Ribozyme expression cassettes were constructed which generate trimmed, trans-acting ribozymes from longer transcripts through the action of a downstream cis-acting ribozyme. This self-processing system produces small, well-defined trans-acting ribozymes with minimal, nonproductive, intramolecular structure. These cassettes also permit direct comparison of different ribozyme expression vectors without the need to compensate for different transcription initiation and termination sequences. Expression cassettes were created that contain a T7 promoter and that encode a single trans-acting ribozyme followed by either a hammerhead, hairpin, or hepatitis delta virus cis-acting ribozyme. All three ribozyme motifs function efficiently when transcribed in vitro, although slight differences are observed in the efficiency of self-processing for the different motifs. When transiently expressed in cultured mouse cells, the same specific cleavage products are observed. In addition, the relative efficiencies of in vitro self-processing between the three ribozyme constructs was maintained in vivo. Thus, the cellular milieu does not differentially alter the activity of the three ribozyme motifs. Detection of ribozyme-catalyzed RNA cleavage products from cultured cells is direct proof of ribozyme action in vivo.

Ribozymes are RNA molecules with enzymatic activity that cleave themselves or other RNA molecules in a sequence-specific manner. Sequence specificity results from the base-pairing of ribozyme sequences with nucleotides around the cleavage site of the target RNA. As with protein enzymes, ribozyme activity is dependent on formation of the correct, three-dimensional folded structure between the ribozyme and its target (Kim and Cech, 1987; Latham and Cech, 1989; Ruffner et al., 1990; Michel and Westhof, 1990; Perrotta and Been, 1991; Berzal-Herranz et al., 1993). Because of their sequence specificity, trans-acting ribozymes show promise as therapeutic agents to down-regulate a given RNA species in the background of cellular RNAs (Haseloff and Gerlach, 1988; Sarver et al., 1990; Ojwang et al., 1992; Sullenger and Cech, 1993). Ribozymes can be synthesized chemically and delivered to target cells exogenously using a variety of delivery vehicles, or they can be produced endogenously through the use of ribozyme expression vectors. The latter approach offers the attractive possibility of providing continual production of trans-acting ribozymes within cells. However, when tested in vitro, transcripts from ribozyme expression vectors frequently show substantially reduced activity compared to simple ribozymes with no extra flanking sequences (Taira et al., 1990; Taylor and Rossi, 1991; Crisell et al., 1993; Ohkawa et al., 1993; Ventura et al., 1993; Bertrand et al., 1994). This is likely due to nonproductive, alternative folding of the ribozyme with flanking sequences (Fedor and Uhlenbeck, 1990). Flanking sequences cannot be completely eliminated from the template given the functional requirements for proper transcription initiation and termination; however, flanking sequences potentially could be removed post-transcriptionally. Thus, one objective of this study was to create expression vectors that produce ribozymes with little or no extraneous sequence, thereby minimizing nonproductive folding of the ribozymes. This objective has been accomplished through the action of downstream, cis-cleaving ribozymes which free the trans-acting ribozymes from extraneous sequences.

A second objective of this study was to compare three different ribozyme motifs for their ability to function as cis-cleaving ribozymes. This comparison was intended to identify the best ribozyme motif for use in future self-processing constructs, especially with respect to the ability of different ribozyme motifs to function in cells. The hammerhead, hairpin, and hepatitis delta virus (HDV) ribozyme motifs consist of small, well-defined sequences that rapidly self-cleave in vitro (Symons, 1992). While structural and functional differences exist among the three ribozyme motifs, it is not clear whether these differences confer any advantage or disadvantage in vivo. Three specific concerns prompted the comparisons described in this paper. First, the HDV ribozyme motif folds into 4 helices which form a pseudoknot structure, with only 3 base pairs predicted for one helix (Perrotta and Been, 1991). Flanking RNA sequences may compete especially well with these pairing interactions to prevent efficient folding and cleavage by the HDV ribozyme. Second, the hairpin ribozyme functions efficiently in both cleavage and ligation (Berzal-Herranz et al., 1992; Chowrira et al., 1993) such that complete cleavage of the target RNA is dependent on rapid dissociation of product. If product dissociation is slowed in vivo, the ligation reaction could be enhanced which would result in reduced cleavage activity by the hairpin ribozyme. Finally, hammerhead ribozyme activity drops rapidly as the magnesium concentration is reduced below 10 mM (Dahn and Uhlenbeck, 1991). In contrast, the HDV ribozyme motif requires less than 1 mM Mg²⁺ for maximal activity (Perrotta and Been, 1990). The hairpin motif demonstrates Mg²⁺ dependence that is intermediate between the other two motifs (Chowrira et al., 1993). While the total concentration of cellular Mg²⁺ is estimated at around 10 mM, the free Mg²⁺ concentration is probably less than 1 mM (Veloso et al., 1973; Murphy et al., 1991; Romani and Scarpa, 1992). Thus, it remains to be deter-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) L35892-L35897.

1 The abbreviations used are: HDV, hepatitis delta virus; DEPC, dimethylsulfoxycarbonate; nt, nucleotide(s).
Self-processing Ribozymes in Mammalian Cells

28557

malyzed whether the magnesium concentration in vivo is sufficient to stimulate all three ribozyme motifs to their maximal levels.

A number of investigators have reported the use of ribozymes to process messages in vivo. Taira and colleagues (Taira et al., 1990, 1991; Yuyama et al., 1992; Ohkawa et al., 1993) have generated plasmids that allow insertion of sequences between pairs of self-processing ribozymes for precise 5' and 3' end generation in yeast and Escherichia coli. Eckner and colleagues (Eckner et al., 1991) reported the use of a hammerhead ribozyme to process the 3' end of histone messages while studying nucleocytoplasmic export of mRNAs in primate cells. Ball and co-workers (Ball, 1992; Pattnaik et al., 1992) utilized an HDV self-processing ribozyme to correctly process the 3' end of a viral RNA in vivo. Grosshans and Cech (1991) have used a hammerhead ribozyme to generate group I introns with defined 3' ends in vitro.

This project was undertaken to generalize the application of ribozymes as self-processing constructs. In that regard: (i) three different ribozyme motifs (hammerhead, hairpin, and HDV) were compared to determine which motif is most active in self-processing, (ii) the effect of flanking sequences on self-processing activity was evaluated, and (iii) the extent of in vivo self-processing was quantitated to more accurately compare ribozyme motifs.

EXPERIMENTAL PROCEDURES

Materials—DNA oligonucleotides were purchased from Midland Certified Regent Co. OST7-1 cell line (Elroy-Stein and Moss, 1991) was obtained from the National Institutes of Health.

Construction of Self-processing Ribozyme Expression Cassettes—To prepare DNA inserts that encode self-processing ribozyme cassettes, partially overlapping top- and bottom-strand oligonucleotides (60-90 nucleotides) were designed to include sequences for the T7 promoter, the trans-acting ribozyme, the cis-cleaving ribozyme, and appropriate restriction sites for use in cloning. The single-strand portions of annealed oligonucleotides were converted to double-strands using Susequence (U. S. Biochemical Corp.). Insert DNA was ligated into pBR322 digested with EcoRI and transformed into E. coli strain DH5a using standard protocols (Maniatis et al., 1982). The identity of positive clones was confirmed by sequencing small-scale plasmid preparations. The sequence of transcripts resulting from these constructs is given in Fig. 1.

Larger scale preparations of plasmid DNA for use in vitro transcription templates and in transfections were prepared using the protocols (Maniatis et al., 1982). The identity of positive clones was confirmed by sequencing small-scale plasmid preparations. The sequence of transcripts resulting from these constructs is given in Fig. 1.

In Vitro Transcription Reactions—Transcription reactions containing linear plasmid templates were carried out essentially as described (Milligan and Uhlenbeck, 1989; Chowilla and Burke, 1991). In order to prepare 5'-end-labeled transcripts, standard transcription reactions were carried out in the presence of 10-20 µCi of [γ-32P]ATP, 200 µCi each NTP, and 0.5 to 1 µg of linearized plasmid template. The concentration of MgCl2 was maintained at 10 mM above the total nucleotide concentration.

To make internally labeled substrate RNA for trans-ribozyme cleavage reactions, a 625-nt region of the UL5 gene was amplified from herpes simplex virus genomic DNA (KOS strain) by polymerase chain reaction using primers that place the T7 RNA polymerase upstream of the amplified sequence. Target DNA was transferred in a standard transcription buffer in the presence of [γ-32P]ATP (Chowilla and Burke, 1991). The reaction mixture was treated with 15 units of ribonuclease-free DNAseI, extracted with phenol followed by chloroform:isoamyl alcohol precipitation was included as the final step.

Primer Extension Assay—Purified cellular RNA (3 µg/reaction) was first denatured in the presence of 5-µl linearized DNA template (100 pmol) by heating to 90°C for 2 min in the absence of Mg2+ and then snap-cooling on ice for at least 15 min. This protocol allows for efficient annealing of the primer to its complementary RNA sequence. The primer was extended using Superscript II reverse transcriptase (8 units/ml; Life Technologies Inc.) in a buffer containing 50 mM Tris-HCl, pH 8.3, 10 mM dithiothreitol, 75 mM KCl, 1 mM MgCl2, 1 mM each dNTP. The extension reaction was carried out at 42°C for 10 min. The reaction was terminated by adding an equal volume of 2× formamide loading buffer and freezing on crushed dry ice. The samples were resolved on a 10% polyacrylamide sequencing gel. The reaction mixture was treated with 15 units of ribonuclease-free DNaseI, extracted with phenol followed by chloroform:isoamyl alcohol precipitation was included as the final step.

The general design of the ribozyme cassettes, as well as specific differences among the cassettes, are outlined below.

A sequence predicted to form a stable stem-loop structure is included at the 5' end of all the transcripts. The hairpin stem contains the T7 RNA polymerase initiation sequence (Milligan...
and Uhlenbeck, 1989) and its complement, separated by a stable tetra-loop (Antao et al., 1991). By incorporating the T7 initiation sequence into a stem-loop structure, we hoped to avoid nonproductive base-pairing interactions with either the trans-acting ribozyme or with the cis-acting ribozyme. The presence of a hairpin at the end of a transcript may also contribute to the stability of the transcript in vivo.

The trans-acting ribozyme used in this study is targeted to a site in the herpes simplex virus UL5 transcript (5’-CUGGAGUC-3’). The 5’-binding arm of the ribozyme, 5’-GAAGGUC-3’, and the core of the ribozyme, 5’-CUGAUGGGCCGAAGGCGCAA-3’, remain constant in all cases. In addition, all transcripts also contain a single nucleotide between the 5’-stem loop and the first nucleotide of the ribozyme. The linker nucleotide was required to obtain the same activity in vitro that was measured with an identical ribozyme lacking the 5’ hairpin (data not shown). Because the three cis-cleaving ribozymes have different requirements at the site of cleavage, slight differences were unavoidable at the 3’ end of the processed transcript. The junction between the trans- and cis-acting ribozyme is, however, designed so that there is minimal extraneous sequence left at the 3’ end of the trans-cleaving ribozyme once cis-cleavage occurs. The only differences between the constructs lie in the 3’ binding arm of the ribozyme, where either 6 or 7 nucleotides, 5’-ACUCCA(sG)+3’, complementary to the target sequence are present, and, where, after processing, two to five extra nucleotides remain.

The cis-cleaving hammerhead ribozyme used in the HH cassette is based on the design of Grosshans and Cech (1991). As shown in Fig. 1A, the 3’-binding arm of the trans-acting ribozyme is included in the required base-pairing interactions of the cis-cleaving ribozyme to form stem I. Two extra nucleotides, UC, were included at the end of the 3’-binding arm to form the self-processing hammerhead ribozyme site (Ruffner et al., 1990) which remains on the 3’ end of the trans-acting ribozyme following self-processing.

The hairpin ribozyme portion of the HP self-processing construct is based on the minimal wild-type sequence (Hampel and Tritz, 1989). Previously, the substrate and the catalytic portion of the hairpin ribozyme (Fig. 1B) has been linked at the end of helix 2 (Berzal-Herranz et al., 1992). Here, however, a tetra-loop at the end of helix 1 (3’ side of the cleavage site) serves to link the two portions and thus allows a minimal 5 nucleotides to remain at the end of the released trans-acting ribozyme following self-processing. Two variants of HP were designed: HP(GU) and HP(GC). The HP(GU) was constructed by incorporating the T7 promoter base pair between G6 and U7; HP(GC) has a Watson-Crick base pair between G6 and C7. A shortened helix 1 (5 base pairs) and a stable tetraloop (GAAA) at the end of helix 1 was used to connect the substrate with the catalytic domain of the hairpin ribozyme (Feldstein and Brauning, 1993; Altschuler et al., 1992). C, HDV transcript containing the trans-acting hammerhead ribozyme linked to a 3’-cis-acting hepatitis delta virus (HDV) ribozyme. The secondary structure of the HDV ribozyme is as proposed by Been and co-workers (Been et al., 1992). The trans-ribozyme domain extends from nucleotides 1 through 48. After 3’-end-processing, the trans-ribozyme contains 5 non-ribozyme nucleotides (UGGCA at positions 50 to 54) at its 3’ end. The 3’-cis-acting ribozyme is comprised of nucleotides 50 through 115. The transcript named HP(GU) was constructed with a potential mobile base pair between G32 and U77; HP(GC) has a Watson-Crick base pair between G32 and C77. A shortened helix 1 (5 base pairs) and a stable tetraloop (GAAA) at the end of helix 1 was used to connect the substrate with the catalytic domain of the hairpin ribozyme (Feldstein and Brauning, 1993; Altschuler et al., 1992). C, HDV transcript containing the trans-acting hammerhead ribozyme linked to a 3’-cis-acting hepatitis delta virus (HDV) ribozyme. The secondary structure of the HDV ribozyme is as proposed by Been and co-workers (Been et al., 1992). The trans-ribozyme domain extends from nucleotides 1 through 48. After 3’-end-processing, the trans-ribozyme contains 2 non-ribozyme nucleotides (AA at positions 49 to 50) at its 3’ end. The 3’-cis-acting HDV ribozyme is comprised of nucleotides 50 through 114. Roman numerals I, II, III, and IV indicate the location of four helices within the 3’-cis-acting HDV and Been (1991). The HDV transcript contains a 31-nucleotide deletion in the HDV portion of the transcript (nucleotides 84 through 115 deleted).
with a G-U wobble base pair in helix 2 (A\textsuperscript{52} \rightarrow G substitution; Fig. 1B). This slight destabilization of helix 2 was intended to improve self-processing activity by promoting product release and preventing the reverse reaction (Berzal-Herranz et al., 1992; Chowriria et al., 1993). The HP\textsubscript{GC} cassette was constructed as a control for strong base-pairing interactions in helix 2 (U\textsuperscript{77} \rightarrow C and A\textsuperscript{52} \rightarrow G substitution; Fig. 1B). Another modification to discourage the reverse ligation reaction of the hairpin ribozyme was to shorten helix 1 (Fig. 1B) by 1 base pair relative to the wild-type sequence (Chowriria and Burke, 1991).

The HDV ribozyme self-processes efficiently when the nucleotide 5' to the cleavage site is a pyrimidine and somewhat less so when adenosine is in that position.\textsuperscript{2} No other sequence requirements have been identified upstream of the cleavage site; however, we have observed some decrease in activity when a stem-loop structure was present within 2 nt of the cleavage site (data not shown). The HDV self-processing construct (Fig. 1C) was designed to generate the trans-acting hammerhead ribozyme with only 2 additional nucleotides at its 3' end after self-processing. The HDV sequence used here is based on the anti-genomic sequence (Perrotta and Been, 1991) but includes the modifications of Been et al. (1992) in which cis-cleavage

\textsuperscript{2} M. Been, unpublished observations.
activity of the ribozyme was improved by the substitution of a shortened helix 4 for a wild-type stem-loop (Fig. 1C).

**RNA Self-processing in Vitro**—To compare the ability of the different ribozyme cassettes to self-process in vitro, each construct was transcribed and allowed to undergo self-processing under identical conditions at 37°C. For these comparisons, equal amounts of linearized DNA templates bearing the various ribozyme cassettes were transcribed in the presence of [γ-32P]GTP to generate 5'-end-labeled transcripts. In this manner, only the full-length, unprocessed transcripts and the released trans-ribozymes are visualized by autoradiography. In all reactions, Mg^2+ was included at 10 mM above the nucleotide concentration so that cleavage by all the ribozyme cassettes would be supported. Transcription templates were linearized at several positions by digestion with different restriction enzymes (Fig. 2A) so that self-processing in the presence of increasing lengths of downstream sequence could be compared. The resulting transcripts have either 4–5 non-ribozyme nucleotides at the 3' end (HindIII-digested templates), 220 nucleotides (NdeI-digested template), or 450 nucleotides of downstream sequence (RcaI-digested template).

As shown in Fig. 2B, all four ribozyme cassettes are capable of self-processing and yield RNA products of expected sizes. The multiple bands observed for each species are due to the presence of extra, non-template-encoded guanosine at the 5' end of the transcript (discussed below). Thus, the shortest RNA in each lane (50 to 54 nt) corresponds to the sizes expected for trans-acting ribozymes released from the 5' end of each transcript by cleavage at the correct sites.

Two nucleotides essential for hammerhead ribozyme activity (Ruffner et al., 1990) have been changed in the HH(mutant) core sequence (see Fig. 1A) and so this transcript is unable to undergo self-processing. This is evidenced by the lack of a released 5' RNA in the HH(mutant) lanes, although the full-length RNAs are present (Fig. 2B). This result indicates that the faster-migrating species of RNA are products of self-processing and not merely a result of transcription termination.

Comparison of the amounts of released trans-ribozyme (Fig. 2C) indicates that there are differences in the ability of these ribozymes to self-process in vitro, especially with respect to the presence of downstream sequence. For the two HP constructs, it is clear that HP(GC) is more efficient than the HP(GU) ribozyme, both in the presence and in the absence of extra downstream sequence. In addition, the activity of HP(GU) falls off more dramatically when downstream sequence is present. The stronger G:C base pair likely contributes to the HP(GC) construct's ability to fold correctly (and/or more quickly) into the productive structure, even when as much as 216 extra nucleotides are present downstream. The HP ribozyme construct is also quite efficient at self-processing and slightly better than the HP(GC) construct even when downstream sequence is present.

Of the three ribozyme motifs, the presence of extra downstream sequence seems to most affect the efficiency of HDV. When no extra sequence is present downstream, HDV is quite efficient and self-processes to approximately the same level as the HH and HP(GC) cassettes. However, when extra downstream sequence is present, the self-processing activity seems to decrease almost as dramatically as is seen with the (suboptimal) HP(GU) cassette.

As mentioned above, we observed heterogeneity toward the 5' end of all T7 transcripts (Fig. 2B). The predominant band and the shortest RNA corresponds to the size predicted for the 5'-processed RNA. However, RNA bands greater than the expected length were also observed. These species appear as a distribution with a regular spacing of 1 nt. Similar heterogeneity is observed in the full-length RNAs, although it is not detectable in Fig. 2B. To determine the source of heterogeneity, RNA transcripts were end-labeled using [γ-32P]GTP. Bands corresponding to the expected length for processed product (n) and the next two slower migrating bands (n + 1 and n + 2) were isolated and sequenced using sequence-specific ribonucleases. The band corresponding to "n" contains the exact sequence encoded in the template, while the other bands were shown to contain additional guanosine residues at their 5' ends (data not shown). It is not clear why the additional guanosine residues are present, although stuttering has been observed in transcripts that begin with multiple Gs (Martin et al., 1988) and the 5' stem loop may exacerbate this behavior. The additional guanosine residues are generated from all the constructs and do not alter the conclusions reported here.

To further compare the relative self-processing efficiencies of the three ribozyme motifs, kinetics of self-processing during transcription were measured. Linear templates were prepared by digesting the plasmids with HindIII so that transcripts contained only four to five vector-derived nucleotides at the 3' end (see Fig. 1). By comparison of the unimolecular rate constant (k) determined for each construct, it is clear that HH is the most efficient at self-processing (Table 1). The HH transcript...
Self-processing Ribozymes in Mammalian Cells

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RNA preincubation with MgCl$_2$

Full Length RNA

3' Cleavage Products

**Fig. 4. RNA self-processing in OST7-1 cells.** In Vitro lanes contain full-length, unprocessed transcripts that were added to cellular lysates prior to RNA extraction. These RNAs were either preincubated with MgCl$_2$ (+) or with DEPC-treated water (−) prior to being hybridized with 5'-end-labeled primers. Cellular lanes contain total cellular RNA from cells transfected with one of the four self-processing constructs. Cellular RNA was probed for ribozyme expression using a sequence-specific primer extension assay. Solid arrows indicate the location of primer extension bands corresponding to Full Length RNA and 3' Cleavage Products.

self-processes 2-fold faster than HDV and 3-fold faster than HP(GC) transcripts. Although the HP(GU) RNA undergoes self-processing, it is at least 6-fold slower than the HP(GC) construct. This is consistent with previous observations that the stability of helix 2 is essential for self-processing and trans-cleavage activity of the hairpin ribozyme (Hampel et al., 1990; Chowrira and Burke, 1991; Joseph et al., 1993). The rate of HH self-cleavage during transcription measured here (1.2 min$^{-1}$) is similar to the rate measured by Long and Uhlenbeck (1994) using a HH that has a different stem I and stem III. Self-processing rates during transcription for HP and HDV have not been previously reported. However, self-processing of the HDV ribozyme (as measured here during transcription) is significantly slower than when tested after isolation from a denaturing gel (Been et al., 1992). This decrease likely reflects the difference in protocol as well as the presence of 5'-flanking sequence in the HDV construct used here.

**Effect of Downstream Sequences on trans-Cleavage in Vitro—**

Does 3'-end-processing improve the activity of a trans-acting ribozyme? To answer this question, transcripts containing the trans-ribozyme with or without 3'-flanking sequences were assayed for their ability to cleave their target in trans. To this end, transcripts from three templates were resolved on a preparative gel, and bands corresponding both to processed trans-acting ribozymes from the HH transcription reaction and to full-length HH(mutant) and ΔHDV transcripts were isolated.

In all three transcripts, the trans-acting ribozyme portion is identical, with the exception of sequences at their 3' ends. The HH trans-acting ribozyme contains only an additional UC at its 3' end, while HH(mutant) and ΔHDV have 52 and 37 nucleotides, respectively, at their 3' ends. A 622-nucleotide, internally-labeled target RNA was incubated, under ribozyme excess conditions, along with the three ribozyme transcripts in a standard reaction buffer. The HH trans-acting ribozyme cleaves the target RNA approximately 10-fold faster than the ΔHDV transcript and greater than 20-fold faster than the HH(mutant) transcript (Fig. 3). The additional nucleotides at the end of HH(mutant) form 7 base pairs with the 3' target-binding arm of the trans-acting ribozyme (Fig. 1A). This interaction must be disrupted (at a cost of 6 kcal/mol) to make the trans-acting ribozyme available for binding the target sequence. In contrast, the additional nucleotides at the end of ΔHDV were not designed to form any strong, alternative base-pairing with the trans-ribozyme. Nevertheless, the ΔHDV sequences are predicted to form multiple structures involving the 3' target-binding arm of the trans-ribozyme that have stabilities ranging from 1–2 kcal/mol. Thus, the observed reductions in activity for the ΔHDV and HH(mutant) constructs are consistent with the predicted folded structures, and it reinforces the view that the flanking sequences can decrease the catalytic efficiency of a ribozyme through nonproductive interactions with either the ribozyme or the substrate or both.
Ribozyme Self-processing in OST7-1 Cells—Since three of the constructs (HH, HDV, and HP(GC)) self-process efficiently in solution, the effect of the mammalian cellular milieu on ribozyme self-processing was next explored. A transient expression system was employed to investigate ribozyme activity in vivo. A mouse cell line (OST7-1) that constitutively expresses T7 RNA polymerase in the cytoplasm was chosen for this study (Elroy-Stein and Moss, 1990). In these cells, plasmids containing a ribozyme cassette downstream of the T7 promoter will be transcribed efficiently in the cytoplasm (Elroy-Stein and Moss, 1990). Circular plasmids encoding HH, HDV, and HP(GC) constructs were transfected into OST7-1 cells. Four hours post-transfection, cells were lysed, and total RNA was isolated. Primer extension analysis was carried out on the total cellular RNA. As shown in Fig. 4, specific bands corresponding to full-length precursor RNA and 3' cleavage products were detected from cells transfected with the self-processing cassettes. All three constructs, in addition to being transcriptionally active, appear to self-process efficiently in the cytoplasm of OST7-1 cells. In particular, the HH and HP(GC) constructs self-processing to greater than 95%. The overall extent of self-processing in OST7-1 cells appears to be strikingly similar to the extent of self-processing in vitro (Fig. 4, In Vitro +MgCl\textsubscript{2} versus Cellular).

Consistent with the in vitro self-processing results, the HP(GU) cassette self-processed to approximately 50% in OST7-1 cells (data not shown). As expected, transfection with plasmids containing the HH(mutant) cassette yielded a primer extension product corresponding to the full-length RNA with no detectable cleavage products (Fig. 4). The latter result strongly suggests that the primer extension band corresponding to the 3' cleavage product is not an artifact of reverse transcription. We were concerned with the possibility that RNA self-processing might occur during cell lysis, RNA isolation, and/or the primer extension assay. Two precautions were taken to exclude this possibility. First, 50 mM EDTA was included in the lysis buffer. EDTA is a strong chelator of divalent metal ions such as Mg\textsuperscript{2+} and Ca\textsuperscript{2+} that are necessary for ribozyme activity (Dahm and Uhlenbeck, 1991; Chowrira et al., 1993; Suh et al., 1993). Divalent metal ions are therefore unavailable to self-processing RNAs following cell lysis. A second precaution involved using primers in the primer extension assay that were designed to hybridize to essential regions of the processing ribozyme. Binding of these primers should prevent the 3' cis-acting ribozymes from folding into the conformation essential for catalytic activity.

Two experiments were carried out to further eliminate the possibility that self-processing is occurring either during RNA preparations or during the primer extension analysis. The first experiment involves primer extension analysis on full-length precursor RNAs that were added to nontransfected OST7-1 lysates after cell lysis. Thus, only if self-processing is occurring at some point after lysis would cleavage products be detected. Full-length precursor RNAs were prepared by transcribing under conditions of low Mg\textsuperscript{2+} (5 mM) and high NTP concentration (total 12 mM) in an attempt to eliminate the free Mg\textsuperscript{2+} required for the self-processing reaction (Michel et al., 1992). The full-length precursor RNAs were gel-purified, and a known amount was added to lysates of nontransfected OST7-1 cells. RNA was purified from these lysates and incubated for 1 h in DEPC-treated water at 37 °C prior to the standard primer extension analysis (Fig. 4, In Vitro -MgCl\textsubscript{2} versus Control). The predominant RNA detected in all cases corresponds to the primer extension product of full-length precursor RNAs. If, instead, the purified RNA containing the full-length precursor is incubated in 10 mM MgCl\textsubscript{2} prior to the primer extension analysis, most or all of the RNA detected by primer extension analysis undergoes cleavage (Fig. 4, In Vitro +MgCl\textsubscript{2}, versus Control). These results indicate that the standard RNA isolation and primer extension protocols used here do not provide a favorable environment for RNA self-processing, even though the RNA in question is inherently able to undergo self-cleavage.

In a second experiment to demonstrate lack of self-processing during work-up, internally labeled precursor RNAs were prepared and added to nontransfected OST7-1 lysates as in the previous control. The internally labeled precursor RNAs were carried through the RNA purification and primer extension reactions (in the presence of unlabeled primers) and analyzed to determine the extent of self-processing. By this analysis, the vast majority of the added full-length RNA remained intact during the entire process of RNA isolation and primer extension (data not shown).

These two control experiments validate the protocols used and support our conclusion that the self-processing reactions catalyzed by HH, HDV, and HP(GC) cassettes are occurring in the cytoplasm of OST7-1 cells.

**DISCUSSION**

We undertook a comparative investigation to evaluate the ability of three different ribozyme motifs to process the 3' end of an RNA transcript both in solution and in mammalian cells. Here we report that all three motifs self-process efficiently in vitro and in mammalian (OST7-1) cells. Although the in vitro analysis reveals minor differences in the rate of self-processing (2-5-fold), all three constructs self-process to 87-95% completion in the absence of 3'-flanking sequences. These numbers represent a conservative estimate of self-processing since new transcripts continued to be produced during the 30-min reaction. In vitro, the self-processing constructs described in this study appear to be significantly more active than those reported in the literature, wherein the extent of self-processing ranged between 15 and 50% (Taira et al., 1990, 1991; Altschuler et al., 1992). Our results suggest that cis-cleaving ribozymes can be used efficiently to truncate RNA molecules at specific sites in vivo.

The ability of the three ribozyme motifs to function efficiently in OST7-1 cells suggests that the cellular milieu does not differentially alter the activity of these ribozyme motifs. Our main concern in this regard was that the cellular concentration of Mg\textsuperscript{2+} available to ribozymes might be closer to the estimated free concentrations (0.1 mM) rather than to the total cellular Mg\textsuperscript{2+} concentrations (10 mM). If that were the case, the HH construct (which requires at least 10 mM Mg\textsuperscript{2+} for optimal in vitro activity) would be expected to show reduced processing relative to the other two constructs. That this was not observed suggests that the available cellular Mg\textsuperscript{2+} concentrations are greater than 1 mM by a substantial amount or that other cellular factors compensate to maintain ribozyme activity. It must be noted that transcription, and therefore self-processing, takes place in the cytoplasm of OST7-1 cells, and that the amount of transcript produced is high relative to cellular RNA (Elroy-Stein and Moss, 1990). It is possible that these conditions titrate out some activating or inhibitory cellular factors that might otherwise alter the rate and extent of self-processing. In addition, the effect of a nuclear environment on ribozyme activity remains unknown. Since the nucleus contains a large number of RNA binding proteins, it is possible that nuclear localization may have a significant effect on ribozyme catalysis (Tsuchihashi et al., 1993). Wong-Staal and co-workers have recently demonstrated the inhibition of HIV replication in human cells by a tRNA-HP ribozyme chimera that is predominantly localized in the nucleus (Ojwang et al., 1992; Yu et al., 1993). It will be of interest, therefore, to study the expression
and self-processing characteristics of these ribozyme cassettes in the nucleus.

Detection of ribozyme-catalyzed RNA cleavage products from cultured cells is direct proof of ribozyme cleavage in vivo. Although a number of researchers have reported the inhibition of targeted gene expression (Cotten and Birnstiel, 1989; Camero and Jennings, 1989; Sarver et al., 1990; Sullenger and Cech, 1993; Yu et al., 1993; Zhao and Pick, 1993), only a few have detected RNA cleavage products in vivo (Saxena and Ackerman, 1990; Steinlecke et al., 1992; Bertrand et al., 1994).

We have successfully used a sensitive primer extension assay to detect 3' products of self-processing in OST7-1 cells. Results from all control experiments strongly support our conclusions that the ribozyme self-processing reaction is in fact taking place in vivo. This is yet another demonstration of ribozyme action in mammalian cells.

We have shown that 3'-flanking sequences can drastically decrease the catalytic efficiency of a ribozyme. In separate studies, the same effects were observed with extra 5'-flanking sequences (data not shown). For the HDV motif, the efficiency of the self-processing reaction decreased 6-fold with an increase in the length of the 3'-flanking sequence. trans-Cleavage reactions are similarly affected by non-ribozyme flanking sequences. These results are consistent with those in the literature, wherein researchers have observed significant inhibition of ribozyme activity as a direct consequence of flanking non-ribozyme sequences (Taira et al., 1990; Taylor and Rossi, 1991; Crisell et al., 1993; Ohkawa et al., 1993; Ventura et al., 1993; Bertrand et al., 1994).

In all cases, the self-processing ribozymes tested here have essentially the same upstream trans-ribozyme sequence and exactly the same downstream vector sequence. A completely different sequence context may well affect each ribozyme differently due to a new set of potential sequence-specific folding interactions. The smaller, more stable ribozymes may be more robust in that they may be more likely to be active when embedded in any extraneous sequence. Moreover, if a self-processing ribozyme is able to fold quickly into its stable, functional structure, cleavage may occur before full-length transcription is complete, and the interference from downstream RNA may be at least partially avoided. Of the three ribozyme motifs, the presence of extra downstream sequence seems to most affect the efficiency of HDV self-processing. HDV is not only the largest ribozyme motif tested here, its structure is also predicted to include a short stem III and a pseudoknot that is required for activity (Perrotta and Been, 1991). Because of these properties, one might expect more interference arising from nonproductive base-pairing of the HDV sequence with flanking non-ribozyme sequences. Although the HDV construct tested here is identical to those ribozymes without a 2',3'-cyclic phosphate terminus. This in-
Self-processing Ribozymes in Mammalian Cells