be essential to halt the disease process.

AAV vectors are attractive in this regard because they are nonpathogenic parvoviruses of human origin, which integrate into chromosomal DNA of a host cell and stably express the therapeutic gene during the entire life span of transduced cells. Second, AAV vectors are less dependent on the proliferative nature of the target cells for efficient transduction. This is supported by many reports indicating that these vectors efficiently infect dividing and non-dividing cells (Kaplit *et al.*, 1994) (Ponnazhagan *et al.*, 1997) (Koeberl *et al.*, 1997). This is critical for gene delivery to synovial fibroblasts and chondrocytes, which are quiescent cells *in vivo*.

In this report, rAAV-mediated expression of -galactosidase was readily detectable in *ex vivo* infected cultures of RSFs and chondrocytes. More importantly, stromelysin-specific ribozymes driven by U1-, U6-, and tRNA-based promoters were efficiently expressed in RSFs. Such expression cannot be assumed as we have previously observed that a similar U1-ribozyme transcription unit was not expressed from a recombinant adenovirus in primary human or rat smooth muscle cells (Macejak *et al.*, 1999). Because the ribozyme transcripts are small RNA molecules (relative to most mRNAs) we were able to express more than one transcription unit from a single rAAV vector. Although the three ribozyme transcription units used here are thought to direct nuclear localization (Bertrand *et al.*, 1997) (Thompson *et al.*, 1995a), their subnuclear localization may be unique. The U1-ribozyme RNA was extremely stable (**Figure 3C**), contained a trimethyl-G "cap" and was bound by SM proteins, consistent with this ribozyme transcript being part of a snRNP. Expression of multiple ribozyme transcription units from one vector may enable the ribozyme transcripts to attack the target RNA at different points along its maturation/transport pathway. Consistent with this hypothesis we observed an additive effect with the U1- and U6-derived ribozymes expressed in combination compared to their sole expression

(Figure 5C). The situation does not appear to be that simple, however, since expression of all three ribozyme transcription units in combination did not generate the most reduction in target RNA. This may be due in part to the lower level of expression of all three ribozyme transcripts compared to their individual expression levels (Figure 5B).

Interestingly, the levels of U1- and U6-derived ribozyme increased in rAAV-infected RSFs upon passaging (Figure 4). There are several possible explanations for this observation. The first is related to the fact that the conversion of ss-DNA of rAAV genome to a double stranded form is essential for the expression of a transgene. This rate-limiting step is reported to be more efficient in dividing cells as compared to growth-arrested cells. Second, genomic integration of rAAV may enhance transgene expression and the formation of double-stranded AAV genomes is indispensable for a stable chromosomal integration. In addition, it is possible that the enhancement of ribozyme expression in dividing RSFs might be due to an increase in the pool of episomally ds-form of viral genome.

The steady state levels of tRNA-ribozyme were much greater than that of either the U1- or U6-derived ribozymes (Figure 5B). Coordinately, the pooled level of stromelysin mRNA in RSF cells infected with rAAV-encoding the tRNA-ribozyme chimera were significantly reduced (60-80%, $p < 0.05$) as compared to the levels in RSF infected with the empty vector or a vector expressing an irrelevant ribozyme (Figure 5C and Figure 6B). Since the expression of NGFR was heterogeneous among the infected RSF populations, it is likely that ribozyme expression from cell to cell is coordinately heterogeneous. Thus, in some cells ribozyme transcript levels may not be sufficient to impact stromelysin RNA, while in those cells with the highest ribozyme expression stromelysin RNA may be virtually absent. The reduction of stromelysin mRNA was observed to be largely due to a ribozyme cleavage mechanism, as the attenuated version of this ribozyme did not show a statistically significant reduction in stromelysin RNA levels compared to the empty vector control (Figure 6B). Recently, Bertrand et al. (Bertrand *et al.*, 1997) analyzed the activity of ribozymes in cells and observed inhibition of a target gene with an mRNA-ribozyme chimera, but not with tRNA, U1, or U6 ribozyme transcripts. However, the tRNA- and U6-derived ribozymes that we tested here were significantly different from those reported by Bertrand et al. and were optimized for cleavage activity and intracellular stability.

It is uncertain whether a 60-80% reduction in stromelysin RNA by the tRNA-ribozyme would be sufficient to halt the disease process *in vivo*. Surprisingly, rAAV infection alone induced the expression of stromelysin RNA in cultured RSFs in the absence of IL-1 treatment. The reason for this is not clear; however, several vectors (rAAV and others) which do not target stromelysin

have a similar effect in cell culture, as do other manipulations such as transfection. The induction of stromelysin mRNA by the transduction itself may be a transient event, and it may subside after a few cell passages. Because RSF cells undergo senescence after 5-7 passages, it is difficult to test this hypothesis. If stromelysin induction due to rAAV infection occurs *in vivo*, the impact of this induction would need to be resolved before considering rAAV as a viable vector strategy for therapeutic intervention in arthritis. Alternatively, additional methods to deliver the tRNA-ribozyme to RSFs *in vivo* can be pursued. In any case, our observations underscore the utility of AAV as a useful vector for the stable, long-term expression of ribozymes in primary mammalian cell lines.

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IV. Materials and Methods

A. Cells and tissues.

Rabbit synovial fibroblasts (RSFs) were surgically removed from the intra-articular lining of rabbit knees and cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) and 100 µg/ml of penicillin and streptomycin. The human chondrocyte sarcoma cell line SW1353 was obtained from ATCC. Monolayer cultures of 293 cells were grown and maintained in DMEM plus 10% FBS.

B. Ribozyme transcription unit plasmids.

The mouse U6 promoter was amplified from plasmid pMU6 (obtained from J. Dahlberg, Univ. of Wisconsin) via PCR with oligonucleotides (AAGTCGACCGACGC CGCCATCTCTA and AAGGAATTCGAGTGCCCAA CAAGGCTTTTCTCCAAGGG) and cloned into the SalI and EcoRI sites of a modified pGEM5Z (Promega). The modified plasmid contained EcoRI and HindIII sites I inserted between the SalI and NcoI sites of the original plasmid. Oligonucleotides (AGCTTGAGTTCGAGTGTTTTTGC and CATGGCAAAAACACTCGAACTCA) encoding an RNA polymerase III termination signal were designed to generate HindIII/NcoI sites upon annealing and were inserted into the modified 5Z plasmid containing the U6 promoter. Oligonucleotides encoding the ribozyme and an unstructured spacer region (AATTCAAGCACAAACACAACGAAGGAAGTATGAGGCCG AAAGGCCGAAAAGATGGCACACACACACAACAA and AGCTTTGTTGTGTGTGTGTGCCATCTTCGGCCTTCGGCCT CATCAGTTTCTTCGTTGTGTTTGTGCTTG) were designed to generate EcoRI/HindIII ends upon annealing and were then inserted into the modified U6 promoter plasmid. The final plasmid was designated "pU6C1049". The human U1 promoter was amplified

from plasmid HU1L (obtained from William Marzloff, Univ. of North Carolina) via PCR with oligonucleotides (GAGCGAATTCAGATCTAGGGCGACTTCTATGTAG and GAGCAAGCTTACGCGTCTAGGTTCTGGGCTCTGCCCGAC). The plasmid HU1L was also amplified with oligonucleotides (AAACCTAGGATACTTACTGCA GGGGAGATACCATGATCACGAAGG and AAAGGATCCTC TAACTTTTAGCACCAAAAATCAGGGGAAAGCGCGAA CGCAGTCCCCACTACCACAAATTATGCAGTCGAGTTTCCACATTTG) to generate sequences encoding a truncated U1 RNA and a mouse U1 RNA processing signal. These two PCR products were cloned into the EcoRI/BamHI sites in a pGEM4 plasmid (Promega) generating AvrII/PstI sites at the transcriptional start site of the U1 gene. Oligonucleotides encoding the ribozyme (CTAGGGAAGGAAGTATGAG GGGCGAAAGGCCGAAAGATGGCCCTGCA and GGCCATCT TTCGGCCTTTCGGCCTCATCAGTTCCTTCC) were designed to generate AvrII/PstI ends upon annealing and were inserted into the pGEMU1 plasmid. Oligonucleotides encoding the ribozyme and a spacer region (GAAGGAACTGATGAGGCCGAAAG GCCGAAAGATGGCAAACTGTTCTGTTTTG and GATCAAAAACAGAACAGTTTTGCCATCTTTCGGCCTTTCGGCCTCATCAGTTCCTTCC) were annealed to generate SacII/BamHI ends and cloned into the TRZ parent plasmid (Thompson *et al.*, 1995a). Oligonucleotides were purchased from The Midland Certified Reagent Company. All ribozyme transcription unit inserts were verified by sequencing.

C. rAAV plasmids.

pLK-1b was a kind gift from Dr. Arun Srivastava (Indiana University School of Medicine, Indianapolis, IN). pLK-1b contains the bacterial LacZ gene with the SV40 poly(A) signal in the 3' untranslated region, driven by the Cytomegalovirus immediate early (CMV) promoter. This CMV-LacZ cassette is flanked by AAV-termini within the pEMBL vector backbone. The recombinant and helper AAV plasmids, pSub201 and pAAV/Ad, respectively, were kindly provided by Dr. R.J. Samulski, University of North Carolina, Chapel Hill, N.C. pSub201 was digested to completion with Xho I and ligated to XhoI-NotI-XhoI linkers (adapters), generating the plasmid pAT19.

Due to the palindromic nature of AAV-ITRs which are prone to deletion in the bacterial host, most of the cloning steps were conducted in a vector backbone devoid of AAV-ITRs, and the final version of the expression cassette for the selective marker and ribozyme was subsequently subcloned into pAT19 containing AAV-ITRs. A truncated cDNA of the NGFR gene (lacking the region encoding the cytoplasmic domain) was subcloned from plasmid E2N as a 1.1 kb SacII-EcoRV fragment into pGEM5Z downstream of the CMV promoter. NotI linkers were then ligated to the purified NsiI – FspI fragment containing the CMV-NGFR-poly (A) expression cassette, and the fragment was subcloned into pBluescript-SK+ at the NotI site. Several restriction sites (SmaI through KpnI) from the pBluescript multiple cloning site (MCS) were deleted. To increase the size of rAAV genome for efficient packaging, a 1.6 kb stuffer fragment originating from the bacterial LacZ gene was inserted immediately upstream of the CMV promoter at the SalI site. An MCS for ribozyme insertion was cloned into the EcoRV site

within the LacZ stuffer fragment. The presence of the correct MCS was confirmed by sequence analysis. The entire expression cassette was then transferred to pAT19 using NotI to generate pAT22, such that the cassette is flanked by the AAV terminal repeats.

Transcription units encoding the stromelysin-specific ribozyme driven by either a modified human U1 sn RNA, mouse U6 sn RNA, or human tRNA promoter (see above) were cloned into the MCS of pAT22 to generate pAT 23, pAT 36, and pAT 42, respectively. The U1- and U6-derived ribozyme transcription units were both cloned into the MCS of pAT22 to generate plasmid pAT43. All three of the U1-, U6-, and tRNA-derived ribozyme transcription units were cloned into the MCS of pAT22 to generate pAT44. For each of these plasmids, the entire expression cassette (for ribozyme and NGFR expression) was transferred to pAT19 using NotI, such that the cassette is flanked by the AAV terminal repeats. Each ribozyme transcription unit insert was verified by sequencing and rAAV plasmids were demonstrated to be functional in an AAV rescue-replication assay performed in 293 cells.

D. In vitro cleavage assay.

Ribozyme plasmid inserts were amplified with oligonucleotides to generate T7 RNA polymerase promoter templates. Ribozyme RNAs were then transcribed *in vitro* (Ambion Megashortscript) and gel purified. Ribozyme RNA was renatured in cleavage buffer (10mM MgCl₂, 50mM Tris-HCl, pH7) for 5 min at 65°C, followed by 10 min at 37°C, prior to incubation with a ³²P-labeled RNA substrate containing the cleavage site. The cleavage reaction was performed under single turnover conditions in ribozyme excess and analyzed by denaturing PAGE.

E. Ribozyme RNA stability analysis.

Plasmids encoding the various ribozyme transcription units were transfected (2 µg DNA/ well of a 6-well plate) into 293 cells by calcium phosphate precipitation. After an overnight transfection, media was replaced and the cells were incubated an additional 24 h. Cells were then incubated in media containing 5 µg/ml Actinomycin D. At various times, cells were lysed in guanidinium isothiocyanate, and total RNA was purified by phenol/chloroform extraction and isopropanol precipitation. RNA was analyzed by Northern blot and the levels of specific ribozyme RNAs were quantified by phosphorimager analysis (Molecular Dynamics PhosphorImager). The level of each RNA at time zero was set to 100%.

F. Viral production and titer determination.

For large scale preparation of rAAV, 293 cells were cotransfected using a calcium phosphate method (Morris, Labrie, and Mathews, 1994) with the helper plasmid pAAV/Ad and each recombinant AAV plasmid using 100 µg of each plasmid per 500 cm² plate. Transfection was carried out for 12-16 hrs, followed by adenovirus dl312 infection (moi 5-10). Cells were processed for rAAV purification when significant adenovirus cytopathic effect was visible microscopically (65-72 hrs p.i). The cell pellet was frozen and thawed 3-4 times at -70°C and 37 °C, respectively, and the cell lysate was centrifuged to collect the supernatant. Virions in this crude preparation were precipitated in 10% polyethylene glycol, and the pellet was resuspended in Buffer C (50 mM Hepes, 10 mM EDTA, 150 mM NaCl pH 7.5 - 8.0). The rAAV particles in this preparation

were further purified by two CsCl equilibrium step gradient centrifugations (1.33 / 1.55 g/cm³ densities). The rAAV band (1.42-1.45 g/cm³ density) located at the junction between 1.33 and 1.5 g/cm³ CsCl densities, just below the visible band of adenovirus (1.33 g/cm³ density), was collected and dialyzed against PBS containing 1% sucrose. rAAVs are designated vAT corresponding to the plasmid used for co-transfection (e.g., pAT22 yields vAT22). The rAAV for LacZ expression was designated vLK-1b. The copy number of rAAV genomes was determined using a previously described slot-blot hybridization method (Kube and Srivastava, 1997). Infectious units per ml were determined by FACS analysis of cell surface NGFR expression.

G. X-gal staining of rAAV-infected cells.

Cell culture media was aspirated and cells were fixed for 5 minutes at 4 °C with 0.74% formaldehyde / 0.05% glutaraldehyde in PBS lacking magnesium. The fixative was then aspirated and X-Gal Staining Solution (5 mM each of ferri-isothiocyanate and ferrus-isothiocyanate containing 1 mg/mL X-Gal and 2 mM MgCl₂) was added. Cells were incubated at 37 °C until color develops.

H. Fluorescence Activated Cell Scanning (FACS) Analysis.

RSF cells were harvested 48 hours after virus or mock infection and washed twice with FACS Buffer (phosphate-buffered saline containing 1.0% BSA and 0.1% azide). 500 cells / µl were incubated for 30 minutes at 4 °C with primary antibody (mouse anti-human NGFR IgG). Cells were then washed twice with FACS Buffer and incubated for 30 minutes at 4 °C with secondary antibody (FITC-conjugated sheep anti-mouse IgG). Cells were washed twice with FACS Buffer and resuspended in FACS Buffer containing 1 µg/µl propidium iodide for analysis. Stained cell preparations were analyzed using a Becton Dickinson FACScan instrument.

I. Northern analysis.

Virus or mock infected cells were lysed at 1x10⁷ cells/mL in Lysis Buffer (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, and 0.5% sarcosyl) to which 0.1 volume of 3 M sodium acetate was added and the mixture was vortexed. Two sequential extractions with chloroform/isoamyl alcohol (25:1) were performed, and RNA in the aqueous phase was precipitated with isopropanol. The pellet was washed with 100% ethanol and resuspended in RNase-free water. Northern analysis was performed as described (Thompson *et al.*, 1995a). Briefly, purified RNA (10 µg per sample) was denatured in loading dye by heating at 95 °C for 3 min and electrophoresed on a 6% acrylamide / 7 M Urea gel in Tris-Borate-EDTA (TBE) buffer. The separated RNA was then transferred to a Genescreen Plus membrane, prehybridized for 1 hour with Church buffer (5% SDS, 0.2 M sodium phosphate) + 0.5% casein, and hybridized overnight at 65 °C with a ³²P-labelled RNA probe (1 x 10⁶ cpm/ml) in the same buffer. The ribozyme probe was obtained by *in vitro* T7 transcription using the pGEM5Z-based plasmid pU6C1049 described above, and contains sequences

complimentary to the poly(U) and ribozyme but not to the U6 promoter. Thus the probe should hybridize to ribozyme transcripts synthesized from the U1, U6, or tRNA promoters with similar efficiencies. Following hybridization, filters were washed and subjected to both autoradiography and phosphorimager analysis. Ribozyme RNA samples were normalized to endogenous tRNA.

J. RNase Protection Analysis (RPA).

A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA probe was used in each of these experiments as an internal control. Template DNA plasmids were transcribed using T7 RNA polymerase in the presence of [³²P]-CTP for 1 hr at 37° C. The reaction was terminated by the addition of DNase I and the volume was increased to 200 µl with TE. Unincorporated ribonucleotides were removed using a G-50 column equilibrated in TE. The stromelysin probe contains sequences corresponding to nucleotide positions 949 - 1201, based on the rabbit (RABSTROM) sequence (accession #M25664). The integrity of the RNA probe was checked by autoradiography following electrophoresis on a 6% denaturing polyacrylamide gel. Each probe (1x10⁶ cpm) was added to crude lysate from 100,000 cells (in lysis buffer, see RNA purification above), and incubated overnight at 37° C. RNase mixture (500ml) containing RNase A (500 U) and RNase T1 (1,000 U) was then added and the reaction was incubated for an additional 45 minutes at 37° C. The reaction was terminated by the addition of SDS and proteinase K and incubation proceeded for 45 minutes at 37°C. The RNA was precipitated with isopropanol and resuspended in loading buffer. The sample was heated to 95°C for 5 min, chilled on ice, and electrophoresed on a 7 M urea / 6% polyacrylamide sequencing gel. The levels of stromelysin and GAPDH RNAs were quantified by phosphorimager analysis.

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