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3′-End Modification of the Adenoviral *VA1* Gene Affects Its Expression in Human Cells: Consequences for the Design of Chimeric VA1 RNA Ribozymes

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ABSTRACT

Polymerase III (pol III)-dependent genes, like the adenoviral *VA1* gene, are of particular interest for expressing small therapeutic RNAs into cells. A new VA1 RNA carrier molecule was generated through the deletion of the VA1 RNA central domain to give rise to the VAδIV RNA vector that was devoid of undesirable physiologic activity (i.e., inhibition of the interferon-induced protein kinase, PKR). This vector was used to express in human cells hammerhead ribozymes targeted against the human immunodeficiency virus (HIV). Eight anti-HIV ribozymes were inserted at the 3′-end of this vector immediately before the four T-residues that serve as a transcription termination signal. Although the constructs were active *in vitro*, they failed to inhibit HIV replication in transient assays. Analysis of the intracellular ribozyme expression in cells revealed several anomalies. First, using mutant derivatives, we showed that the presence of two or three consecutive T-residues in the ribozyme portion was sufficient to promote the release of anomalous short transcripts. Second, when the ribozyme did not contain T-rich sequence, full-length transcripts were produced, but these transcripts were very unstable and were retained in the cell nucleus. In contrast, insertion of the ribozyme in place of the central domain of VA1 RNA led to production of full-length transcripts that were stable and located in the cytoplasm but that were not found to be active *in vitro*. Taken together, these results have important consequences for the future design of active intracellular ribozymes based on the use of pol III-transcribed genes.

INTRODUCTION

THE POSSIBILITY OF MODULATING GENE EXPRESSION via the intracellular delivery of small therapeutic RNAs has emerged recently as a promising approach for the future treatment of various diseases. Several RNA-based molecular tools have been developed, including RNA decoys, antisense RNAs, and ribozymes. Particularly, the intracellular expression of modified RNAs designed to inhibit human immunodeficiency virus (HIV) replication is one direction for AIDS gene therapy (Yu et al., 1994; Bertrand and Rossi, 1996). One potential advantage over conventional drug delivery is the ability of expressing these therapeutic RNAs continually in pathogenic cells after stable gene transfer. In addition, RNA-based approaches may be of particular interest because it is unlikely that cells expressing therapeutic RNAs serve as targets for the host immune response that is responsible for the clearance of cells expressing a foreign therapeutic protein.

Among various eukaryotic gene promoters, those recognized by RNA polymerase III (pol III) offer an interesting alternative approach for driving modified RNA expression in cells (Jennings and Molloy, 1987; Cotten and Birnstiel, 1989; Cagnon et al., 1995; Thompson et al., 1995; Prislei et al., 1997). Indeed, pol III-transcribed genes are easy to manipulate, as these genes are usually small and contain internal or external well-defined promoter regions (Galli et al., 1981; Geiduschek, 1988; Goodrich and Tjian, 1994). In addition, the 3′-end of the gene is characterized by the presence of a run of four to six consecutive T-residues that act as a strong transcription termination signal (Galli et al., 1981; Geiduschek, 1988). The *VA1* gene of the adenoviruses (for review, see Matthews and Shenk, 1991) is efficiently transcribed by pol III to give rise to a highly ordered

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RNA molecule that is accumulated to high levels in the cell cytoplasm of adenovirus-infected cells (Mellits et al., 1990; Ghadge et al., 1994; Rahman et al., 1995; Ma and Matthews, 1996). This small RNA interacts with an interferon (IFN) induced protein kinase (PKR) to prevent its activation, which would otherwise shut down translation through the phosphorylation of eukaryotic initiation factor 2 (eIF-2) (Thimmappaya et al., 1982; Akusjarvi et al., 1987; Matthews and Shenk, 1991).

In a previous study (Cagnon et al., 1995), we showed that VA1 RNA may provide a suitable gene cassette for the intracellular expression of a short antisense RNA directed against HIV. To extend these previous observations, we envisioned the use of the *VA1* gene as a cassette suitable for the expression of chimeric anti-HIV VA1 ribozymes (VA1 RBZs) in human cells. Ribozymes are small catalytic RNA molecules that can be designed to specifically bind an RNA target by base pairing and, subsequently, to cleave it (Haseloff and Gerlach, 1988; Marschall et al., 1994; Symons, 1994). However, the intracellular activity of ribozymes is highly variable, and the parameters controlling their efficiency *in vivo* are not clearly defined. It has been shown that the association step between the ribozyme and its target is the determining step inside the cells (Sullenger and Cech, 1993; Bertrand and Rossi, 1996; Bertrand et al., 1997). Thus, intracellular ribozyme concentration has to be as high as possible, and the ribozyme should reach the same subcellular localization as its target RNA (Bertrand et al., 1997; Hormes et al., 1997). In addition, intracellular folding of the ribozyme is critical for its activity. It is thus important to carefully optimize the design of the RNA molecule used to carry the ribozyme into cells, as this will affect all these parameters (Thompson et al., 1995; Prislei et al., 1997).

In the present study, we show that intracellular expression of chimeric VA1 RNAs that contain hammerhead ribozymes appended at their 3′-end reveal several anomalies in RNA termination, stability, and localization. The nature and the position of the ribozyme may profoundly affect expression of the VA1 RNA in mammalian cells. We conclude that regulation of a pol III-transcribed gene is a complex process involving more than the intragenic promoter. These results will be useful for the future design of intracellular active ribozyme.

MATERIALS AND METHODS

Construction of modified VA1

The *VA*δIV gene was obtained in a two-step polymerase chain reaction (PCR). First, two independent PCRs, PCR5′ and PCR3′, were carried out using 2 ng of adenovirus type 2 (Ad2) genomic DNA (Sigma Chimie, L'isle-d'Abeau Chemes, France) as template and 250 ng of each specific primer. PCR5′ generated a DNA fragment corresponding to the 5′ moiety of the Ad2 *VA1* gene deleted of the sequence forming the central domain (from nt 93 to 118). For PCR5′, primers were (from 5′ to 3′): VA5′, GTTATCGATCTAGACCGT GCAAAAGGAGAGCC, localized upstream of the Ad2 *VA1* transcription unit, and VAδIV5′, GTCGCACACCTGGAT ATCACGGCGGACGGCCGGATC, which is respectively complementary to the Ad2 VA1 RNA, from nt 133 to 117 and from nt 92 to 72 (underlined). In addition, a mutation was introduced (C replaced by A) at nt 119 (bold) to create a new restriction site (Eco RV). PCR3′ generated the 3′ moiety of the Ad2 *VA1* gene. For PCR3′, primers were VAδIV3′, CAG GTG TGC GAC GTC AGA CAA CGG from nt 122 to nt 145 in the Ad2 sequence, and VA3′, AAA ATC GAT CGC AGC AGC CGC CGC GCC TGG AAG, localized downstream from the Ad2 *VA1* transcription unit. Second, a new PCR was carried out using 2 μl from each PCR5′ and PCR3′ mixture added with VA5′ and VA3′ as primers. From this last PCR, we obtained the *VA*δIV gene, which was purified from agarose gel using a Spin-X column (Costar, Brumath, France) and was cloned into the Cla I restriction site of the pVV2 plasmid (Cagnon et al., 1995). All PCRs were carried out through 40 cycles of 94°C, 56°C, and 72°C for 1 minute each. The final 72°C cycle lasted 10 minutes. Two units of *Taq* DNA polymerase (AmpliTaq, Perkin Elmer, Courtaboeuf, France) were used in a total volume of 100 μl.

The eight anti-HIV ribozymes were obtained through the specific annealing of two complementary synthetic oligonucleotides and cloned into a blunted restriction site of the *VA*δIV (Eco47 III or Eco RV). Cloning efficiency was improved by adding 3 U of the corresponding restriction enzyme into the ligation mixture (Ligation Kit, Stratagene, La Jolla, California). All constructs were sequenced. All the oligonucleotide sequences used to construct the anti-HIV ribozymes cloned in the Eco47 III restriction site of the *VA*δIV gene are shown in Figure 1. When ribozymes were inserted in an internal position of the *VA*δIV gene (Eco RV), the oligonucleotide sequences were as follow: RBZ10int, GGGATCGCCCACATTTGGCTCTGAT-GAGTCCGTGAGGACGAAACCTGGTAATCAGGATCCC.

In vitro *transcription assays*

The various chimeric VAδIV RBZ RNA molecules and their HIV RNA substrates were obtained by *in vitro* transcription assays using the AmpliScribe™ T7 Transcription Kit (Epicentre Technologies, Le Perray, France). Labeled RNAs were synthesized by adding 50 μCi of [α-^{35}S]UTP (3000 Ci/mmol) to the reaction mix. DNA templates used to synthesize the various ribozymes *in vitro* were obtained after amplification by PCR of the corresponding VAδIV RBZ gene. Each PCR was carried out using a common upstream primer (42 nt), VApT7, AAAT**TAATACGACTCACTATA**GGGCACTCTTCCGTG-GTCTGG, that contains the T7 promoter (17 nt in bold) as well as additional sequences complementary to the first 21 nt of the Ad2 *VA1* gene (underlined) and a downstream primer specific either to the ribozyme sequence or to the 3′-end of *VA*δIV gene, VA3′ end, AAAAGGAGCGCTCCCCCGTTG. PCRs were carried out as described, and 1 μg of gel-purified PCR product was used as DNA template for the *in vitro* transcription assay. Preparation of template DNAs by PCR permits a total flexibility in the selection of the 5′-end and 3′-end of the transcript, which allows synthesis *in vitro* of large amounts of RNAs identical to those produced *in vivo*.

Template DNAs used for the production of HIV RNA substrates were obtained through the cloning of two subfragments of the HIV-1_{BRU} Gag sequence (GagA and GagB) in the pCRScript™SK(+) vector using the pCRScript™SK(+) Cloning Kit (Stratagene). GagA and GagB sequences were from HIV-1_{BRU} sequence (nt 251–950 and nt 914–1920). Corresponding DNA fragments were obtained by PCR using the following primers:

GagAu, GCTGAAGCGCGCACGGCA, GagAd, GATGGTC-TCTTTTAACAT, GagBu, CATCAAGCAGCCATGCAA, and GagBd, TCTGCTCCTGTATCTAAT. Purified PCR products were cloned into pCRScript vector before sequencing. After *in vitro* synthesis, labeled RNAs GagA (833 nt) and GagB (1006 nt) were purified on RNAse-free Quick Spin™ Columns (Boehringer Mannheim, Meylan, France) and quantified. A specific activity of 50,000–100,000 cpm/μg was usually obtained.

In vitro *ribozyme assays*

For *in vitro* cleavage assays, ribozymes or [^{35}S]-labeled substrate RNAs solubilized in TE buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) were heated at 95°C for 5 minutes and renatured by slowly cooling to 37°C. After addition of $MgCl_2$ (20 mM), cleavage reactions were initiated by mixing together an equal volume (5 μl) of ribozyme and labeled substrate RNA. The reactions were stopped at various times by adding EDTA (50 mM) and precipitated by ethanol with 10 μg yeast TRNA as carrier. For determination of the kinetic parameters, ribozyme concentration (500 nM) was tenfold greater than substrate concentration (50 nM). Labeled cleavage products were treated at 65°C for 5 minutes with 1.1 M formaldehyde/10 mM sodium phosphate, pH 6.5. Samples were electrophoresed in a 2% agarose gel containing 1.1 M formaldehyde/10 mM sodium phosphate, pH 6.5, and 2 mM EDTA. RNAs were alkali transferred to a Hybond N+ nylon membrane (Amersham, Arlington Heights, IL), which was directly exposed to XAR5 films (Kodak, Marne la Vallée, France). The densitometric analysis of autoradiographed film was obtained by scanning (Gel Analyst System, Iconix, France). Molecular size was determined by comparison with nucleic acids molecular weight standards (Boehringer Mannheim), including VA1 RNA (160 nt), VA_crev RNA (188 nt), and VAδIV RNA (134 nt).

Cellular RNA extraction and analysis

Human embryonic kidney 293 cells (ATCC, CRL 1573) and simian COS-7 cells (ATCC, CRL1651) were grown in DMEM (Gibco-BRL, Life Technologies, Cergy Pontoise, France) containing 7% fetal bovine serum (FBS) (Biowhittaker, Emerainville, France), 100 U/ml penicillin, and 50 μg/ml streptomycin. Transfections of 293 cells were carried out through $CaPO_4$ precipitation procedure using the MBS Mammalian Transfection Kit (Stratagene). One day before transfection, cells were plated in a 6-well cluster (3 × 10^5 cells per well) precoated with laminin (Boerhinger Mannheim). RNA extraction and analysis were performed as previously described (Cagnon et al., 1995). For Northern blot analysis, membranes were probed with radiolabeled oligonucleotides complementary either to the 5′-end of the VA1 RNA, VAS2, GCCATGATACCCTTGCGAATT-TATCCACCAGACCA, or to the central domain of VAδIV RNA, VASM, CACGGCGGACGGCCGGATCCGGGG. As indicated, the pCMV β-gal control plasmid furnished with the transfection kit (a plasmid harboring the β-galactosidase gene under the CMV promoter) was cotransfected (0.5 μg) with plasmids encoding for the different ribozymes (5 μg). Northern blots were subsequently probed with a VA1 or a β-gal-specific probe. The level of expression of the β-gal mRNA was used as an internal control to normalize transfection efficiency. The half-life ($t_{1/2}$) determination of VA1-modified RNAs was determined as previously described (Cagnon et al., 1995).

In situ *hybridizations*

In situ hybridizations were performed as previously described (Bertrand et al., 1997) except that cells were fixed for 10 minutes in 4% paraformaldehyde, 10% acetic acid, 1× PBS and that hybridization was carried out overnight at 37°C. The RNA probe used in this study was complementary to the VA1 sequence and was internally labeled with digoxigenin during *in vitro* transcription. After hybridization, slides were incubated with an antidigoxigenin antibody conjugated to alkaline phosphatase and stained with NBT/BCIP (Bertrand et al., 1997).

Prediction of RNA secondary structure

The predicted pattern of folding VA1-modified RNAs was obtained with Mac DNASIS Pro V3.6 software (Hitachi), using the Zuker algorithm (Zuker and Stieger, 1981).

RESULTS

Construction of VAδIV and VAδIV RBZ *genes and* in vitro *analysis of ribozyme catalytic activity*

Wild-type Ad2 VA1 RNA was modified by deleting most of the nucleotides forming its central domain to create a new RNA carrier molecule termed VAδIV RNA (Fig. 1). Thus, the VA1 RNA was devoid of its ability to block the activation of PKR, as the integrity of the VA1 RNA central domain is required to retain this function (Mellits et al., 1990; Rahman et al., 1995). As shown, the VAδIV RNA is shorter than the parental Ad2 VA1 RNA (134 nt instead of 160 nt) and consists essentially of an extended double-strand axis containing few mismatches (Fig. 1A). In addition, a new internal restriction site (Eco RV) was created to facilitate subsequent addition of exogenous sequences inside the VA1 central domain.

To inhibit replication of HIV-1, eight chimeric VAδIV RNAs were constructed that contained hammerhead ribozymes designed to cleave conserved regions of the HIV-1_{BRU} Gag encoding mRNA. Each anti-HIV hammerhead ribozyme may be divided into six functional domains (Fig. 1B). The consensus sequence for the catalytic core was initially described by Haseloff and Gerlach (1988), and the variable flanking sequences involved in the specific recognition of the target HIV RNA, which were termed specifier I and II, contained either 7 or 8 nucleotides. These ribozymes were introduced into the Eco47 III cloning site of VAδIV, very near its 3′ end (Fig. 1B). Thus, it was expected that the ribozymes could fold properly into active conformation, as they appeared to be relatively free of intramolecular hybridization with the nonribozyme sequence (not shown).

Ribozyme catalytic activity for each anti-HIV VAδIV RBZ was tested by analyzing *in vitro* the efficiency of convertion of HIV RNA substrates into the corresponding cleavage products (Fig. 2A,B). The length of the cleavage products was shown to correspond to their expected size. Each construct displayed activity that varied considerably (Fig. 2C): VAδIV RBZ36 and 33 were inactive, VAδIV RBZ20 was the most active, VAδIV

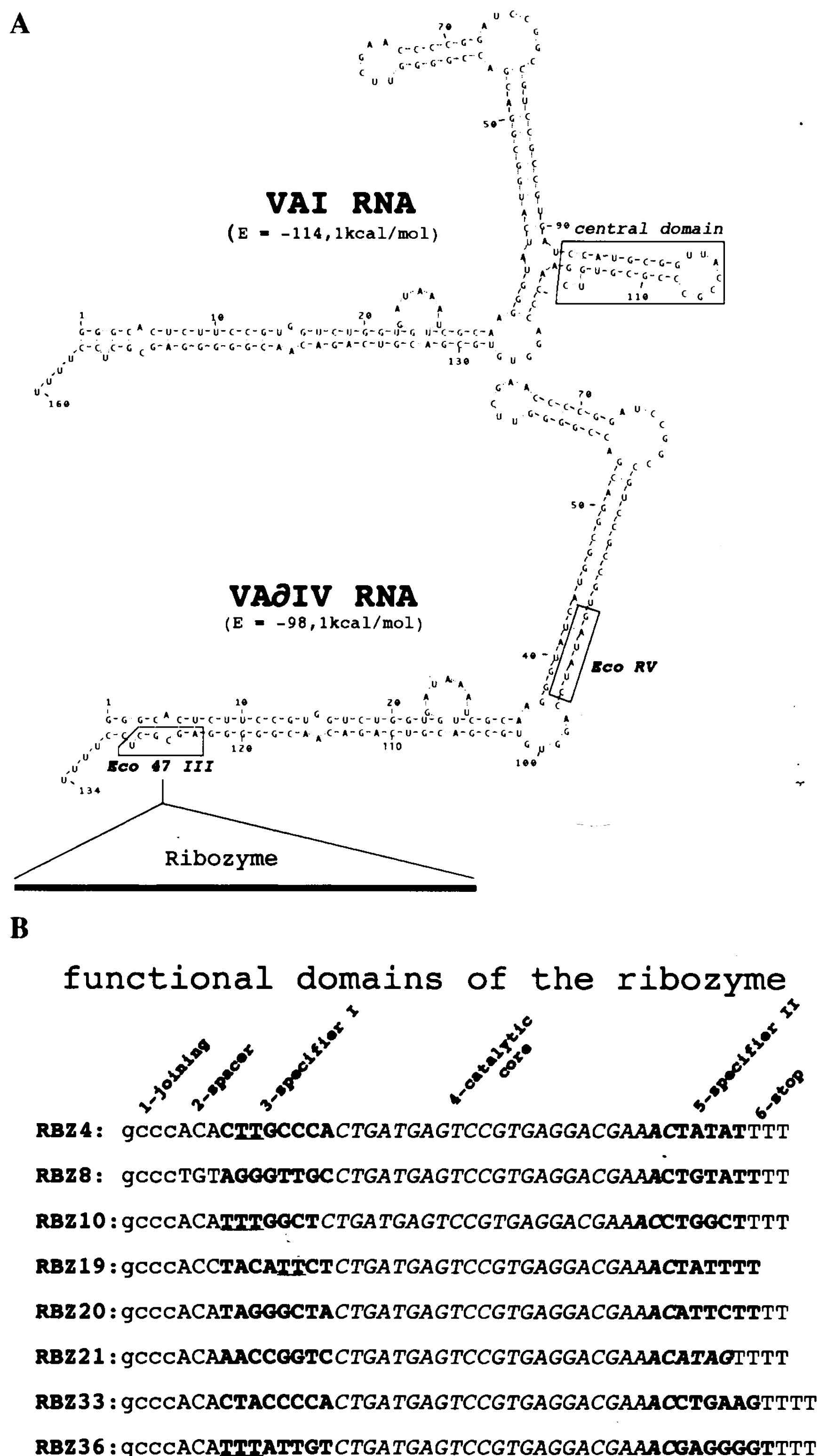

FIG. 1. Design of the *VAδIV* RNA carrier molecule and construction of chimeric anti-HIV VAδIV-RBZs. **(A)** The central domain of the adenoviral type 2 VA1 RNA was deleted (from nt 93 to 117, surrounded sequence) to create the VAδIV RNA carrier molecule. In addition, a mutation was introduced at nt 119 (A by C) to generate a new cloning site (Eco RV, boxed) localized in the central part of VAδIV RNA. The sequences and predicted RNA secondary structures of wild-type VA1 RNA and VAδIV RNA are shown. As indicated, the Eco47 III restriction site (boxed) was used to add different ribozymes at the 3′-end of the VAδIV RNA molecule. **(B)** The sequence of the eight anti-HIV ribozymes developed for this study are represented as divided into six functional domains: *1*, the joining domain (small characters) is likely to close the terminal stem of the chimeric VAδIV RBZ by base pairing with the 5′-end; *2*, the spacer domain is added to separate the ribozyme sequence from the carrier molecule; *3* and *5*, the specifier I and II (bold characters) are sequences that are complementary to the target HIV-1_{BRU} Gag RNA: *4*, the catalytic core (italic characters) represents the hammerhead ribozyme consensus sequence; *6*, a run of 4 T-residues acts as a strong signal stop.

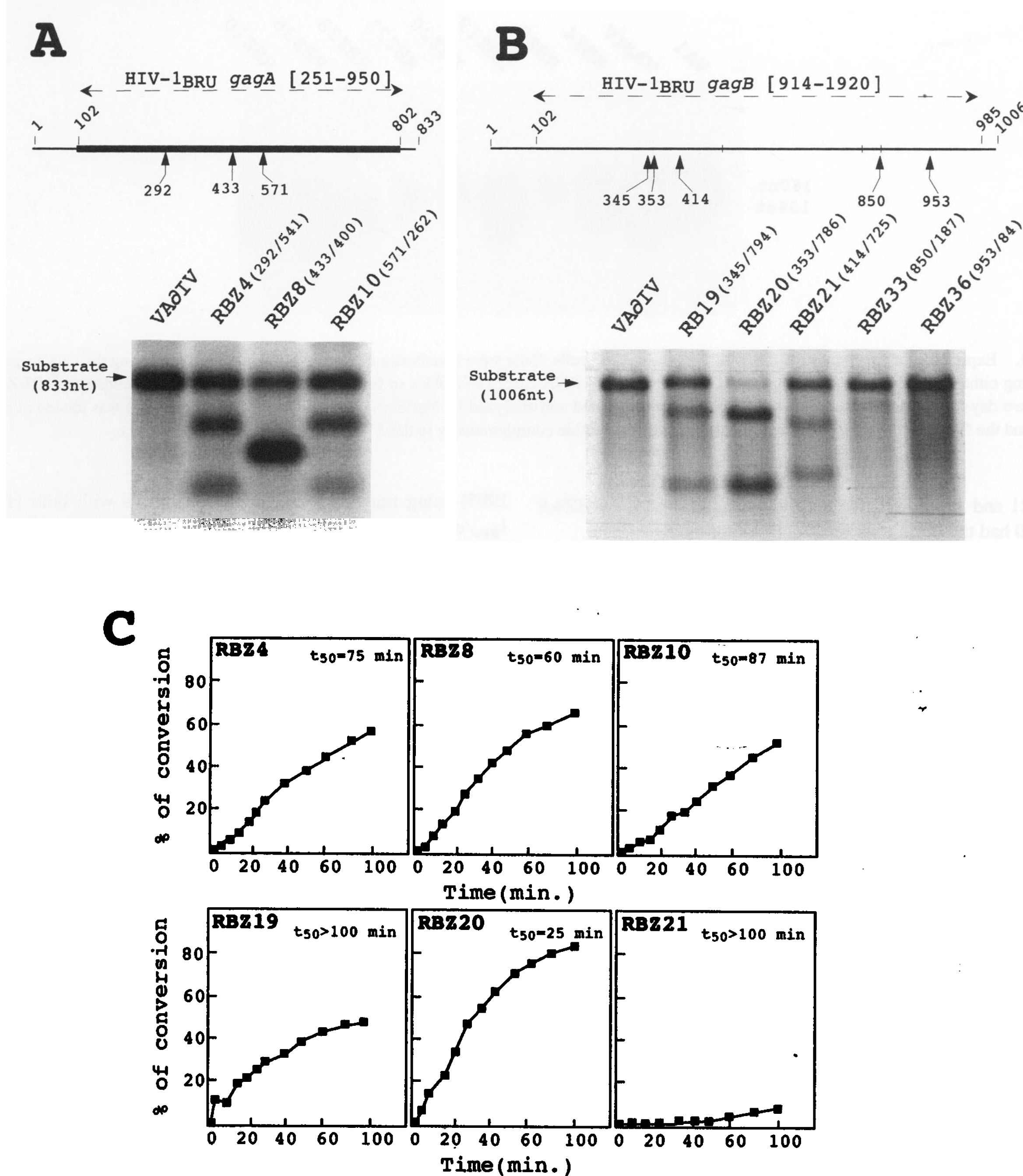

FIG. 2. Analysis of the *in vitro* cleavage activity of the chimeric anti-HIV VAδIV RBZs. **(A,B)** Two HIV-cloned template DNAs were used to obtain HIV-1_{BRU} gag RNA substrate from *in vitro* transcription assays: GagA and GagB were from HIV-1_{BRU} sequence spanning, respectively, from nt 251 to 950 and from nt 914 to 1920 (large brackets). Bold lines represent HIV sequence, and thin lines were transcribed from the plasmid template. The HIV-1_{BRU} GagA RNA substrate (833 nt) was used to study the *in vitro* cleavage activity of chimeric VAδIV RBZs 4, 8, and 10 **(A)** and HIV-1_{BRU} GagB RNA substrate (1006 nt) for VAδIV RBZ19, 20, 21, 33, and 36 **(B)**. The cleavage site is shown by an arrow, and the expected length of cleavage products is given between small brackets. [^{35}S]-labeled HIV RNA substrates (100 nM) were incubated 2 hours at 37°C in the presence of each ribozyme (200 nM), and the appearance of cleavage products was electrophoresed in denaturing conditions. The electrophoretic mobility of cleavage products was compared with molecular weight standards. **(C)** [^{35}S]-Labeled HIV substrates (50 nM) were incubated for the indicated times with ribozymes (500 nM), and the products of the reaction were analyzed by electrophoresis. Autoradiographs were analyzed by densitometric scanning, and values are expressed as percentage of substrate conversion into cleavage products. For each ribozyme, the time required to hydrolyze 50% of the substrate (t_{50}) was indicated.

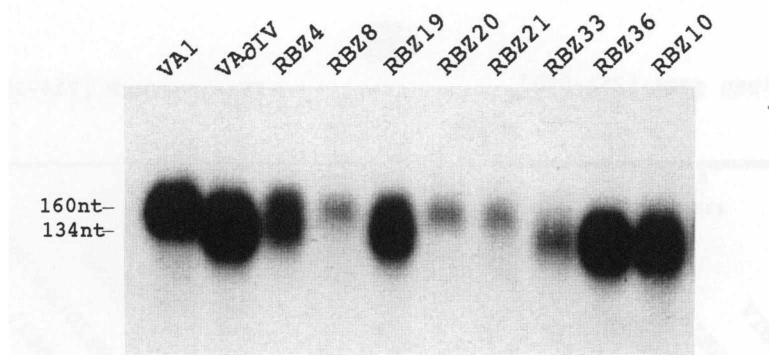

FIG. 3. Expression of the chimeric VAδIV RBZs genes in 293 cells. Cells were transitorily transfected with plasmids harboring the various genes encoding either for wild-type VA1 RNA (VA1), for the VAδIV RNA carrier (VAδIV), or for the eight chimeric ribozymes constructs (RBZ4 to 36). Two days after transfection, cytoplasmic RNAs were isolated and analyzed by Northern blot analysis. Total RNA (5 μg) was loaded in each lane, and the filters were probed with a [^{32}P]-labeled oligonucleotide complementary to the 5′-end of VA1 RNA (VAS2 probe).

RBZ21 and 19 showed the least activity, and VAδIV RBZ4,8 and 10 had the same efficiency in cleaving their substrate.

Analysis of the intracellular expression of the anti-HIV VAδIV RBZs

The ability of the eight chimeric VAδIV RBZs to inhibit HIV viral spreading in human cells was investigated. This experiment was conducted as previously described (Cagnon et al., 1995) using transiently transfected 293 cells with both HIV-1_{BRU} encoding plasmid and one of the eight different plasmids harboring a ribozyme gene. Despite several attempts, no specific inhibition of HIV replication was observed (data not shown).

To explain this failure, we further investigated the expression of the different ribozyme cassettes in cells. The different constructs were transiently transfected in 293 cells, and RNAs were isolated for subsequent Northern blot analysis (Fig. 3). Despite

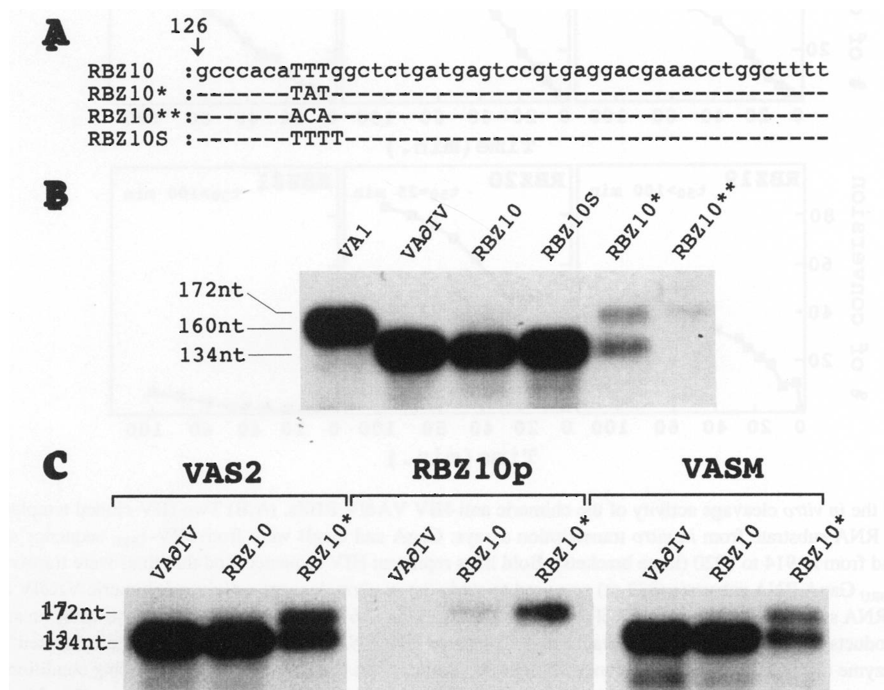

FIG. 4. Analysis of the expression of the VAδIV RBZ10 mutants. **(A)** Three mutants of the VAδIV RBZ10 (RBZ10*, RBZ10**, and RBZ10S) were constructed by adding the mutated ribozyme sequence downstream of the VAδIV RNA sequence at position 126 (site Eco47 III). Only the mutated ribozyme sequences are shown. **(B)** 293 cells were transfected with plasmids encoding different VAδIV·RBZ10 mutants. Two days after transfection, cytoplasmic RNAs were isolated and analyzed by Northern blot analysis. Total RNA (5 μg) was loaded on each lane, and the filters were probed with a [^{32}P]-labeled oligonucleotide complementary to the 5′-end of VA1 RNA (VAS2). **(C)** Cells were treated as in **(B)**, but identical Northern blots were probed either with VAS2 or RBZ10p, which is specific for ribozyme 10 sequence, or with VASM probe, which is complementary to the central part of VAδIV RNA.

deletion of the VA1 RNA central domain, the *VAδIV* gene was abundantly expressed. However, by adding the various ribozymes at the 3′-end of the *VAδIV* gene, the intracellular expression of this gene was greatly modified (Fig. 3). It was obvious that both size and level of expression were altered. Some constructs (i.e., VAδIV RBZ4, 10, 19, and 36) showed a high level of expression close to that of the parental *VAδIV* gene. However, for these constructs, the transcripts appeared to be shorter, with a length of approximately 130–140 nt instead of the expected 172 nt (Figs. 3 and 4). Other constructs (i.e., VAδIV RBZ8, 20, and 21) were weakly expressed but had a correct length of 172 nt. VAδIV RBZ33 was characterized by a low level of expression and displayed an apparently heterogeneous size. The possibility that transfection efficiency may explain the different levels of ribozyme expression was ruled out, as these results were reproduced at several times in independent experiments. In addition, cotransfection experiments using the pCMV β-gal as an internal control of transfection have revealed that β-gal mRNA was expressed to an identical level in all conditions (data not shown).

A detailed analysis of the length of the VAδIV RBZ10 transcript confirmed that this RNA was shorther than expected (Fig. 4B). Its apparent electrophoretic mobility in denaturing conditions was comparable to that of the VAδIV RNA (134 nt) and greater than the mobility of the wild-type VA1 RNA (160 nt). Moreover, despite a high level of expression (Fig. 4C, VAS2), VAδIV RBZ10 transcript was not detected when the same Northern blot was probed with a ribozyme-specific probe (Fig. 4C, RBZ10p). Thus, the lack of ribozyme moiety was sufficient to explain that the VAδIV RBZ10 was shorter than expected. These observations and further investigations led us to conclude that highly expressed chimeric VAδIV RBZ genes were all shorter than their expected size, whereas the VAδIV RBZs that gave rise to full-length transcripts were weakly expressed.

Identification of sequence elements responsible for the short length of the chimeric VAδIV RBZs

Because the eight anti-HIV ribozyme constructs differ from each other only by the variable specifier sequences, it was hypothesized that these sequences might contain signals responsible for the aberrant expression of these pol III-transcribed genes. A sequence comparison has revealed that most of the chimeric genes that gave rise to anomalous short transcripts (i.e., RBZ4, 10, 19, and 36) contained several T-residues in the specifier I (see Fig. 1, underlined sequences). Therefore, we hypothesized that the presence of a run of T-residues could be responsible for the release of incomplete transcripts. This hypothesis was tested by generating three VAδIV RBZ10 mutated genes that contained a modified run of T-residues (Fig. 4A). For RBZ 10* the run of three T-residues was interrupted by replacing the second T by A, RBZ 10** was devoid of T-residues, and for RBZ10S, the G-residue that follows the run of T was replaced by a T-residue to introduce a wild-type pol III transcription termination signal (four consecutive T-residues).

The intracellular expression of these different VAδIV RBZ10 mutated genes was analyzed in transiently transfected 293 cells through Northern blot analysis (Fig. 4B,C). First, we observed that the addition of a strong stop signal does not change the electrophoretic mobility of the VAδIV RBZ10S transcript as compared with VAδIV RBZ10 and VAδIV RNA. This confirmed that the size of these three RNA molecules was the same and, thus, that the VAδIV RBZ10 RNA ended at the run of three T-residues. Second, by mutating only one T-

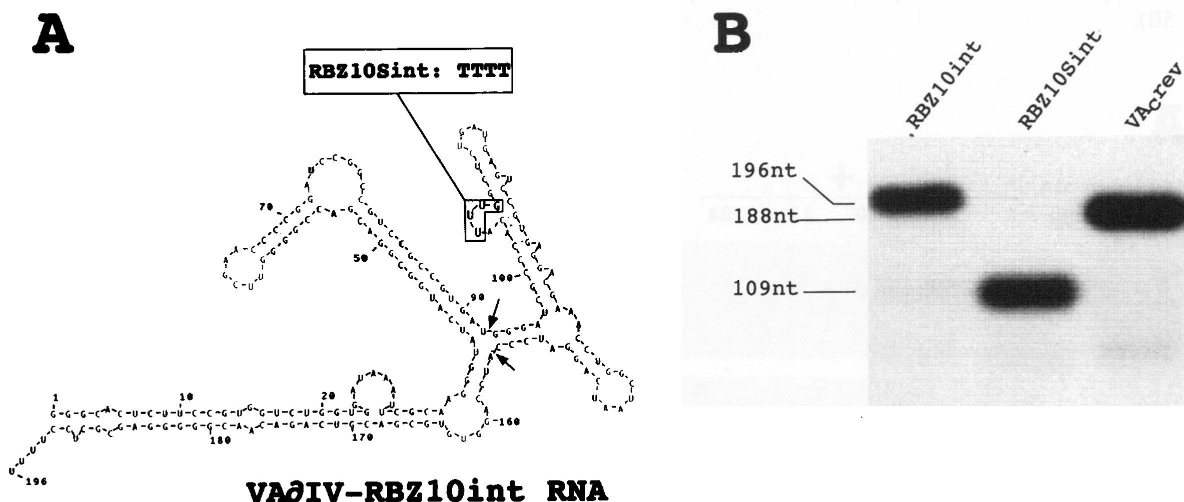

FIG. 5. Design and expression of chimeric VAδIV RBZ genes containing the RBZ10 sequence localized in an internal position (VAδIV RBZ10int). **(A)** The carrier VAδIV RNA was modified by adding the RBZ10int in the Eco RV internal restriction site of the *VAδIV* gene (arrows). The sequence and the predicted RNA secondary structure of this chimeric ribozyme are shown. In addition, the sequence TTTG found in RBZ10int (boxed) was replaced by a run of four T-residues to generate the RBZ10Sint (inset). **(B)** 293 cells were transiently transfected with plasmids encoding various VAδIV RBZ10int genes shown in (A). Total RNAs were analyzed by Northern blot analysis. Total RNA (5 μg) was loaded onto each lane, and the filters were probed with a [^{32}P]-labeled oligonucleotide complementary to the 5′-end of VA1 RNA (VAS2). VA_crev (188 nt) and molecular weight molecular RNAs were used for calibrating the electrophoretic mobility of the various transcripts.

residue into an A-residue (RBZ10*), the expression pattern of the resulting transcript was strongly modified. In addition to a first short transcript (134 nt), a second one appeared with a larger size. This additional band was likely to correspond to the full-length VAδIV RBZ10* transcript (172 nt), as this molecular RNA species was detected by Northern blot using the specific ribozyme probe (Fig. 4C, RBZ10p). Third, results obtained with RBZ10** constructs (Fig. 4B) extended these observations by showing that in the absence of T-residue in specifier I, only a larger full-length transcript was detected. These results argued that the run of three T-residues, located at the beginning of the RBZ10 sequence, played a critical role in the intracellular production of anomalously incomplete transcripts lacking the ribozyme sequence.

This result, however, seemed to contradict observations we made in a previous study (Cagnon et al., 1995). In this work, a short antisense sequence ($_c$rev sequence) was added inside the central domain of the VA1 RNA to give rise to chimeric antisense RNA, termed VA$_c$rev. It was shown that despite the fact that this sequence contained a run of three T-residues, the appearance of shortened transcripts was never observed. To explain this discrepancy, we generated new constructs that contained ribozyme 10 derived-sequences substituted for the VA1 RNA central domain (Fig. 5A) (RBZ10int and RBZ10Sint). Thus, a new family of chimeric VA1 RBZ genes, refered as VAδIV RBZint genes, was generated. 293 cells were transiently transfected with these constructs, and RNA expression was analyzed using Northern blot (Fig. 5B). It was shown that the RBZ10int transcript was strongly accumulated in cells and was 196 nt long, as was expected for the full-length transcript. Therefore, the run of three T-residues of the RBZ10 was unable to promote the release of short transcripts when located at the internal position. On the other hand, the wild-type pol III transcription termination signal (four T) located in the same context (RBZ10Sint) promoted the transcription termination, showing that transcriptional arrest at this position was indeed possible (Fig. 5B).

Because the VAδIV RBZint10 RNA was stable and abundantly expressed in cells, we investigated further its ability to cleave the HIV-1$_{BRU}$ Gag RNA. Analysis of the *in vitro* ribozyme catalytic activity of this construct was conducted as described but was unsuccessful, as no cleavage activity was revealed (data not shown).

Analysis of the intracellular stability of the chimeric VAδIV RBZ

The low expression level of some chimeric VAδIV RBZ genes (i.e., RBZ8, 20, 21, and 33) was somewhat surprising, as the *VAδ*IV gene cassette used to drive ribozyme expression was highly expressed to give rise to a stable RNA product (Fig. 3). We hypothesized that the long 3′-end extension formed by the ribozyme could destabilize wild-type VAδIV RNA. To test this hypothesis, the mutated VAδIV RBZ10* was of particular interest, as this gene gave rise to short and long transcripts in the same cell. Therefore, direct comparison of half-life ($t_{1/2}$) for long and short transcripts was made in 293 cells transfected with this construct (Fig. 6). After transfection, the cells were treated (or not) with actinomycin D to block all endogeneous transcriptional activities. RNAs were extracted at different times and analyzed by Northern blot to quantify the expression level of the VAδIV RBZ10*. The stability of the two RNA species was very different. As shown in Figure 6, the full-length transcript was very unstable ($t_{1/2} < 1.5$ hours) compared with the short one ($t_{1/2} = 7.5$ hours). Concurrently, the $t_{1/2}$ measured for the VAδIV RBZ10 transcript was comparable to those of the short VAδIV BZ10* transcript ($t_{1/2}$ around 7 hours) (Fig. 6B) and of the parental VAδIV RNA carrier ($t_{1/2}$ around 6.5 hours) (data not shown).

Subcellular localization of RNAs expressed from various cassettes derived from the VA1 *gene*

In addition to its abundance, the ribozyme ideally should be cytoplasmic in order to target mRNA (Bertrand et al., 1997;

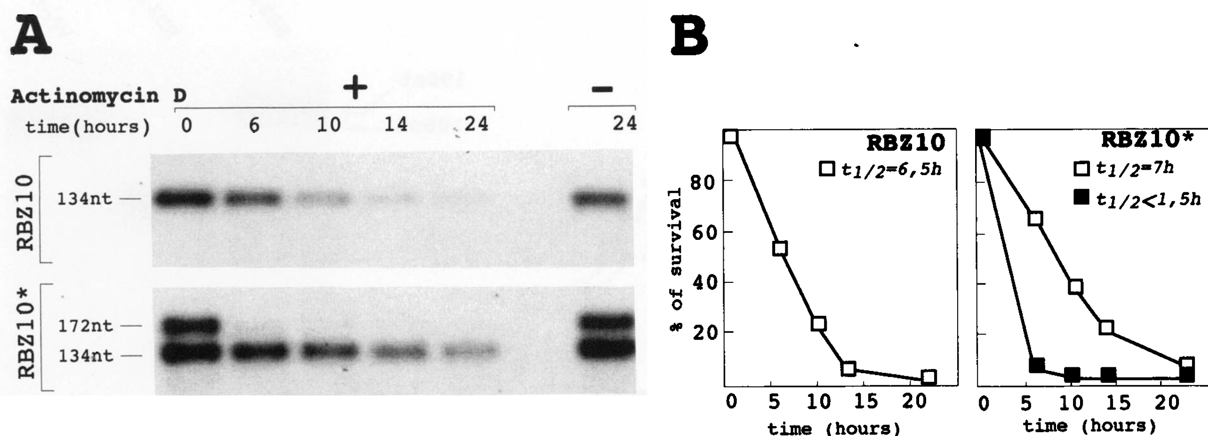

FIG. 6. Characterization of the intracellular stability of chimeric VAδIV RBZ10 RNA. **(A)** 293 cells were transfected with plasmids harboring either the VAδIV RBZ10 or VAδIV RBZ10* genes. Cells treated (or not) with 4 μg/ml actinomycin D for the indicated times were collected, and total RNA was isolated. Total RNA (5 μg) was analyzed by Northern blot analysis, and the filters were probed with a [^{32}P]-labeled oligonucleotide complementary to the 5′-end of VA1 RNA (VAS2). **(B)** After scanning of the autoradiograph in (A), the percentage of survival was calculated with reference to the zero time sample. For RBZ10*, open and filled squares represent, respectively, the kinetics of decrease measured for the smaller RNA species (196 nt).

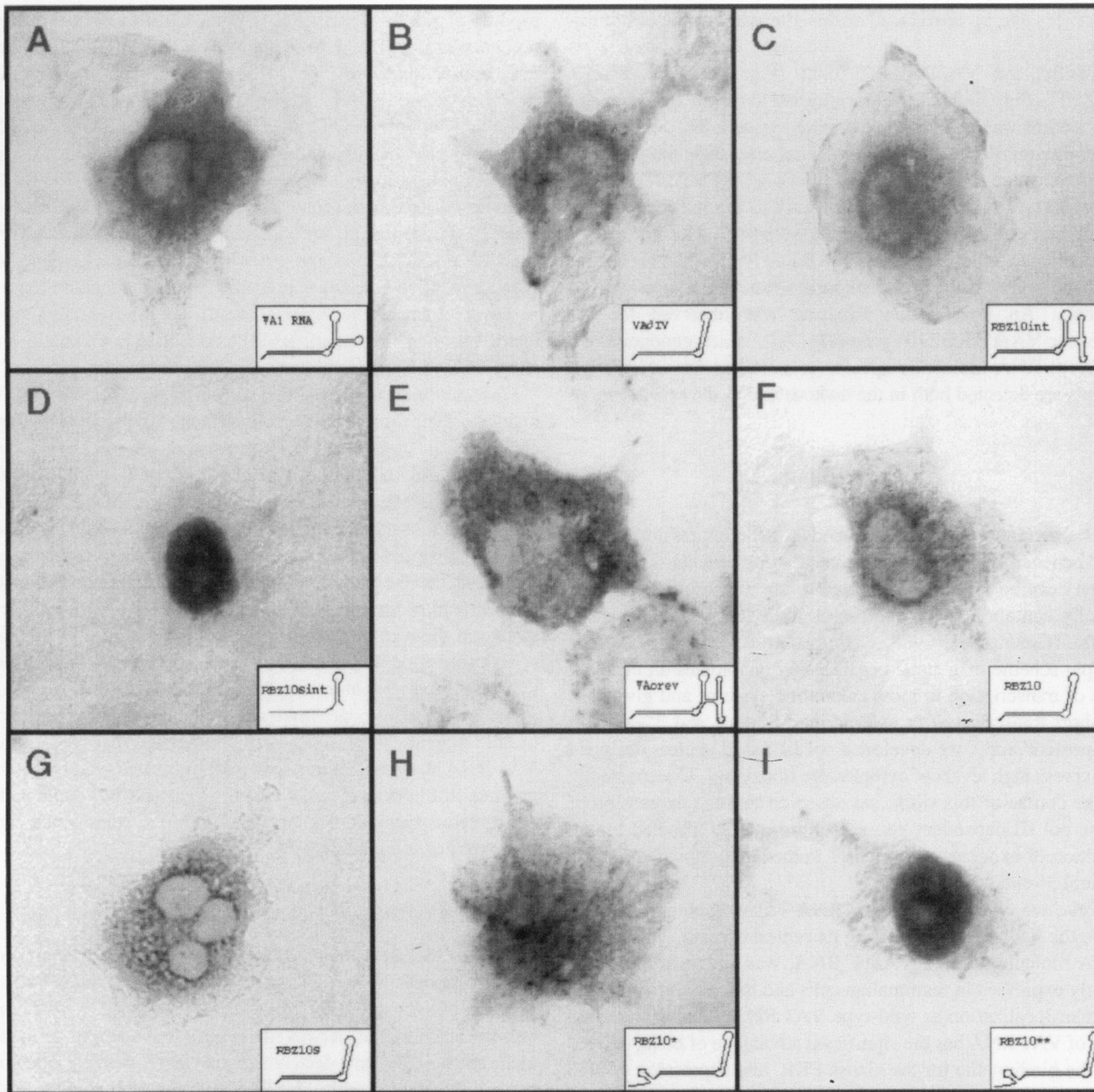

FIG. 7. *In situ* localization of the various ribozymes derived from VAδIV cassette expression. Transiently transfected COS cells expressing the different constructs developed for this study were hybridized *in situ* with an RNA probe specific for VA1 RNA. Probe detection was performed using the alkaline phosphatase color reaction as described in Materials and Methods. Cells were transfected with wild-type VA1 RNA **(A)** and VAδIV RNA **(B)** expression plasmids, and the following ribozymes expression cassette: VAδIV RBZ10int **(C)**, VAδIV RBZ10Sint **(D)**, VAδIV RBZ10*int **(E)**, VAδIV RBZ10 **(F)**, VAδIV RBZ10S **(G)**, VAδIV RBZ10* **(H)**, and VAδIV RBZ10** **(I)**.

Hormes et al., 1997). The VA1 RNA is cytoplasmic, but it was not known if the various modifications we introduced would modify its localization. Because 293 cells are relatively small cells, it was difficult to obtain a good spatial resolution for compartmentalization studies. To overcome these difficulties, COS cells were used, although similar results were obtained using 293 cells. COS cells were transiently transfected with the various cassettes derived from the VA1 RNA gene, including mutants of the RBZ10, and processed for *in situ* hybridizations (Fig. 7). As expected from a previous study, wild-type Ad2 VA1 RNA accumulated in the cytoplasm (Fig. 7A). By deleting the VA1 RNA central domain, this localization was not modified, as the VAδIV RNA was also found in cytoplasm (Fig. 7B). Similarly, chimeric genes in which the ribozyme was appended at the 3′-end of the VAδIV and which produced an anomalous short transcript that is almost identical to the VAδIV RNA (VAδIV RBZ10 and VAδIV RBZ10S) produced a cytoplasmic transcript (Fig. 7F,G). More interestingly, constructs in which the added sequence RNA was inserted instead of the central domain (VAδIV RBZ10int and VA$_c$rev) were also cytoplasmic (Fig. 7C,E). This shows that the central domain was not required for cytoplasmic export of the VA1 RNA and that many

sequences can be introduced at this site without inhibiting export.

Finally, the VAδIV RBZ10Sint (Fig. 7D) and VAδIV RBZ10** (Fig. 7I) RNAs were restricted to the nucleus. VAδIV RBZ10Sint was abundantly expressed in cells but was devoid of its natural 3′-end VA1 RNA sequence because the presence of a strong stop signal at position 109. VAδIV RBZ10** was expressed at a low level to give rise only to the full-length transcript that contained the ribozyme sequence. The apparently high expression level of VAδIV RBZ10** (Fig. 7I) did not reflect the reality, as different exposure times were used during staining. An intermediate situation was observed for the chimeric VAδIV RBZ10* gene (Fig. 7H), which generated two different RNA molecular species in cells and for which transcripts are detected both in the nucleus and in the cytoplasm.

DISCUSSION

The regulation of pol III-dependent gene expression is usually believed to involve simple regulator elements. Their promoters can be internal or external to the transcription unit but usually contain a small number of short functional consensus motifs (Geiduschek, 1988; Campbell and Setzer, 1992). This simple scheme of genetic organization guarantees an efficient rate of transcription in most eukaryotic systems and gives rise to short transcripts with well-defined 5′-ends and 3′-ends. In the present study, we developed pol III-based vectors designed to express high levels of cytoplasmic ribozymes. Unexpectedly, in the course of this work, we observed that the expression of these pol III-dependent genes was profoundly affected by the addition of exogeneous sequence immediately upstream of the natural 3′-end of the gene.

First, we constructed a new RNA carrier molecule derived from the VA1 RNA by deleting its central domain. This shuttle RNA molcule, termed VAδIV RNA, was shown to be abundantly expressed in mammalian cells and to have the same subcellular localization as wild-type VA1 RNA. This new derivative of VA1 RNA has the significant advantage of being deleted for the binding site for the kinase PKR, and expression of high levels of this RNA should, therefore, not affect the physiology of the cell.

Second, anti-HIV ribozymes were appended at the 3′-end of the VAδIV RNA by cloning at the Eco47 III restriction site. By adding the ribozyme at this external position, we wished to reduce intramolecular interactions between ribozyme and nonribozyme sequences to obtain the highest ribozyme activity in the chimeric molecule. This was indeed the case, as shown through analysis of *in vitro* cleavage assays. However, when the intracellular expression of these chimeric genes was assayed, we observed a number of anomalities that we analyzed in further detail.

Premature release of incomplete transcript

Some chimeric constructs are abundantly expressed, but they give rise to anomalous short transcripts devoid of the ribozyme sequence (i.e., RBZ4, 10, 19, and 36). The formation of these short transcripts could be due to a premature transcriptional arrest, or, alternatively, they could represent a stable degradation product of the full-length RNA. First, we show that this phenomenon is sequence dependent, as the presence of only two or three consecutive T-residues in the specifier I appears to be sufficient in promoting this phenomenon. This is demonstrated by showing that mutation of this critical run of T-residues (RBZ10 construct) promotes the appearance of the full-length transcript and results in a profound decrease in overall expression (8–10 times less). However, sequences that can favor an aberrant release of the transcript are complex, as illustrated through the use of VAδIV RBZs 8 and 19, which despite the presence of two consecutive T-residues in exactly the same position (e.g., 7 nt downstream of the terminal stem) are differentially regulated. On the other hand, RBZ33 that does not contain any string of T-residues is shorter but weakly expressed.

One can thus conclude that in addition to the run of T-residues, the primary sequence in which this run is embedded also plays an important role in promoting the release of short transcripts. Second, the position of the run of T-residues inside the *VA1* gene also seems to be critical, as the release of short transcripts does not occur when the same T-rich region is localized in the central part of the VA1 RNA. It is tempting to speculate that the long terminal stem, located either upstream or downstream of the run of T-residues, could represent a critical structural element involved in the correct processing of transcripts generated by pol III. It has been shown that nuclear proteins that bind to double-stranded RNA, such as the La protein (Maraia et al., 1994), are involved in regulating the termination of Pol III genes. Thus, it may be hypothesized that the terminal VA1 RNA stem can act as a structural motif involved in the fixation of different nuclear proteins, which facilitates either the downstream transcription termination or the stabilization and the export of small nuclear RNAs.

Instability of the full-length transcripts

We report that a major difference between full-length and short transcripts is their intracellular stability. The level of expression of each ribozyme is indirectly confirmed by ribozyme stability in cells. Full-length ribozymes are shown to be unstable, and thus they are detected at low level, whereas short ribozymes that are stable strongly accumulated in cells. Full-length transcripts are characterized by the presence of an additional unfolded RNA sequence appended to the terminal stem. It has been shown recently that in *Xenopus* oocytes, similar chimeric ribozyme constructs accumulate 8–10 times less compared with wild-type VA1 RNA (Prislei et al., 1997). Undoubtedly, the presence of an unfolded RNA sequence that terminates the molecules may favor the intervention of exoribonucleases. However, to occur, this degradation process appears to require additional determinants. First, in the Prislei et al. study (1997), it was shown that stability of the chimeric transcripts was low despite the presence of a stem-loop structure added upsteam to poly(U) termination site to stabilize the 3′-end. Second, we do not observe degradation of the VAδIV RBZ10Sint, which is likely to terminate also by an unfolded sequence. Third, full-length transcripts are unstable independent of the ribozyme primary sequence used and, thus, of the ribozyme folding. It may be hypothesized that the instability determinant is composed of the terminal single-stranded region located downstream from the terminal stem. In view of the production of

VA1 RNA or other similar cellular pol III-RNAs, such a mechanism could have evolved to allow for destruction of aberrant read-through transcripts that do not terminate at the correct site.

Intracellular localization of the VAδIV RBZ

Wild-type VA1 RNA is efficiently exported into the cell cytoplasm. Because VAδIV RNA has an identical intracellular distribution, we can conclude that the central domain of VA1 RNA is not required for cytoplasmic export. More interestingly, foreign sequences can be introduced in place of the central domain without perturbing export. Only two constructs, RBZ10** and RBZ10intS, give rise to RNA molecules that are retained in the nucleus. Further investigations are needed to explain why these transcripts are not exported to the cytoplasm. However, these two transcripts are characterized by an aberrant 3′-end that either lacks the wild-type 3′-end of VA1 RNA (RBZ10Sint) or displaces it 30 nt downstream of its natural location (RBZ10**). This shows that the presence of an additional sequence located just downstream of the wild-type VA1 RNA terminal stem altered the export of this RNA. Thus, our results suggest that the wild-type location of the VA1 3′-end, immediately after the terminal stem is required for efficient cytoplasmic export of the RNA.

Design of chimeric VA1 RBZ RNA

These results underscore that the termination, the stability, and the intracellular localization of VA1 RNA and of its derivatives are greatly influenced by the nature of the sequence found at the 3′-end of this pol III-transcribed gene. In particular, the terminal stem of VA1 RNA could be an important determinant of the fate of the transcript. On one hand, it is likely to favor the premature release of incomplete transcripts, and on the other hand, it may be a determinant in regulating the post-transcriptional events that govern export and stability of full-length RNA. In view of using VA1 RNA as a carrier molecule to express therapeutic ribozymes, our results argue that appending the ribozyme at the 3′-end of the terminal stem does not fulfil the expected requirements.

In contrast, replacing the central domain by the ribozyme sequence is a design that meets most of the required critiera. First, the chimeric molecule is stable and highly expressed in human cells. Second, we show that this type of chimeric transcript is efficiently exported to the cell cytoplasm. This is of interest if one recalls that molecular targets of ribozyme are mRNAs, which are mainly localized in the cell cytoplasm. Third, the VAδIV cassette was designed to be easy to manipulate for subsequent cloning. Furthermore, it does not interact with undesired cellular components, such as the kinase PKR. The only unknown fact is if such chimeric ribozyme molecules retain a high cleavage actvity. Some of our preliminary results suggest that under these conditions, the ribozyme may undergo structural constraints that impair its catalytic activity (data not shown) (Thompson et al., 1995; Prislei et al., 1997). It is likely that when the RBZ is added inside the VAδIV RNA molecule, it does not fold into a proper conformation that favors its catalytic activity. However, the structure of these chimeras can be further optimized to allow for higher ribozyme activity (Thompson et al., 1995), work currently in progress in our laboratory.

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