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The Selection In Vivo and Characterization of an RNA Recognition Motif for Spectinomycin

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Abstract—Ribonucleoprotein (RNP) complexes participate in almost all macromolecular processes, including RNA processing, protein synthesis, and the signal recognition of proteins targeted for export. An understanding of these processes requires detailed knowledge of interactions at the molecular level, which has evidently been difficult due to the size and complexity of the particles. Fragmentation of large RNP complexes into functional subdomains is proven to be a successful in vitro strategy to probe ligand interactions at the molecular level. We reasoned that RNA molecules expressed in vivo may fold in such a manner as to mimic a drug binding site present on the intact ribosome. If expressed at sufficient levels, the RNA would sequester the antibiotic thereby permitting the continued function of the ribosome and consequently allow the cell to survive in the presence of the drug. Evidence is presented here in support of this RNA fragment-rescue concept following the selection and characterization of RNA fragments that confer resistance to the antibiotic spectinomycin. © 1997 Elsevier Science Ltd.

Introduction

The translation apparatus is essential to all living cells and represents one of the major targets for antibiotics. An understanding of the mechanism of drug action is dependent on detailed knowledge at the molecular level, of the structure and function of the ribosome and its associating factors. Such information is clearly difficult to obtain when faced with the size and complexity of the ribosome. However, several lines of evidence support the notion that the ribosome can be fragmented into smaller, functional subdomains that retain organizational and ligand-binding properties characteristic of the intact particle.[1] The small subunit ribosomal RNA (rRNA) from *Escherichia coli* (16S rRNA) is organized into three major domains: the 5′, central and 3′ domains and rRNA fragments representing each of these subdomains can reassemble with specific subsets of ribosomal proteins.[2,3,4] In vitro assembly of intact 30S subunits reflect two to three nucleation events, thus providing a further indication for the existence of independent assembly domains.[5] A 16S rRNA fragment encompassing the 3′ domain (nucleotides 923–1542) can be reconstituted together with eight ribosomal proteins, and results in the formation of a compact particle that resembles the head of the 30S subunit.[4] This particle retains the property of being able to bind the antibiotic spectinomycin which specifically protects the N-7 position of G1064 from attack by dimethyl sulfate in both 30S subunits and the subparticle.[4,6] The degree of protection to both particles shows the same dependence on drug concentration, indicating that spectinomycin binds with similar affinity to each particle. What is the precise nature of the spectinomycin binding/recognition motif within this domain? Resistance mutations have been mapped to structural changes in either ribosomal protein S5[7] or within helix 34 of 16S rRNA formed by base pairing between nucleotides 1046–1065 and 1191–1211 (Fig. 1).[8,9] The binding of spectinomycin is independent of S5 indicating that the rRNA is the major determinant of the binding site.[4] The G1064–C1192 base pair is likely directly involved in the binding of spectinomycin as revealed by the chemical footprinting data and the existence of resistance mutations that reflect either a disruption of the base pair or replacement of the base pair.[8] Mutations in *E. coli* 16S rRNA that confer spectinomycin resistance include C1192U,G,A, G1064U,C,A, G1064U-C1192A, G1064-C1192G, G1064A-C1192U, and C1066U.

The major effect of spectinomycin in vitro is proposed to inhibit the translocation of peptidyl-tRNAs from the A-site to the P-site by preventing the binding of elongation factor G (EF-G) to the ribosome.[10] Helix 34 has been proposed to melt during the elongation cycle and spectinomycin exert its inhibitory effect by stabilizing the helix.[8] It is interesting to note that helix 34 has the potential to exhibit two structural conformers similar to the phylogenetic model, without disruption of the overall base pairing arrangement (Fig. 2).[11] The conformers reflect the alternate availability of either an upper (1199–1201) or lower (1202–1204) 5′-UCA-3′ triplet.

In an attempt to further investigate the spectinomycin binding site and its precise mode of action, we have developed a novel in vivo approach to select for RNA molecules that bind spectinomycin. To date, characterization of the interaction between drug and rRNA has been based on in vitro approaches, for example, ligand binding to an oligoribonucleotide analogue of the decoding region located near to the 3′ end of 16S rRNA.[12] This RNA fragment interacts with both antibiotic

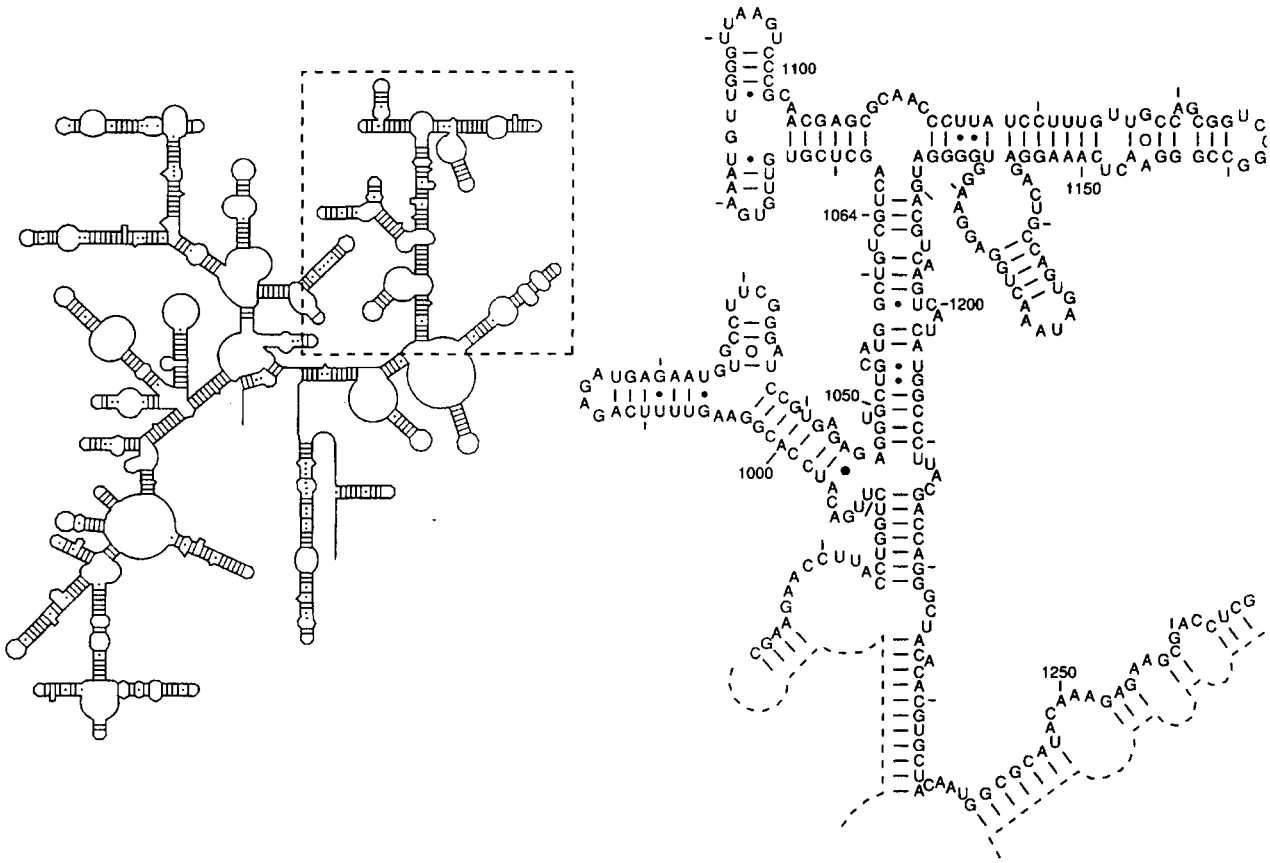

Figure 1. *E. coli* 16S rRNA secondary structure. Expression of the rRNA fragment (nucleotides 972–1266) encompassing helix 34 (nucleotides 1046–1065 and 1191–1211) confers resistance in vivo to spectinomycin.

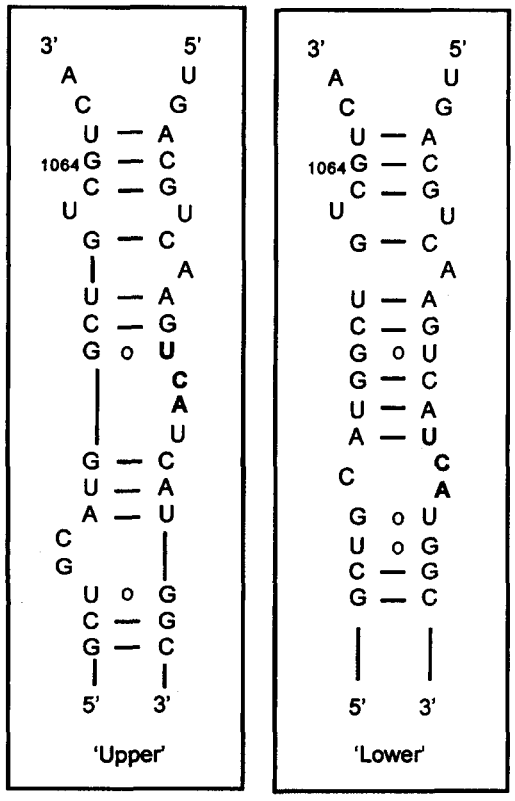

Figure 2. Proposed secondary structure conformers of wild-type helix 34. The conformers reflect alternate availability of either the 'upper' (left) or 'lower' (right) 5′-UCA-3′ triplets.

(neomycin) and RNA ligands (tRNA and mRNA) of the 30S subunit in a manner that resembles normal subunit function. We reasoned that RNA molecules expressed in vivo may fold in such a manner as to mimic a drug binding site present on the intact ribosome. If the RNA molecules are expressed at sufficient levels, then they will sequester the antibiotic thereby permitting the continued function of the ribosome and consequently allow the cell to survive in the presence of the drug. To validate this RNA fragment-rescue concept, two approaches have been undertaken. A specific rRNA fragment encompassing helix 34 was cloned and the transformants examined for tolerance to growth in the presence of spectinomycin. The second approach was to generate a random library of rRNA fragments and select for transformants present within the entire library that were resistant to spectinomycin. Both approaches resulted in the identification of RNA fragments that either encompassed the wild-type helix 34 or a molecule that could be predicted to fold at the secondary structure level in a manner that resembles helix 34.

Results

pGEMR

In an attempt to maximize the stability of rRNA fragments expressed in vivo, a vector was designed for

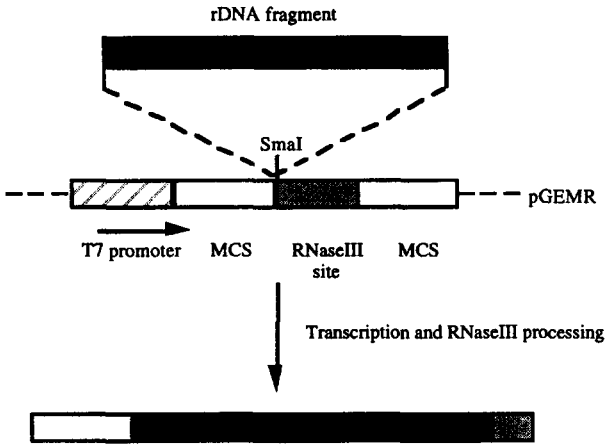

Figure 3. Schematic representation of the fragment library constructs in pGEMR.

the expression of RNA molecules with a flanking RNaseIII cleavage site sequence characteristic of that present in the precursor rRNA transcripts.[13,14] Accordingly, the RNA fragment transcripts were predicted to be processed by the nuclease, generating a 'native' 3′ terminus equivalent to that on the mature rRNAs. This was achieved by annealing two synthetic, complementary oligodeoxyribonucleotides that encode the RNase III cleavage site and directionally cloning into the EcoR1 and BamHI restriction sites within the multicloning region present on pGEM3Z (Promega). The resulting vector, designated pGEMR, was additionally designed to include a unique SmaI restriction site immediately upstream of the RNase III cleavage site (Fig. 3). DNA fragments encoding the RNA molecules of interest were blunt end ligated into the SmaI site and expressed from the upstream T7 promoter.

A 16S rRNA fragment confers spectinomycin resistance

Chemical footprinting studies have shown that spectinomycin interacts with G1064 in helix 34 (nucleotides 1046–1065 and 1191–1211).[6] Furthermore, a series of spectinomycin-resistant mutations have been constructed within this region.[8,9] Therefore, the in vivo expression of an RNA fragment encompassing helix 34 was predicted to sequester spectinomycin and consequently permit the continued functioning of the ribosome and thereby confer drug resistance to the whole cell. To examine this concept, the *E. coli* 16S rDNA ApaI-SmaI fragment (nucleotides 932–1384) was isolated and further restricted with BstUI to release the fragment encompassing nucleotides 972–1266. The BstUI fragment was cloned into pGEMR generating pGEMR294 and confirmed by sequence analysis. *E. coli* strains DH5α[15] and JM109,[16] each harboring pGP1-2,[17] a pACYC derivative encoding the T7 polymerase, were transformed with pGEMR (control) and pGEMR294, selecting in the presence of ampicillin and kanamycin. The resulting transformants were further challenged for their ability to grow on plates at 37 °C in the presence of increasing concentrations of spectinomycin. Transformants of pGEMR failed to grow on plates containing spectinomycin above 5 μg/mL. However, transformants of pGEM294 grew on plates containing spectinomycin at 20 μg/mL. Plasmids isolated from the transformants and used to retransform each strain, confirmed that resistance was indeed plasmid dependent. Therefore, the simplest interpretation of the result was that the rRNA fragment encompassing helix 34 was expressed at a sufficient level and formed a structure able to bind spectinomycin thereby conferring drug resistance.

In vivo selection of RNA fragments that confer spectinomycin resistance

The potential of the in vivo RNA fragment-rescue approach was further investigated by screening for spectinomycin-resistant transformants from amongst a population of cells encoding a library of rRNA fragments. The entire *rrnB* operon encoding all three rDNAs (16S, 23S, and 5S) and the spacer regions, was fragmented at random using a combination of one or more restriction endonucleases selected for the frequency of cleavage sites that occur within the target operon. The enzymes AluI, HaeIII, and DpnI, release fragments that can be cloned in either orientation into the SmaI site on pGEMR. *E. coli* strain DC2[18] (harboring pGP1-2), was transformed by the RNA library and transformants selected on ampicillin and kanamycin. DC2 harbors a mutation that affects membrane permeability and therefore exhibits an increased sensitivity to antibiotics. To establish that the pool of transformants harbored pGEMR encoding a mixed population of rRNA fragments, the plasmids were isolated, linearized with HindIII and transcribed in vitro with T7 RNA polymerase in the presence of ^{32}PαUTP. The products were resolved by denaturing gel electrophoresis and visualized by autoradiography. The library evidently consisted of a large number of transcripts, varying in size with an average of 200–500 nucleotides in length (data not shown).

The library of DC2 transformants was spread on LB agar containing ampicillin, kanamycin, and increasing concentrations of spectinomycin (10, 20, 40, and 80 μg/mL) and incubated at 37 °C. Spectinomycin resistant colonies grew slowly, developing after 48 h incubation on plates containing up to 40 μg/mL spectinomycin. Plasmids were isolated from these spectinomycin-resistant transformants and used to retransform DC2 (containing pGP1-2) to confirm that the resistance phenotype was indeed attributable to the plasmid. The plasmids were subsequently characterized by sequence analysis.

Eight transformants each encoded identical RNA fragments generated by the insertion of two rRNA fragments. The 16S rDNA AluI fragment encompassing nucleotides 862–1068 was ligated immediately upstream of the 23S rDNA AluI fragment nucleotides 998–1099. The 16S rDNA fragment includes the 5′ half of helix 34 (nucleotides 1050–1067). The 23S rDNA fragment

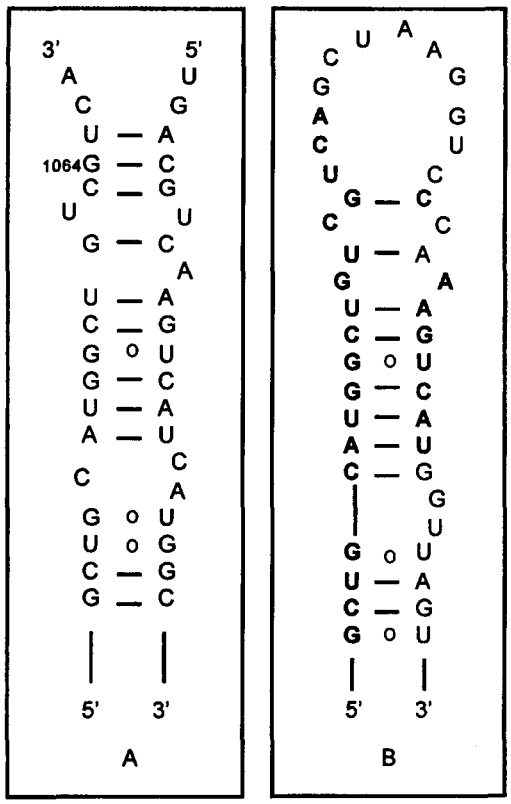

Figure 4. RNA fragments that confer resistance to spectinomycin. (A) Secondary structure encompassing helix 34. (B) Predicted secondary structure of the 16/23S rRNA hybrid molecule: 16S rDNA AluI fragment (nucleotides 862–1068) and 23S rDNA AluI fragment (nucleotides 998–1099). Nucleotides common to helix 34 are in bold type.

encoded a nucleotide sequence identical to that present on the 3′ half of helix 34 (nucleotides 1196–1202) thereby generating a hybrid helix 34 molecule (Fig. 4). The simplest interpretation of this result is that the hybrid molecule is expressed at a sufficient level and is able to fold in such a manner as to bind spectinomycin. Furthermore, it is apparent that primary sequences flanking helix 34 are not essential to the drug binding. It is interesting to note that the hybrid molecule has the potential to retain the equivalent of the G1064–C1192 base pair implicated in binding spectinomycin, and is flanked by a non-Watson–Crick pair and bulged A residue also present in the wild-type helix 34. Similarly, both molecules have the potential to form the lower helix (relative to the bulged A residue) whereas formation of the upper helix present in the wild-type helix 34 is not obvious for the hybrid molecule.

A further spectinomycin-resistant transformant was isolated and shown to harbor the 23S rDNA fragment encompassing nucleotides 2082–1877. The fragment was inserted in the reverse orientation and therefore the transcript represented the reverse complement to the rRNA sequence. There is no primary sequence similarity to either helix 34 or the hybrid molecule. In the absence of chemical and enzymatic probing studies, no firm conclusions can be drawn in terms of the mode of drug resistance.

Discussion

Dissection of large RNA molecules into smaller functional domains is a potent strategy towards understanding RNA–ligand interactions at the molecular level.[1] Reports to date have focused on in vitro studies[12] whereas we present here a whole cell strategy. The RNA fragment-rescue assay is based on the premise that the in vivo expression of RNA fragments able to bind and thereby sequester a drug, will permit the continued functioning of the intact enzyme and thus ensure cell viability. This concept has been validated by the expression of a specific 16S rRNA fragment (nucleotides 97–1266), encompassing the spectinomycin binding domain in helix 34, and demonstrating that this conferred drug resistance. The potential of this approach is further emphasized by the selection of transformants encoding additional RNA fragments that confer resistance to spectinomycin from amongst a large and random pool of RNA fragments. The 16S/23S hybrid molecule (Fig. 4) lacks the sequences characteristic of those flanking helix 34 in 16S rRNA, indicative of their not being essential to the formation of the spectinomycin binding site.

We speculate that the drug binds in vivo to the naked RNA fragment rather than an RNP complex for the following reasons. The RNA fragments are constitutively expressed by comparison to the expression of the r-proteins and intact rRNAs which are strictly coordinately regulated in response to the growth-rate. Secondly, although r-proteins S2 and S3 footprint to helix 34, the patterns are identical and are believed to reflect a conformational change in the RNA that facilitates the binding of r-protein S5.[19] The hybrid molecule lacks the sequences flanking helix 34 that show unique protection patterns in the presence of S2 and S3. Spectinomycin resistance mutations have been mapped to S5, and crystallographic studies indicate an interaction between S5 and helix 34,[20] although this protein has been additionally crosslinked and footprinted to the central domain of 16S rRNA.[19] Work is in progress to determine whether the RNA does form a stable RNP. It is possible that a transient RNP complex is formed by, for example, one or more of the translation factors. The functional activities of the translation termination release factors RF1 and RF2 are influenced by point mutations within helix 34[21] and their location according to immunoelectron microscopy is consistent with this site.[22,23] Further, spectinomycin has been proposed to affect translocation by inhibiting the binding of elongation factor G to the ribosome.[10] Mutations within helix 34 and in S5 decrease the inhibitory effect of the drug on EF-G during peptide elongation. Although EF-G does not footprint to helix 34, similar studies show the eukaryotic homologue EF-2 to protect residues within the 18S rRNA equivalent to

helix 34.[24,25] It is intriguing to note that the 23S fragment of the 16/23S hybrid molecule includes, at least in part, a region that is known to influence the interaction of both the RFs and EF-G to the ribosome.

The precise RNA recognition motif for spectinomycin binding remains elusive. The 16/23S hybrid molecule is *predicted* to share common structure motifs with helix 34 including a potential non-Watson–Crick base pair (helix 34 U-U; 16/23S hybrid C-C) and an unpaired A residue. The formation and functional significance in terms of spectinomycin binding, if any, of these features represent the current focus of our research. Chemical probing studies indicate that the upper stem region of helix 34 present in naked 16S rRNA becomes disrupted during assembly of the 30S subunit.[26,27] Mutations that potentially stabilize this upper stem are deleterious and implicate the importance of this disruption for ribosome function.[8] Spectinomycin is proposed to stabilize the upper stem, and thereby exert its inhibitory action. The lower stem of helix 34 also has the potential to undergo a conformational change without disruption of the overall base-pairing arrangement (Fig. 2). By contrast, the predicted helical stem in the 16/23S hybrid molecule is apparently stable. Transformants expressing the 16/23S RNA exhibit a higher level of spectinomycin resistance than those encoding the wild-type helix 34 fragment molecule. Although this latter observation may reflect alternate stabilities of the two RNA fragment types, it is tempting to speculate that the drug binding site may be influenced by the nature and stability of the lower helix and that the conformational flexibility of the lower stem is important for protein synthesis.

Experimental

Strains and plasmids

E. coli strains DC2,[18] DH5α,[15] and JM109[16] were transformed by pGP1-2, a pACYC-derivative encoding T7 RNA polymerase,[17] selecting on LB plates containing kanamycin (50 μg/mL). Plasmid pGEMR is a derivative of pGEM3Z (Promega) in which complementary oligomers encoding the RNase III processing signal[14] were directionally cloned into the EcoRI and BamHI restriction sites downstream of the T7 promoter. The oligomer sequence included a unique SmaI site upstream of the RNase III site for insertion of DNA encoding the RNA fragments. Transformants of pGEMR were selected on LB agar containing ampicillin (200 μg/mL). Spectinomycin-resistant transformants were recovered at 37 °C following selection on LB agar containing ampicillin, kanamycin, and increasing concentrations of spectinomycin (5, 10, 15, 20, and 40 μg/mL). Plasmids were isolated using the Qiagen Tip-systems according to manufacturer's recommendations. Sequence analysis was as according to the fmol sequencing system (Promega) using forward and reverse primers complementary to the T7 promoter and RNase III cleave site. pGEMR294 was constructed by insertion of the 16S rDNA Bst UI fragment encompassing nucleotides 972–1266.

RNA fragment library

Random rDNA fragments were generated by restriction endonuclease digestion of the *rrnB* operon using either AluI, HaeIII or DpnI, or a double digestion with AluI/DpnI or HaeIII/DpnI. The fragments were ligated into the SmaI site on pGEMR and used to transform the respective strains. The diversity of RNA fragments within the library was determined by in vitro transcription of radiolabeled transcripts using the RiboMax system (Promega), resolution by denaturing gel electrophoresis and autoradiography according to standard procedures.[28]

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References

1. Schroeder, R. *Nature (London)* **1994**, *370*, 597.
2. Noller, H. F.; Woese, C. R. *Science* **1981**, *212*, 402.
3. Zimmerman, R. A. *Ribosomes*; Nomura, M.; Tissieres, A.; Lengyel, P., Eds.; Cold Spring Harbor Laboratory: New York, 1974; pp 225–269.
4. Samaha, R. R.; O'Brien, B.; O'Brien, T. W.; Noller, H. F. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7884.
5. Nomura, M.; Traub, P.; Guthrie, C.; Nashimoto, H. *J. Cell Physiol.* **1969**, *74*, 241.
6. Moazed, D.; Noller, H. F. *Nature (London)* **1987**, *327*, 389.
7. Bollen, A.; Davies, J.; Ozaki, M.; Mizushima, S. *Science* **1969**, *165*, 85.
8. Brink, M. F.; Brink, G.; Verbeet, M. Ph.; de Boer, H. A. *Nucleic Acids Res.* **1994**, *22*, 325.
9. Johanson, U.; Hughes, D. *Nucleic Acids Res.* **1995**, *23*, 464.
10. Bilgin, N.; Richter, A. A.; Ehrenberg, M.; Dahlberg, A. E.; Kirland, C. G. *EMBO J.* **1990**, *9*, 735.
11. Prescott, C. D.; Kleuvers, B.; Goeringer, H. U. *Biochimie* **1991**, *73*, 1121.
12. Purohit, P.; Stern, S. *Nature (London)* **1994**, *370*, 659.
13. Dunn, J. J.; Studier, F. W. *J. Mol. Biol.* **1983**, *166*, 477.
14. Bram, R. J.; Young, R. A.; Steitz, J. *Cell* **1980**, *19*, 393.
15. Hanahan, D. *J. Mol. Biol.* **1983**, *166*, 557.
16. Yanisch-Perron, C.; Vierra, J.; Messing, J. *Gene* **1985**, *33*, 103.
17. Tabor, S.; Richardson, C. C. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *83*, 1074.
18. Clark, D. *FEMS Microbiol. Lett.* **1984**, *21*, 189.
19. Stern, S.; Pace, T.; Changchien, L. M.; Noller, H. F. *Science* **1989**, *244*, 783.
20. Ramakrishnan, V. V.; White, S. W. *Nature (London)* **1992**, *358*, 768.

21. Prescott, C. D.; Kornau, H. C. *Nucleic Acids Res.* **1992**, *7*, 1567.

22. Moffat, J. G.; Timms, K. M.; Trotman, C.; Tate, W. P. *Biochimie* **1991**, *73*, 1113.

23. Tate, W. P.; McLaughan, K. K.; Kastner, B.; Trotman, C.; Stoffler-Meilincke, M.; Stoffler, G. *Biochem. Int.* **1988**, *17*, 179.

24. Moazed, D.; Robertson, J. M.; Noller, H. F. *Nature (London)* **1988**, *334*, 362.

25. Dumont-Miscopein, A.; Lavergne, J.-P.; Guillot, D.; Sontag, B.; Reboud, J.-P. *FEBS Lett.* **1994**, *356*, 283.

26. Baudin, F.; Ehresmann, C.; Romby, P.; Mougel, M.; Colin, J.; Lempereur, L.; Bachellerie, J. P.; Ebel, J. P.; Ehresmann, B. *Biochemie* **1987**, *69*, 1081.

27. Laughrea, M.; Tam, J. *Biochemistry* **1992**, *31*, 12035.

28. Sambrook, J.; Fritsch, E. F.; Maniatis, T. In *Molecular Cloning*; Nolan, C., Ed.; Cold Spring Harbor Laboratory: New York, 1989.

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