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Identification of Defined Sequences in Domain V of E.coli 23S rRNA in the 50S Subunit Accessible for Hybridization with Complementary Oligodeoxyribonucleotides

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Identification of defined sequences in domain V of *E.coli* 23S rRNA in the 50S subunit accessible for hybridization with complementary oligodeoxyribonucleotides

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ABSTRACT

The accessibility of specific sequences in domain V of <u>E. coli</u> 23S rRNA in the 50S subunit to complementary oligodeoxyribonucleotides (cDNA) has been investigated. The apparent percentage of subunits engaged in complex formation was determined by incubation of radiolabeled cDNA probe with 50S subunits, followed by nitrocellulose membrane filtration of the reaction mixtures and measurement of the bound radiolabeled cDNA probes by liquid scintillation counting of the filters. The site(s) of hybridization were determined by digestion of the RNA in the RNA/DNA heteroduplex by RNase H.

The results of this study indicated that single-stranded sequences, 2058-2062, 2448-2454, 2467-2483, and 2497-2505 were available for hybridization to cDNA probes. Bases 2489-2496, which have been postulated to be base paired with 2455-2461 were also accessible for hybridization.

INTRODUCTION

Several techniques are now being employed to determine the accessibilty of rRNA both within the individual subunits and the intact 70S ribosome. The data accumulated to date have been obtained primarily from ribonuclease digestion (1,2,3,4), chemical modification (5), and cDNA hybridization studies (6,7). Although considerable work has been done on the accessibility of 16S rRNA in the 30S subunit of <u>E. coli</u>, the picture of accessible 23S rRNA regions in the 50S subunit is not well defined. The sequence of 23S rRNA has been determined for several organisms and secondary structure maps have been postulated (8,9,10). These models are in close agreement.

It is now apparent that rRNA is involved in subunit association (6,11), antibiotic binding (12), tRNA interactions (13,14,15), and mRNA binding and alignment (16,17). The rRNA involved in these interactions is single-stranded and in most cases highly conserved (18). In line with the postulate that these conserved, single-stranded sequences of rRNA are directly involved in ribosomal functions, it follows that these sequences should be exposed to solvent and available for interaction.

We have begun probing the accessibility of single-stranded 23S rRNA sequences to complementary oligodeoxyribonucleotides. Accessibility to these probes provides evidence for the potential of particular sites to participate in ribosomal functions in which surface location and solvent exposure is necessary.

There are two advantages to oligodeoxyribonucleotide probing. First, probing with cDNA is highly specific because these probes are designed to interact with defined rRNA sequences, whereas ribonuclease digestion and chemical modification result in either digestion or modification of several sites within the molecule, which may perturb conformation throughout the ribosome. Second, the conditions employed in cDNA probing are essentially physiological, thus minimizing secondary effects as a result of solvent conditions which can cause alterations in the native conformation of the ribosome.

In this paper we report on the accessibility of several sequences in domain V of E. coli 23S rRNA within the 50S subunit to cDNA probes. This study focuses on single-stranded sequences which occur between bases 2043 through 2625 (Figure 1). This central loop of domain V comprises the major rRNA portion of the peptidyltransferase center, a region postulated to be highly active (18).
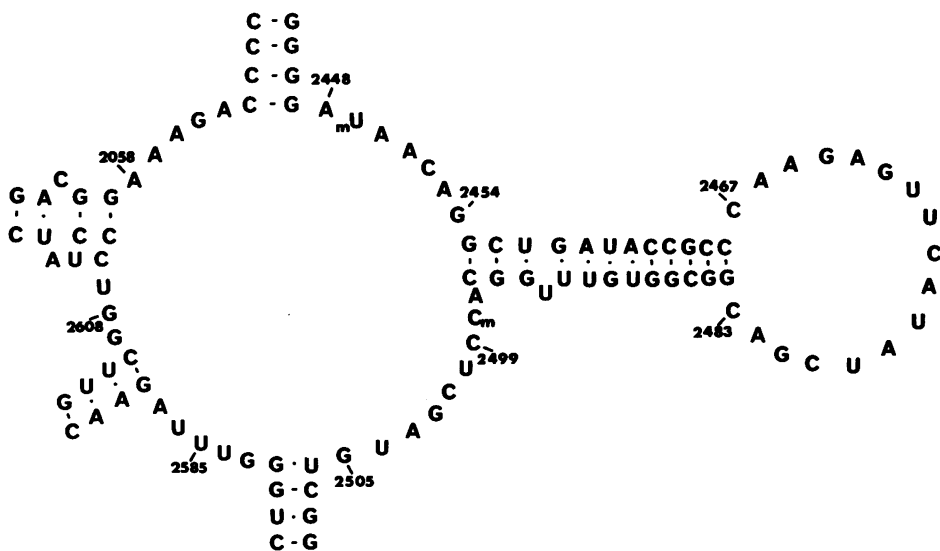

Figure 1. A portion of the secondary structure map of E. coli 23S rRNA containing domain V.

MATERIALS AND METHODS

Synthesis and Purification of Oligodeoxyribonucleotides

Oligodeoxyribonucleotides were synthesized on a Biosearch model 8600 Automated
DNA Synthesizer utilizing beta-cyanoethyl diisopropylamino phosphoramidite
chemistry. All reagents were obtained from Biosearch with the exception of
HPLC grade acetonitrile and methylene chloride (Baker). The cDNA oligomers
were deblocked according to the manufacturers protocol and purified both
before and after removal of the 5' dimethoxytrityl (DMT) blocking group by
reverse phase high performance liquid chromotography (RP-HPLC). A Column
Engineering, C-18 ODS, 5 uM, 25 cm column was employed. The gradient used ran
from 100% buffer A (10 mM TEA-Ac, pH 7.2) to 70.8% buffer B (10mM TEA-Ac/50%
acetonitrile) at a flow rate of 1 ml/min.

Purified DNA oligomers were then 5' end labeled by using [gamma-32P]ATP
(New England Nuclear) and T4 polynucleotide kinase (United States Biochemical)
according to the method of Chaconas and van de Sande (19).

Enzymatic Sequencing of cDNA Probes

The sequences of the synthesized probes were determined by an enzymatic
method developed by Black and Gilham (20). The cDNA probes were digested with
spleen phosphodiesterase II (Pharmacia) to yield a nested set of fragments, 5'
end labeled with [gamma 32-P]ATP using polynucleotide kinase and seperated by
thin layer chromotography (TLC) on polyethyleneimine cellulose sheets
(Brinkman). A subsequent treatment with Bal 31 (Bethesda Research Labs)
produced labeled mononucleotides which were identified by TLC in the second
dimension.

Isolation of Ribosomes and Ribosomal Subunits

E. coli strain, MRE 600 was grown in standard trypticase soy broth (BBL
Microbiology Systems), at 37 C with vigorous shaking. Cells were harvested at
an A-600 value of 0.5-0.6 corresponding to the mid to late log phase of
growth. The cultures were quickly chilled on ice and pelleted by
centrifugation at 5000 RPM for 5 mins. in a Sorvall GSA rotor. Alumina (Sigma)
was added to the cell paste at a ratio of 2:1 (wt/wt) and the cells were hand
ground for one hour in the cold. Buffer A (10 mM MgCl2, 100 mM KCl, and 10 mM
Tris-HCl, pH 7.4) was added as needed to form a slurry. The alumina was
removed by centrifugation at 16,000 RPM for 1 hour in a Sorvall SS-34 rotor.
The supernatant was spun at 60,000 RPM for 2.5 hours in a Beckman Ti-70 rotor,
pellets were resuspended overnight in a minimal volume of buffer A at 4 C, and
the low speed spin was repeated followed by a second high speed spin as
before. Pellets were resuspended in buffer B (1.5mM MgCl2, 100mM KCl, and 10mM

Tris-HCl, pH 7.4) to the desired concentration.

Separation of the subunits was accomplished by the procedure of Tam and Hill (21) with some modifications. After separation of the subunits by zonal centrifugation the 50S and 30S fractions were pelleted separately by centrifugation at 60,000 RPM in a Beckman Ti-70 rotor for 6 hours and 8 hours respectively. Pellets were resuspended in buffer B and then repelleted in the Ti-70 rotor at 60,000 RPM for 4 hours for the 50S particles and 6 hours for the 30S particles. Pellets were resuspended in buffer B, dialyzed against 2x4 liters of buffer B at 4 C over a 24 hour period and stored in 500 ul. aliquots at -70 C. The 50S subunit preparations were checked for 30S contamination and subunit integrity by sedimentation velocity centrifugation using schlieren optics in a Spinco Model E analytical ultracentrifuge.

<u>Binding of Probes to 50S Subunits</u>

The binding of cDNA probes to 50S subunits was assayed by incubating the subunits with 5' end labeled probe (with specific activities between 250-1000 cpm/pmol) for 2-24 hours at 4 C on ice in 50 uls of binding buffer (5-15mM MgCl2, 100-150mM KCl, and 10 mM Tris-HCl, pH 7.4). After incubation the reaction mixtures were diluted to 1 ml with binding buffer and filtered through HAWP 0.45 uM nitrocellulose filters (Millipore) followed by two 1 ml washes of the filter with binding buffer. The amount of complex formation between radiolabeled probe and subunit was quantified by liquid scintillation counting of the dried filters. All reactions were performed in triplicate in order to correct for variations in the retention of subunits on the filters.

Sucrose gradient centrifugation was also used to assay complex formation. Radiolabeled probes (500,000 CPM) were incubated with 55 pmol of 50S subunits in 50 uls of binding buffer at 4 C (on ice) for 4.5 hours. Binding reactions were then layered onto a 5-20% (wt/vol) sucrose gradient in binding buffer and centrifuged in a Beckman SW-60 rotor at 54,000 RPM for 1.75 hours at 4 C. The gradient fractions were assayed spectrophotometrically at 260 nm for subunit migration and by liquid scintillation to monitor probe migration.

The reversible nature of probe binding was determined by competition analysis. Increasing amounts of cold probe were added to preincubated labeled probe/50S subunit complexes and the initial incubation period was repeated. Radiolableled probe binding was assayed by nitrocellulose membrane filtration and liquid scintillation counting.

<u>Binding Specificity</u>

To determine the site(s) of hybridization 25 pmol of 50S subunits were incubated with 50 pmol of probe in 50 uls of RNase H buffer (40mM Tris-HCl,

Table 1. Probe sequences and binding data.

PROBE		PROBE SEQUENCE (5'to 3')	APPARENT MAXIMUM % BINDING	SATURATION RATIO (PROBE/50S)
1)	2058-2062	TCTTT	11	20:1
2)	2448-2454	CTGTTAT	21	30:1
3)	2450-2455	CCTGTT	16	30:1
4)	2467-2476	TGAACTCTTG	13	34:1
5)	2468-2482	TCGATATGAACTCTT	25	24:1
6)	2469-2481	CGATATGAACTCT	23	28:1
7)	2472-2481	CGATATGAAC	23	40:1
8)	2489-2499	GGTGCCAAACA	4	13:1
9)	2497-2501	CATCG	20	15:1
10)	2497-2505	CATCGAGGT	32	30:1

10mM MgCl2, and 60mM KCl, pH 7.4) with 2.4 units of RNase H (Pharmacia) for 18 hours at 4 C. After incubation, the reactions were phenol extracted 3x's with an equal volume of equilibrated phenol, three volumes of absolute ethanol was added to the aqueous phase, followed by centrifugation for 1 hour at 10,000 RPM in a Sorvall HB-4 rotor. The ethanol was aspirated off and the pellets were washed with 70% ethanol/4 mM NaCl and centrifuged as before. The digestion products were dissolved in 7M urea, 0.025% xylene cyanol, and 0.025% bromophenol blue and analyzed by electrophoresis on a 7M urea, 5% polyacrylamide gel (35:1 acrylamide/bis, 89mM Tris-borate, 1 mM EDTA, pH 8.3) for 5 hours at a constant current of 12.5 mA. RNA size markers (Bethesda Research Labs) were treated according to the manufacturers protocol.

RESULTS

Oligodeoxyribonucleotide Synthesis and Purification

The secondary structure map proposed by Noller (18) was used to design the sequence of several probes which would complement the desired target sites. The probe sequences are listed in Table 1. In all cases but one, the target sites were proposed to be single-stranded (Fig.1). The probes have been assigned numerical codes based on the sequence to which they are complementary. Selected probes were sequenced in order to certify proper functioning of the automated synthesizer (data not shown). In all cases the sequence obtained was correct.

Binding of Oligodeoxyribonucleotides to 50S Subunits.

The percentage of 50S subunits which formed probe/subunit complexes was assayed by a nitrocellulose membrane filtration. Apparent binding ranged from 4% for probe 2489-2499, which has a binding site proposed to occur in a double
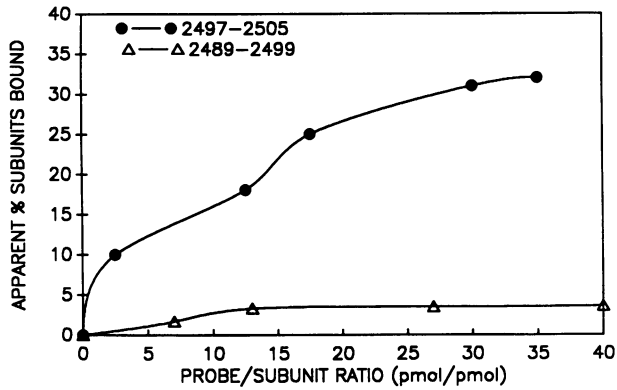
Figure 2. Binding curves for probes, 2497-505 and 2489-499. Conditions were as described in Materials and Methods.

stranded conformation, to 32% for probe 2497-2505, which has a single-stranded binding site (Fig. 2). Probe binding saturated when between 13-40 fold excess of probe was added to the 50S subunits (pmol/pmol). Table 1 lists the apparent maximum percent binding for each probe and the ratio of probe/subunit required to saturate.

The binding of probes to the 50S subunits was also assayed by sucrose gradient centrifugation. Figure 3 shows a typical profile obtained by this type of analysis. All of the probes assayed by this technique demonstrated comigration of radiolabeled probe with 50S subunits.
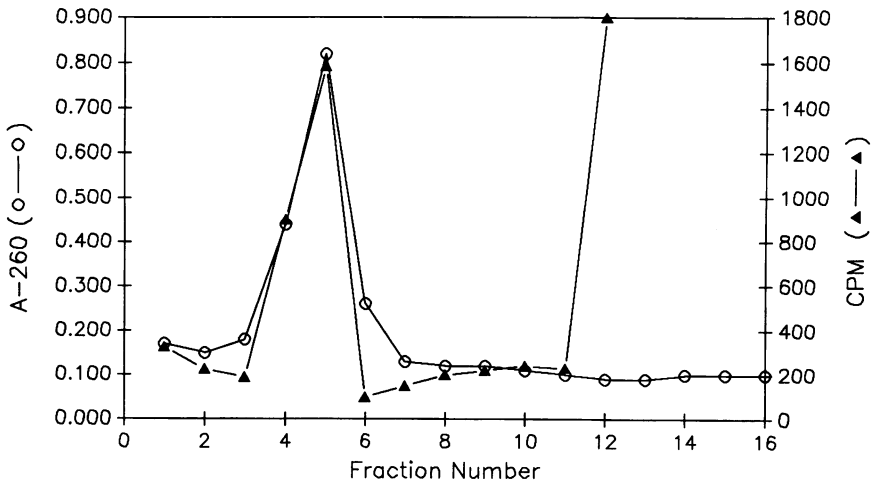

Figure 3. Sucrose gradient binding analysis of probe 2448-454 and 50S subunits. Conditions were as described in Materials and Methods.
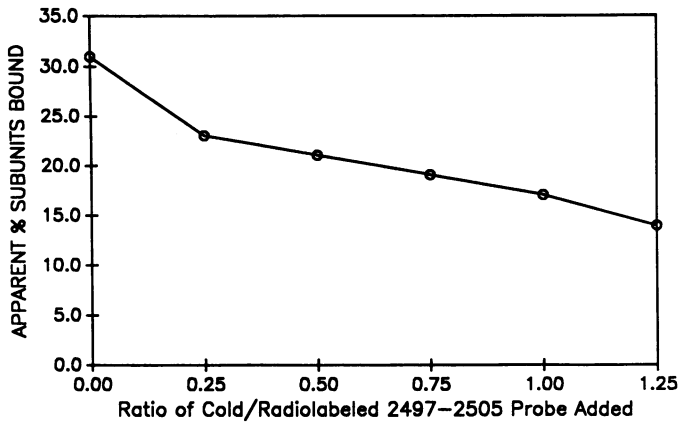
Figure 4. Probe competition assay. Cold 2497-505 was added to reactions containing preformed radiolableled 2497-505/50S subunit complexes (see Materials and Methods for conditions)

The effects of magnesium concentration on probe binding were tested for the 2497-2505, 2448-2454, and 2468-2482 probes. The apparent maximum percent binding did not vary when concentrations of 5, 10, and 15 mM MgCl2 were used. The KCl concentration was maintained at 100 mM in these studies. Increasing the KCl concentration to 150 mM while the Mg concentration was held at either 10 or 15 mM did not have an effect on the apparent maximum percent binding.

The reversibilty of probe binding was demonstrated by a competition assay in which increasing amounts of identical cold probe were added to radiolabeled probe/50S subunit complexes. As the concentration of the cold probe is increased the amount of radiolabeled probe bound is seen to decrease (Figure 4).

Specificity of Probe Binding.

In addition to the target site, to which a particular probe is designed to interact, there may be several other sites which share either complete or partial homology. For all of the probes studied, computer searches revealed no sites, other than the desired target site, which were completely complementary. There were however additional sites which shared partial complementarity.

To ascertain target site specificity, RNase H digestion assays were performed. In all cases, when cleavage occurred, only fragments of the expected size were observed. Figure 5 shows some representative RNase H digests of several of the sites investigated. Table 2 summarizes the RNase H
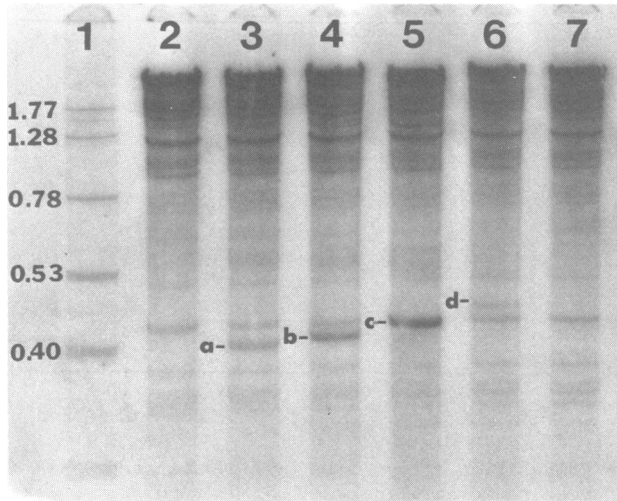
Figure 5. RNase H digestions of probe/subunit complexes. Lane 1: RNA size markers (in kb's). Lanes 2 through 6 are the RNase H digestion products obtained by coincubation of RNase H, 50S subunits and the corresponding probes (see Materials and Methods for conditions). The 3' digestion fragments are indicated by letters a-d. Lane 2: 2058-062, lane 3: 2497-505(a), lane 4: 2489-499(b), lane 5: 2469-478(c), lane 6: 2448-454 (d). Lane 7: control 23S rRNA. For the control, 50 pmol of 50S subunits were treated identically to the digestion reactions. Samples were electrophoresed for 5 hours in a 7M urea, 5% polyacrylamide gel at a constant current of 12.5 mA.

Table 2. RNase H digestion results.

PROBE	TARGET SITE STRUCTURE*	POTENTIAL SINGLE-STRANDED BINDING SITES**	# OF CLIPS OBSERVED	APPROX. SIZE OF OBSERVED FRAGMENT***
1) 2058-62	SS	217-221,478-482 503-507,1084-1088	0	- - -
2) 2448-54	SS	714-718	1	454
3) 2450-55	SS	714-718	Not Done	- - -
4) 2467-76	HP	NONE	1	430
5) 2468-82	HP	1885-89,2267-72 2384-88	1	430
6) 2469-81	HP	2267-72,2384-88	1	430
7) 2472-81	HP	2384-88	1	430
8) 2489-99	DS	NONE	1	410
9) 2497-501	SS	NONE	1	405
10) 2497-505	SS	47-51,1375-80	1	400

* SS (single-stranded), HP (hairpin loop), DS (double-stranded)
** All 23S rRNA single-stranded sites of 5 (or greater) base complementarity are listed. The intended target site is not included.
*** Fragment sizes were estimated to within +/- 10 bases, based on RNA size markers.

data obtained and the positions of other potential probe binding sites. The size of the digestion products was determined through the use of RNA size markers. Computer search analysis of the sequence of E. coli 23S rRNA revealed only one region in 23S rRNA which could result in the observed differential migration pattern when digests of the 2448-2454, 2467-2476, 2489-2499, and 2497-2505 sites are compared. This region corresponds to domain V which contains the desired target sites.

DISCUSSION

The results obtained in this study demonstrate the accessibility of bases 2448-2454, 2468-2483, and 2489-2505 to oligodeoxyribonucleotides of defined sequence. While the 2058-2062 sequence appears to be available for hybridization we were unable to demonstrate specificity of probe binding for this site and therefore we cannot draw a conclusion concerning its accessibility to cDNA probes. All of the sites chosen in this study, with the exception of the 2489-2499 site, exhibit a high degree of phylogenetic conservation (18) which is presumed to indicate the importance or necessity of these sequences in either structural or functional roles. Functional roles such as the binding of tRNA, mRNA, or other translational factors would require that the rRNA be accessible for interaction.

The rRNA within domain V has been implicated in the binding of several antibiotics which have been shown to inhibit the peptidyltransferase reactions (22). Base substitutions at positions 2447, 2503 (23), 2504, 2451 (24), and 2452 (25) result in chloramphenicol resistance. The proposed secondary structure of E. coli 23S rRNA brings these two sites in close proximity (Fig. 1).

Substitutions at 2611, result in resistance to both erythryomycin and spiramycin (26) suggesting that these antibiotics bind, at least partially within domain V. In addition, crosslinking of puromycin (27) and tRNA derivatives (13) to nucleotides in domain V suggests that the tRNA must position itself in close proximity to the rRNA of the peptidyltransferase center. These studies support the notion that the rRNA of this area should be accessible to solvent and thus capable of interaction with cDNA probes.

The apparent percentage of subunits participitating in probe/subunit complex formation, when assayed by nitrocellulose filtration, ranged from 4% to 32% for the 2489-2499 and 2497-2505 sites respectively. Sucrose gradient centrifugation also provided a qualitative approach in analyzing complex formation, but the dilution of probe concentration as the probe/subunit

complexes sedimented resulted in a low level of complex formation. However, this technique illustrated that the probes were binding equally throughout the entire subunit population and not to a subset defined by gross structural or conformational alterations. Less prominent subsets, if they exist, may not be detected. This approach also demonstrated that the binding of probes does not result in gross conformational changes.

The lengths of the probes used ranged from 5 to 15 bases. Within a particular site, increased probe length generally resulted in an increased level of binding. Probe 2497-2501 (5 bases) bound at a level of 20% and saturated at a ratio of 20:1 (probe/subunit) while probe 2497-2505 (9 bases) bound at 32% and saturated at a ratio of 30:1. Similiar results were observed for the hairpin loop which extends from base 2467 to base 2483. Probe 2467-2476 (10 mer) binds at a maximum level of 13%. In comparision, probe 2468-2482 (15 mer) bound at 25%. It is difficult to accurately assess the role which probe length may play in contributing to the observed maximum percent binding and in determining the equilibrium of complex formation since several additional factors come into play, such as rRNA sequence, secondary structure, tertiary interactions, and the effects due to RNA/protein interactions. In view of these variables, the level of binding cannot strictly be used as an absolute gauge to monitor the exposure of one site relative to another.

The reversible nature of probe binding was demonstrated by competing unlabeled probe with prebound radiolabeled probe. The addition of unlabeled 2497-2505 probe to preformed 2497-2505/50S complexes at a ratio of 1:1 (unlabeled/labeled) resulted in a decrease of radiolabeled probe binding from 31% to 17%. This illustrates that complex formation is a dynamic process and that the maximum percent binding obtained for each site is a direct reflection of the association constant.

The number of different rRNA sites involved in hybridization with the cDNA probes can be determined by digestion of the rRNA within the DNA/RNA heteroduplexes with RNase H. RNase H digestion requires a minimum of at least 4 adjacent base-pairs in a DNA/RNA heteroduplex (28). Although most of the sites studied possess sequences which occur at one or more other positions in 23S rRNA, we observed no digestion products which could be attributed to these sites. The cleavage patterns are indicative of probe specificity. The 2448-2454 2497-2505 sites should generate 3' fragments of approximately 450 and 400 bases, respectively. Sites within the 2468-2483 hairpin will generate 3' fragments ranging in size from 420 to 433 bases. Upon electrophoresis the fragments from each cleavage site migrate with similiar rates. The migration

difference between the smallest digestion fragment, obtained from the 2497-
2505 site, and the largest, from the 2448-2454 site, indicate a size
difference of approximately 50 bases (+/- 10) under our conditions (Fig.5).

The migration pattern provides solid evidence for cleavage occurring at
the desired target sites. A computer search shows that no other region in the
23S rRNA, other than domain V, possesses a series of potential binding sites
which could generate fragments of the appropriate size (about 400-450 bases)
with a similiar pattern of differential migration.

Furthermore, RNase H will only clip where the RNA/DNA heteroduplex is in
a true hydrogen bonded helix (29). This rules out the possibility of cleavage
due to non-base pairing interactions at sites which could by chance generate a
similiar migration pattern. From these arguments we conclude that in all cases
where cleavage occurred, the probes were binding to the intended target sites.

The results of the RNase H digestion assays were as expected in all
cases but two. One exception occurred at the 2489-2499 site. Bases 2489
through 2496 are postulated to be base paired with 2455 through 2461. This
postulated helix is composed primarily of G-U base pairs with a bulged U at
position 2493. This is one of the few helical regions of 23S rRNA which is not
considered proven (18). Probe bound to this site at an apparent level of only
4%, and saturated at a molar ratio of 13:1 (probe/50S subunit). Initially this
was interpreted to represent nonspecific background binding. Increasing the
ratio up to 40:1 did not increase the maximum percent binding. However,
ribonuclease H cleaved this site as well as others in this study, even though
the measurable binding was low. This suggests the this postulated helix, if it
exists may be very weak and thus susceptible to melting by the probe. It is
possible that the postulated helical interaction is a transient one and may
undergo a "flexing" or "breathing" type of transition between a single-
stranded and double-stranded conformation. Similiar transitions between single
and double-stranded conformations have been postulated to occur in 16S rRNA
(18). Such conformational "breathing" could presumably displace bound probe
resulting in the observed low level of binding. RNase H digestion would
require only a transient interaction thus explaining the observed cleavage.
Although the probe extends 3 bases into a single-stranded region on the 3'
side of this postulated helix, this would not provide a suitable recognition
site for RNase H which has been demonstrated to require at least a 4 base pair
RNA/DNA heteroduplex.

A second suprising result was seen for the 2058-2062 site. While this
site bound probe at an apparent maximum level of 11%, digestion by RNase H was

not observed. It is probable that the access of RNase H to this site could be impeded by ribosomal proteins or by the folding of rRNA. We are currently developing alternative methods to clip the RNA in such shielded regions in order to demonstrate specificity.

The RNase H data suggest that the number of accessible 23S rRNA sites in the 50S subunit is limited. Such a model is supported by chemical modification studies (31). The accessibility of single-stranded, conserved sequences, may indicate a potential functional involvement of these sequences in the translational process. Although each of the target sites probed in this study have partially homologous sequences elsewhere in 23S rRNA, these sequences apparently were not accessible for hybridization with cDNA probes.

Complementary DNA probes can provide a powerful tool for investigating the accessibility and functionality of specific sequences of rRNA. By blocking a particular site, thus impeding its ability to interact with various macromolecules involved in the translational process, or with other portions of the ribosome, the possible function of rRNA in this process may be elucidated.

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