Volume 322, number 1, 21–24 FEBS 12378 May 1993

In vitro cleavage of HPV16 E6 and E7 RNA fragments by synthetic ribozymes and transcribed ribozymes from RNA-trimming plasmids

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Received 18 February 1993; revised version received 9 March 1993

A RNA-trimming plasmid pRG523 is constructed, in which three Rz genes, GR5(5′-*cis*-Rz gene), HR2G(*trans*-Rz gene) and GR3(3′-*cis*-Rz gene), are arranged in the order from 5′ to 3′ downstream from the T7 promoter. In vitro transcription of this plasmid shows that the *trans*-Rz can be trimmed to definite lengths by the *cis*-Rz on both sides of the *trans*-Rz. In vitro cleavage of HPV16 E6 and E7 RNA fragments of different lengths by synthetic Rz and that of E7 RNA with a length of 171 nt by synthetic Rz and transcribed Rzs with different lengths of flanking sequences is studied. The results show that the non-base-pairing flanking sequences on both Rz and target RNA can affect the cleavage reaction.

Ribozyme; Human papillomavirus type 16; RNA-trimming plasmid; In vitro cleavage; In vitro transcription

1. INTRODUCTION

The RNA molecule possessing a catalytic activity was named for the first time as ribozyme by Cech and his colleagues [1] after their discovery that the ribosomal RNA intervening sequence of *Tetrahymena* had some enzyme-like properties. Later, it was the Symons' Rz, known as 'hammerhead type Rz', that aroused the interest of scientists, since this kind of Rz was relatively simple in primary and secondary structures, and could be designed and synthesized in a lab [2]. In 1988, Haseloff and Gerlach first described the cleavage of target RNA at specific sites by designed hammerhead Rzs [3]. Since then, many researchers have reported the in vitro cleavage of target RNA by synthetic or transcribed hammerhead Rzs [4–6]. Sarver et al. found in 1989 that the harboring of the Rz gene by CD4+ HeLa cells against HIV gag mRNA could prevent the infection of HIV virus [7]. Furthermore, the Rz could inhibit gene expression in procaryotic and eucaryotic cells [8–10]. Thus, the Rz might be potentially applied for anti-virus infection and design of anti-virus drugs. However, the Rzs transcribed in vivo usually contain LFS (the sequences beyond those pairing with target RNA) that might affect the cleavage reaction. In this paper, we report the in vitro cleavages by synthetic Rzs and transcribed Rzs of E6 and E7 RNA of the HPV16 which is considered to be correlated with the cervical cancers. The effect of LFS of different lengths on the cleavage reaction is also studied. The results show that the LFS of both the target RNA and the Rzs affects the in vitro cleavage reaction. In order to produce Rz with shorter LFS, we have constructed a RNA trimming plasmid. The LFS of the *trans*-Rz will be cleaved off due to the presence of two *cis*-acting Rzs at both sides of the *trans*-Rz and thus, a Rz free from LFS can be produced in vitro or in vivo.

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Abbreviations: HPV, human papillomavirus; Rz, ribozyme; LFS, long flanking sequence; nt, nucleotide.

2. MATERIALS AND METHODS

2.1. *Synthesis of Rzs and Rz genes targeting HPV16 E6 and E7 RNA*

Two 38 mer Rzs (HR2 and HR7) are synthesized using ABI 391 EP DNA Synthesizer with RNA synthesizing reagents from Peninsula Laboratories Inc. The cleavage sites are designed at 240 of HPV16 genome (E6 protein coding region) and at 597 of HPV16 genome (E7 protein coding region). Since these sites are at the proximity of the initiation codon of the E6 and E7 RNA, the cleavage at these sites may lead to a stronger inhibition on the expression of the related genes. The genes corresponding to the HR2 (HR2G) and HR7 (HR7G) are also synthesized on the same machine with the DNA synthesizing reagents. For constructing the RNA-trimming plasmid, two genes for the *cis*-cleavage Rzs (GR5 and GR3) are also synthesized. To facilitate the cloning procedure, restriction sites are added to the ends of the synthesized genes.

2.2. *Construction of the RNA trimming plasmids*

The cloning strategy of the plasmid is shown in Fig. 1. Both strands of the *trans*-Rz genes and the *cis*-Rz genes are phosphorylated by T4 polynucleotide kinase and then annealed. The annealed Rz genes are ligated to pTZ19R (Pharmacia) or its derivatives linearized with indicated enzymes. Competent *E. coli* JM105 cells are transformed, and positive colonies are identified by restriction enzyme analysis and confirmed by sequence analysis as described by Sambrook et al. [11].

2.3. *Construction of the recombinant plasmid for in vitro transcription of E6 or E7 RNA*

The E6 and E7 genes are cut out from plasmid pME6SN and pME7SN, respectively [12], with *Xba*I and inserted into *Xba*I site of pTZ19R. After transformation, the positive colonies are selected and

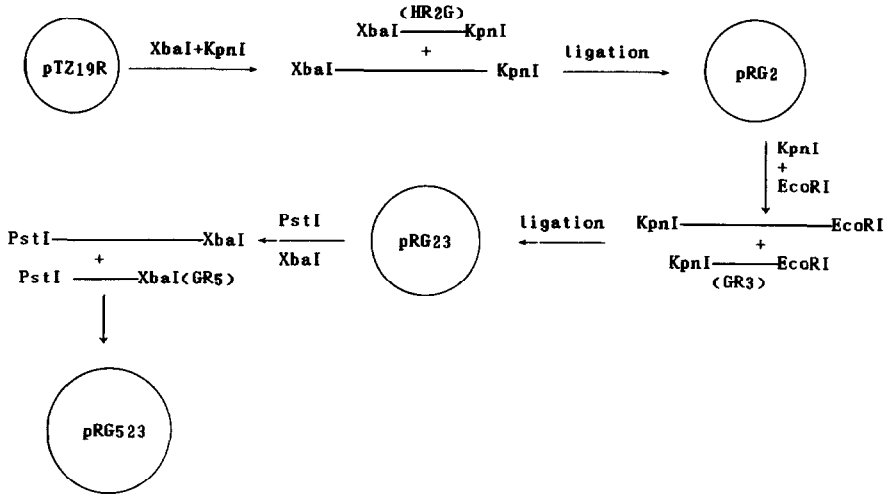

Fig. 1. The cloning strategy of plasmid pRG523.

the orientation of the inserted fragments are identified and named as pTZE6R or pTZE7R.

2.4. *Transcription of the RNA-trimming plasmid pRG523 and the self-cleavage of the transcripts*

In vitro transcription is carried out in a 20 μl solution containing 40 mM Tris-Cl, pH 7.9, 5 mM DTT, 2 mM spermidine, 8 mM $MgCl_2$, 0.5 mM ATP, GTP, CTP, and 0.2 mM UTP, 10 μCi [α-^{32}P]UTP, 1.0 μg circular or *Eco*RI-linearized template pRG523 DNA, 25 U T7 RNA polymerase. The reaction mixture is incubated at 37°C, and each aliquot of 3.5 μl of the mixture is taken at 5 min, 15 min, 30 min, 60 min and 120 min respectively. The gel is autoradiographed after running 6% PAGE. The self-cleaved products are cut from the gel and soaked in NES (0.5 M NH_4Ac, 0.1 M EDTA, 0.1% SDS) overnight. The products are precipitated by ethanol, washed once by 70% ethanol and dissolved in 50 μl of water. For getting larger amounts of the transcribed Rzs, transcription without isotope is set up as described above except that the cold UTP used is also 0.5 mM. The RNA is separated from NTP by gel filtration on Sephadex-G25, and measured by spectrophotometer.

2.5. *In vitro transcription of target RNAs*

A number of different endonuclease linearized plasmids, pTZE6R and pTZE7R, are used as the template for in vitro transcription (Fig. 2) as described above.

2.6. *In vitro cleavage of the target RNA by synthetic and transcribed Rzs*

The cleavage reaction is carried out in 5 μl solution containing 50 mM Tris-Cl, pH 7.5, 20 mM $MgCl_2$, 20 mM NaCl, 0.04 μM of Rz and target RNA. The mixture is incubated at temperatures indicated. After polyacrylamide gel electrophoresis and an overnight autoradiography, the bands are cut and the radioactivity is counted.

3. RESULTS AND DISCUSSION

3.1. *Cleavage of the HPV16 E6 and E7 RNA of different lengths by HR7 and HR2*

Fig. 2 shows the cleavage sites and the cleavage percentage of HPV16 E6 and E7 RNA fragments of different lengths by HR7 and/or HR2. The results show that the cleavage percentage of the short target RNA by synthetic Rz is higher than that of longer target RNA. HR2 cleave E7 RNA fragments of 171 nt and 360 nt long with the cleavage percentage of 64% and 25%, respectively; but the cleavage is inhibited when the E6-E7 RNA is transcribed together (686 nt long). When the 686 nt E6-E7 target RNA is first denatured by heating to 95°C for 3 min and then quickly cooled to 0°C, it can be cleaved by HR2, although there is some nonspecific degradation (data not shown). The cleavage of the E6 RNA fragments by HR7 gives a similar pattern although the difference is not as big as that of E7 RNA. The results show that the LFS on the target RNA can affect the cleavage reaction. It is demonstrated that the cleavage sites in RNA substrates with longer sequences may be covered by LFS, and the stereostructure of RNA substrates may also give a hindrance effect on the entrance of Rz to the cleavage sites. When denatured, the cleavage site will be exposed to Rz. Another possible

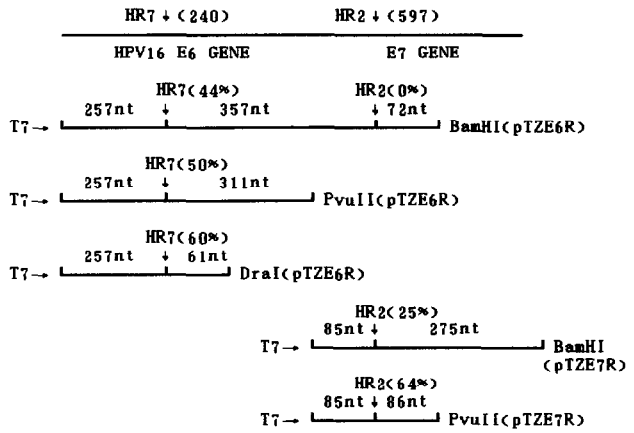

Fig. 2. Diagram of the cleavage of E6 and E7 RNA of different lengths with HR2 and HR7. The restriction enzyme used to linearize the plasmid was shown at the end of each fragment. The numbers shown in the parentheses were the cleavage percentage of the corresponding RNA fragment by ribozyme indicated. The reaction condition was described in section 2 and the reaction was incubated at 37°C for 90 min.

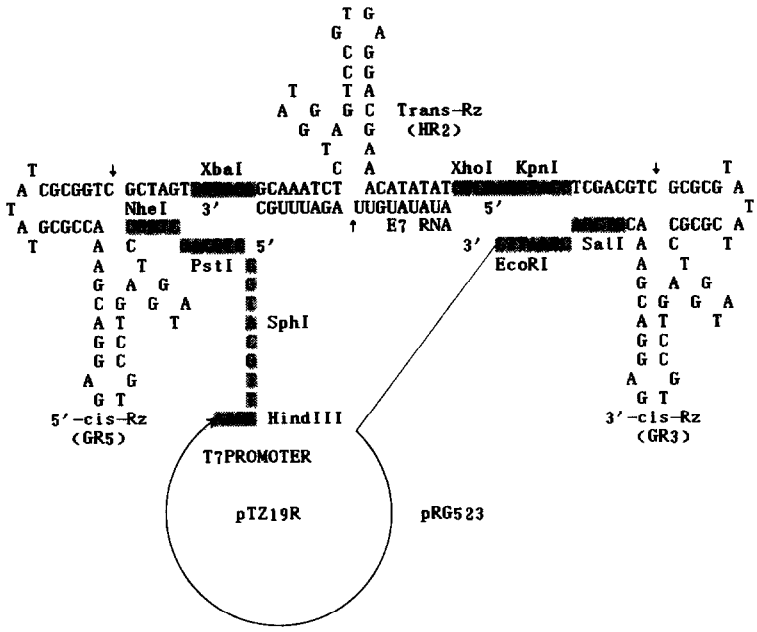

Fig. 3. The map of the RNA-trimming plasmid pRG523. The shadow area shows the sequences of indicated restriction endonucleases.

explanation is that the LFS might be paired with the sequence around the cleavage site to form an incomplete helix which also prevents the Rz from cleaving the target RNA.

3.2. *Transcription of Rz HR2 and the self-cleavage by* cis-*cleavage Rzs*

In plasmid pRG523, three Rz genes (GR5, HR2G, and GR3) are arranged in the order from 5′ to 3′ downstream from the T7 promoter (Fig. 3). Two other plasmids, designated as pRG23, containing HR2G and GR3 without GR5, as well as pRG2, containing HR2G only, are also constructed (data not shown). Two *cis*-Rzs (Rz GR5 and GR3) are designed to trim the 5′ and 3′ LFS of transcribed HR2 Rz respectively. So, after transcription from these three plasmids in vivo or in vitro, HR2 with LFS of different lengths will be produced: the Rz without any process from pRG2, the Rz 3′ trimmed from pRG23, and the Rz both 5′ and 3′ trimmed from pRG523. There are several restriction endonuclease sites in pRG523, and it is easy to clone these Rz genes into eucaryotic expression vector and produce Rzs with LFS of different length in the transfected cells.

To characterize *cis*-cleavage of the 5′ and 3′ processing Rzs during transcription and, in particular, to estimate the relative rate of the transcription and cleavage reaction, aliquots of the transcription mixture are taken at different times (Fig. 4). When the RNA transcripts from *Eco*RI linearized templates pRG523 are examined at 5 min of transcription, the full-length RNA transcripts are found to be present only in trace amounts, and nearly all of the RNA transcripts are processed to give three fragments, i.e. 69 nt, 63 nt and 50 nt. The transcripts from the circular template are also processed to give two definite bands, 69 nt and 63 nt, and the 3′ fragment was found to be longer. From the results we can deduce that the self-cleavage of transcripts by the 5′ and 3′ *cis*-Rz is very efficient. In 1991, Taira et al. constructed a RNA trimming plasmid [13], but compared to our results, the self-cleavage rate is slow. This may be due to the sequence we used in the double helix around the cleavage site of the *cis*-acting Rzs which is

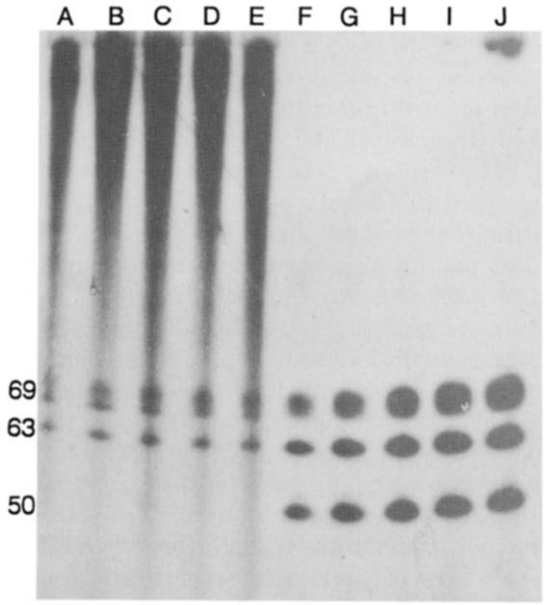

Fig. 4. The transcription of circular (A–E) and *Eco*RI linearized (F–J) plasmid pRG523 and self-cleavage of the transcripts. Lane A and F, 5 min; lane B and G, 15 min; lane C and H, 30 min; lane D and I, 60 min; lane E and J, 120 min.

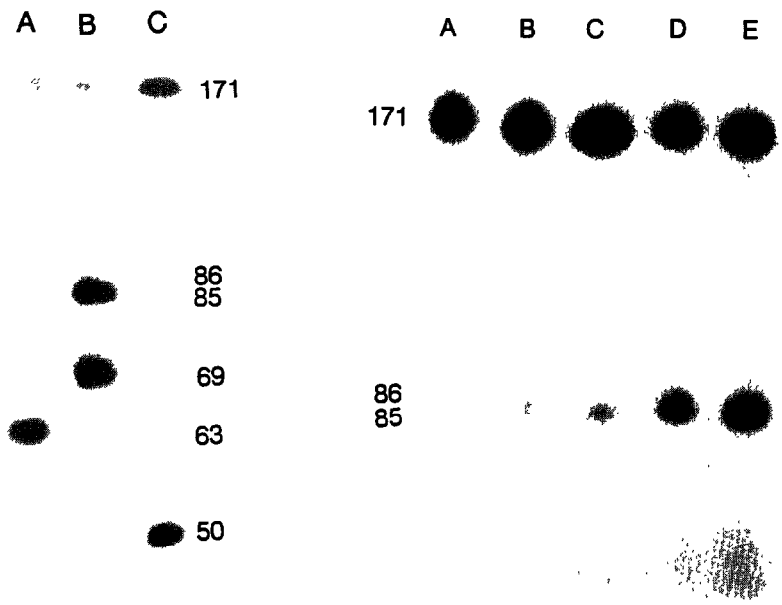

Fig. 5. (A) The cleavage of the E7 RNA by transcribed Rz from pRG523. The E7 RNA was incubated at 37°C for 90 min with 69 fragment (Rz69, lane B) 63 nt fragment (lane A); 50 nt fragment (lane C). (B) Comparison of cleavage of E7 RNA by synthetic Rz (lane E), and transcribed Rz from plasmids pRG2 (lane B), pRG23 (lane C), pRG523 (lane D). Lane A was the control without adding Rz. The reaction was incubated at 37°C for 60 min.

GC rich, whereas the folding back loop is AT rich. This arrangement may make it easy for the *cis*-acting Rz to form a hammerhead structure.

3.3. *In vitro cleavage of E7 RNA by synthetic Rz and transcribed Rz of different lengths*

Fig. 5A shows the cleavage of the E7 RNA by the transcribed Rzs from linearized pRG523. It is found that only the 69 nt (named Rz69) fragment which contains the *trans*-Rz can cleave the target RNA, whereas the 63 nt and 50 nt fragments which contain the 5′ and 3′ *cis*-acting Rz cannot cut the target RNA.

After in vitro transcription and processing, the transcribed Rzs from *Eco*RI linearized pRG2, pRG23, and pRG523 are 94 nt, 91 nt, and 69 nt, respectively. The comparison of cleavage efficiency of the synthetic Rz and transcribed Rz is shown in Fig. 5B. The results indicate that the cleavage activity of HR2 and the transcribed Rz69 is nearly the same. The Rz with shorter LFS has a higher activity. Only in higher concentration can the Rz with longer LFS attain the same cleavage percentage as HR2 and Rz69 (data not shown). The results indicate that the LFS of the Rzs may also hinder or cover the pairing sequences on Rzs and affect the cleavage reaction. This may be the reason why transcribed Rzs do not work well in cells [14,15]. Our plasmid may partially solve this problem when introduced into cells since they possess the *cis*-acting Rzs which will cleave the LFS off. However, we do not know the stability of these Rzs in the cells. In order to study the efficiency of these Rzs in vivo, the *trans*-Rz gene with or without *cis*-acting Rz genes is cloned into eucaryotic vectors. The investigation of their in vivo functions is under way.

Acknowledgements: We thank Prof. Nong-an Chen for helping design the Rzs and Ms. Min Zhuang for technical assistance in synthesizing oligonucleotides. This work was supported by Hi-Tech Development Programme in China.

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