Ribosomal Components Neighboring the 2475 Loop in *Escherichia coli* 50S Subunits†

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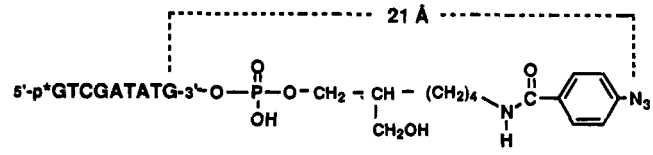
ABSTRACT: We report the synthesis of a radioactive, photolabile oligodeoxyribonucleotide probe and its exploitation in identifying 50S ribosomal subunit components neighboring its target site in 23S rRNA. The probe is complementary to 23S rRNA nucleotides 2475−2483, a single-stranded sequence (the 2475 loop) near the peptidyltransferase center of *Escherichia coli* ribosomes. On photolysis in the presence of 50S subunits, it site-specifically incorporates into proteins L1, L13, L16, L32, and L33 and into 23S rRNA nucleotides G2470, A2471, and G2472. These results provide clear evidence that C2475 in 23S rRNA is within 21 Å (the distance between C2475 and the photogenerated nitrene) of proteins L1, L13, L16, L32, and L33. The implications of these results for the evolving model of the internal structure of the 50S subunit are considered.

In recent articles (Muralikrishna & Cooperman, 1991, 1994; Cooperman et al., 1993; Alexander et al., 1994) we have described the use of radioactive, photolabile, complementary oligoDNA probes as photoaffinity labels to identify ribosomal components that neighbor functionally important single-stranded sequences of rRNA. One such sequence is nucleotides 2475−2483 in 23S rRNA. This sequence forms part of a loop (which we denote the C2475 loop) connected by a stem to the central loop of domain V, which forms part of the peptidyltransferase center (Cooperman et al., 1990). The C2475 loop is available within 50S subunits for the binding of complementary oligoDNAs. Such binding is inhibited in the presence of poly(U) (Hill et al., 1990), suggesting that the C2475 loop may be at or near the 30S−50S interface.

In this work we convert the oligodeoxyribonucleotide 5′-GTCGATATG-3′, complementary to 23S rRNA nucleotides 2475−2483, into a radioactive photoaffinity labeling reagent, *N*-[5-(hydroxymethyl)-6-(3′-pGTATAGCTGp*)hexyl]-*p*-azidobenzamide (p*2483−2475HHABA,[1] **1**; the asterisk indicates the presence of radioactivity), and use this reagent to identify ribosomal components within 21 Å of C2475. Twenty-one angstroms is the maximum distance between C2475 and the nitrene photogenerated from p*2483−2475HHABA.



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EXPERIMENTAL PROCEDURES

Materials. Except as specified below, all materials were obtained as described (Muralikrishna & Cooperman, 1991, 1994; Alexander et al., 1994).

Synthesis and Purification of Oligodeoxyribonucleotides. cDNA 2483−2475, having the sequence 5′-GTCGATATG-3′, and the corresponding mismatched oligonucleotide 5′-GTCCTTATG-3′ (MM-cDNA 2483−2475) were synthesized using phosphoramidite chemistry on a Milligen Biosearch Cyclone automated DNA synthesizer and deblocked according to the manufacturer's protocol. The photolabile, radioactive cDNA p*2483−2475HHABA was synthesized as follows. 3′-Amino modifier C7 CPG (1-((dimethoxytrityl)-oxy)-6-((((fluorenylmethoxy)carbonyl)amino)hexane-2-methyl-*O*-succinyl−long-chain alkylamino-CPG) was used to generate cDNA 2483−2475 derivatized at its 3′-end with a primary amine according to the protocol supplied by Glen Research (Sterling, VA). After deblocking and RP-HPLC purification, the 3′-amino-derivatized cDNA 2483−2475 was first reacted with HSAB to yield 2483−2475HHABA, using a procedure described earlier (Muralikrishna & Cooperman, 1991), and then phosphorylated at the 5′-end with [γ-^{32}P]-ATP and polynucleotide kinase (Sambrook et al., 1989). Purification using Sep-pak (C-18) cartridges (Sambrook et al., 1989) yielded p*2483−2475HHABA. cDNA 2483−2475 labeled at its 5′-end, p*2483−2475, was prepared similarly.

Methods. The following methods were carried out as described previously (Muralikrishna & Cooperman, 1991, 1994; Alexander et al., 1994): noncovalent binding of p*2483−2475 to 50S subunits by Millipore filtration, RNase H digestion of complexes of cDNA with either 50S subunits or 23S rRNA, PAGE analyses of RNase H digestion mixtures, photoincorporation of p*2483−2475HHABA or 2483−2475HHABA into 50S subunits, and localization of photoincorporation sites within 23S rRNA by RNase H and reverse transcriptase analyses. Proteins were prepared from labeled 50S subunits by acetic acid extraction and acetone precipitation in the usual fashion (Kerlavage & Cooperman,

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[1] Abbreviations: p*2483−2475HHABA, *N*-[5-(hydroxymethyl)-6-(3′-pGTATAGCTGp*)hexyl]-*p*-azidobenzamide; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; TP50, total protein from 50S subunits.

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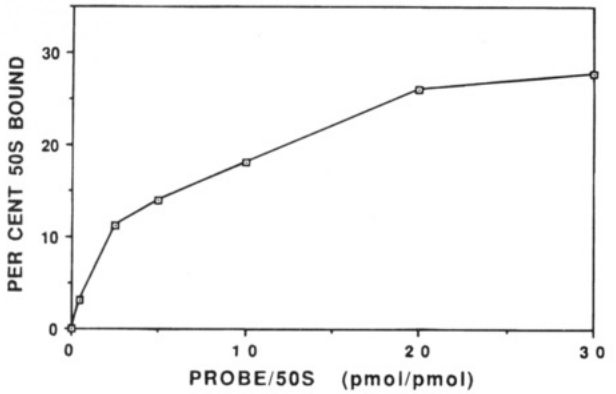


FIGURE 1: Binding of p*2483−2475 to 50S subunits. 50S subunits (12.5 pmol) were incubated with varying amounts of p*2483−2475 (300−500 cpm/pmol) in 25 μL of TKM0.3 buffer (40 mM Tris-HCl, pH 7.4, 60 mM KCl, and 0.3 mM $MgCl_2$) at 37 °C for 5 min and left on ice for 15 min, after which the $MgCl_2$ concentration was raised to 10 mM and the incubation on ice was continued for an additional 2 h. The reaction mixtures were then diluted with 1.0 mL of cold TKM0.3 containing 10 mM $MgCl_2$ (binding buffer) and filtered through HWAP 0.45-μm nitrocellulose filters (Millipore), which was followed by three 1-mL washes of the filters with binding buffer. The amount of filter-bound oligonucleotide was determined by liquid scintillation counting of the dried filters.

1986). Labeled proteins were identified by SDS−PAGE coupled with autoradiographic analysis (Muralikrishna & Cooperman, 1991), RP-HPLC analysis (Kerlavage et al., 1984), and agarose antibody affinity chromatography analysis (Gulle et al., 1988).

Photoincorporation of p*2483−2475HHABA into 23S rRNA or 5S rRNA was monitored by phenol extraction of labeled 50S subunits followed by urea−PAGE and autoradiographic analyses of the rRNA fraction (Muralikrishna & Cooperman, 1991).

RESULTS

Noncovalent Binding of Deoxyoligonucleotides. The noncovalent binding of cDNA p*2483−2475 to 50S subunits reached a plateau value of 28% of 50S subunits at a probe:50S subunit molar ratio of 25:1 (Figure 1). This result is similar to that reported earlier (Hill et al., 1990; Marconi et al., 1990) for the binding of a probe complementary to nucleotides 2468−2482. That at least some of the binding of p*2483−2475 to 50S subunits occurs specifically to its complementary region in 23S rRNA is shown by the ~425-nt RNA fragment that is released following RNase H treatment of the complex (Figure 2). The same fragment is obtained on RNase H digestion of the complex formed between 50S subunits and the photolabile probe 2483−2475HHABA, but is not formed in the absence of either RNase H (lane 1) or cDNA probe (lane 2).

*Proteins L1, L13, L16, L32, and L33 Are Specifically Labeled on Photolysis of the 50S:p*2483−2475HHABA Complex.* Proteins extracted from 50S subunits labeled by p*2483−2475HHABA in the presence or absence of cDNA 2483−2475 or of MM-cDNA 2483−2475 were analyzed by SDS−PAGE and autoradiography (Figure 3). While it is clear from Figure 3 that many proteins are photolabeled by p*2483−2475HHABA, only a limited number of protein bands (i-iv) appear to be labeled specifically from the target site, as judged by the decrease in labeling on addition of cDNA 2483−2475 but not on addition of MM-cDNA 2483−

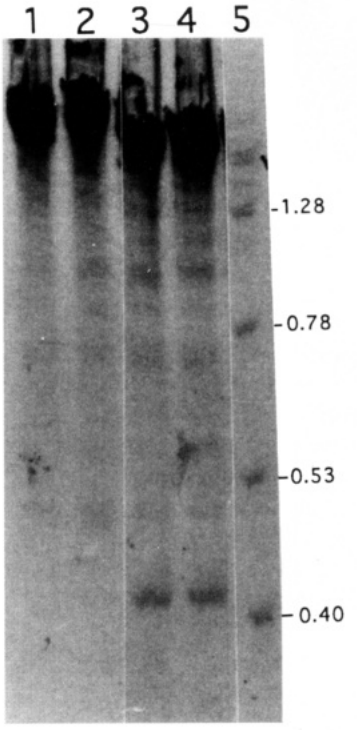


FIGURE 2: RNase H digestions of 50S subunits with cDNA 2483−2475 and with 2483−2475HHABA. Solutions of 50S subunits (50 pmol) and either cDNA 2483−2475 or 2483−2475HHABA (100 pmol) in a volume of 25 μL were digested with RNase H (5 units), and the phenol-extracted rRNA was analyzed. RNA bands were visualized by staining with methylene blue. Lane 1, cDNA 2483−2475 without RNase H; lane 2, RNase H without added oligodeoxyribonucleotide; lane 3, RNase H with cDNA 2483−2475; lane 4, RNase H with 2483−2475HHABA; lane 5, RNA size markers (in kilobases).

2475 (Muralikrishna & Cooperman, 1991, 1994). In typical experiments in which 50S subunits were present in stoichiometric excess over p*2483−2475HHABA, 6−7% of the probe was photoincorporated into ribosomal protein; 2−3% was photoincorporated site-specifically. As previously noted (Cooperman et al., 1993), nontarget site-specific photoincorporation into ribosomal protein appears to be a general phenomenon for photolabile oligoDNA probes, most likely reflecting nonspecific binding interactions with ribosomal proteins in the intact subunit.

Ribosomal protein labeled with an oligoDNA migrates in SDS−PAGE with an apparent molecular mass corresponding to the sum of the protein and oligoDNA (taken as 3.3 kDa) masses (Muralikrishna & Cooperman, 1991, 1994). In what follows we distinguish between actual molecular mass, calculated from a knowledge of ribosomal protein primary structure (Giri et al., 1984), and apparent molecular mass, calculated by comparison of observed band migration with a best-fit line through a plot of band migration vs the logarithm of actual molecular masses of TP50. Band i (apparent molecular mass of 28.8 kDa) clearly corresponds to labeled L1 (apparent molecular mass of 24.8 kDa; actual molecular mass of 24.6 kDa). Band ii (apparent molecular mass of 19.3 kDa), most probably corresponds to labeled L13 (apparent molecular mass of 16.2 kDa; actual molecular mass of 16.0 kDa), but labeled L9 (apparent molecular mass of 14.8 kDa; actual molecular mass of 15.7 kDa) cannot be ruled out. The potential candidates for band iii (apparent molecular mass of 17.7 kDa) are L15 (apparent molecular mass of 14.8 kDa; actual molecular mass of 15.0 kDa), L16

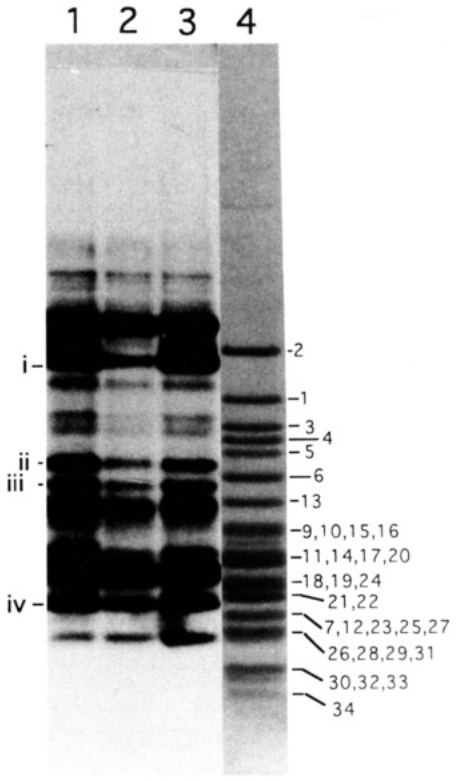


FIGURE 3: Autoradiogram of an SDS−PAGE analysis of proteins photolabeled with p*2483−2475HHABA. Reaction mixtures containing 50S subunits (75 pmol) and p*2483−2475HHABA (7.5 pmol) in the absence or presence (500 pmol) of either cDNA 2483−2475 or MM-cDNA 2483−2475 (total volume, 75 μL) were subjected to photolysis with 3000-Å lamps (Rayonet) for 3 min. The low stoichiometry of p*2483−2475HHABA maximizes the specificity of photoincorporation. Lanes 1−3, 67% acetic acid soluble fraction from 50S subunits photolyzed in the presence of p*2483−2475HHABA. Lane 1, no additions; lane 2, plus cDNA 2483−2475; lane 3, plus MM-cDNA 2483−75. lane 4, TP50 stained with Coomassie Blue, with migration of unlabeled proteins indicated. Gels were prepared with 18% acrylamide/0.09% bis-(acrylamide) in 0.75 M Tris-HCl (pH 8.8)/0.1% SDS.

(apparent molecular mass of 14.8 kDa; actual molecular mass of 15.3 kDa), L11 (apparent molecular mass of 12.1 kDa; actual molecular mass of 14.9 kDa), and L17 (apparent molecular mass of 12.1 kDa; actual molecular mass of 14.4 kDa). For band iv (apparent molecular mass of 10.0 kDa) labeled proteins L29−L34 (actual molecular masses of 5.4−7.3 kDa) are all potential candidates.

Labeled TP50 samples identical to those utilized for lanes 1−3 in Figure 3 were also analyzed by RP-HPLC (Figure 4A). The labeling patterns displayed were obtained in the presence and absence of cDNA 2483−2475. The largest peak of radioactivity, peak A, elutes with proteins L2, L3, L13, and L21 but shows no differential labeling. Smaller peaks B (not fully resolved from A and eluting with proteins L17, L22, L23, and L29), C (eluting with proteins L5, L9, L15, and L16), and D (eluting with proteins L11, L1, and L6) are all differentially labeled. Labeling of these peaks by p*2483−2475HHABA was little affected by addition of MM-cDNA. Unfortunately, photoincorporation into proteins L27 and L32−L34 was obscured by background radioactivity from nonincorporated, photolyzed p*2483−2475HHABA.

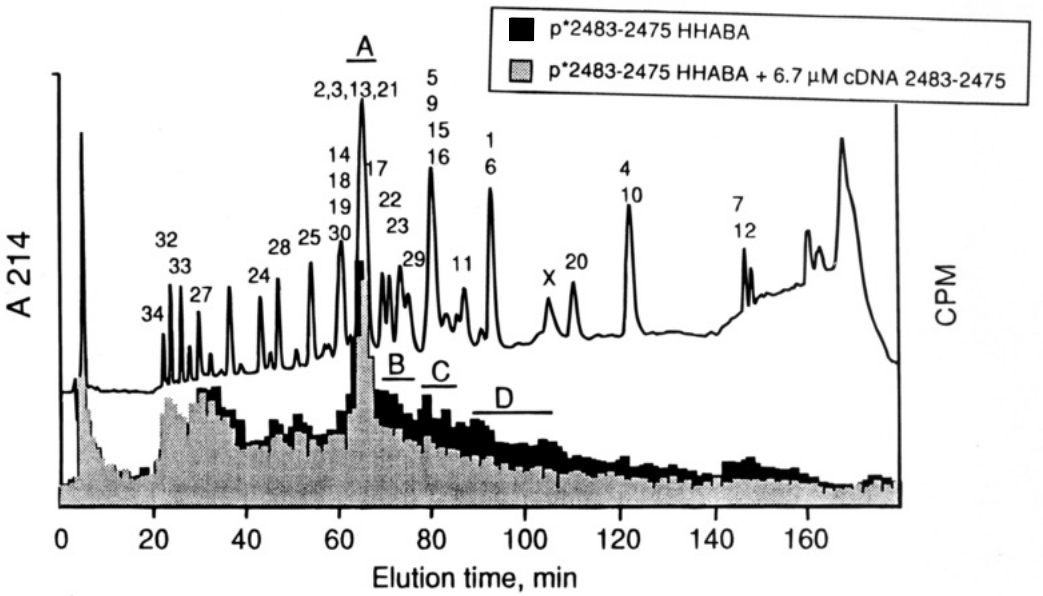
All fractions of the RP-HPLC analysis were combined into pools, and each pool was subjected to SDS−PAGE analysis. The results for pools corresponding to peaks A−D, for samples prepared from 50S subunits labeled with p*2483−2475HHABA in the presence or absence of cDNA 2483−2475 or of MM-cDNA 2483−2475, are presented in Figure 4B. From these results, taken together with the results in Figure 3, it is clear that peaks B−D contain the following proteins specifically labeled from the target site: peak B, L13 (band ii; a trace amount is also seen in peak A); peak C, L15 or L16 (band iii); peak D, L1 (band i). Most of the radioactivity in peak A corresponds to labeled L2, migrating with an apparent mass of 32.9 kDa (L2 has an apparent and actual mass of 29.7 kDa), but as is clear from Figure 4, such labeling is nonspecific. SDS−PAGE analysis of the pools corresponding to proteins L34, L32, L33, and L27 (fractions 21−35, Figure 4A) gave unclear results, due to the background radioactivity noted above. SDS−PAGE analysis of all other pools yielded no evidence of site-specific labeling of any other protein.

The identification of the labeled protein(s) in band iii as L16 and those in band iv as L32 and L33 was completed by agarose antibody affinity chromatography analysis of proteins extracted from these bands (Figure 5).

*Photoincorporation of p*2483−2475HHABA into 50S rRNA.* Urea−PAGE and autoradiographic analysis of the rRNA fraction prepared from 50S subunits photolyzed in the presence of p*2483−2475HHABA showed that incorporation proceeded into 23S rRNA but not 5S rRNA (data not shown). Incorporation of p*2483−2475HHABA into 23S rRNA is light-dependent, almost completely abolished on addition of cDNA 2483−2475, and only slightly reduced by the addition of MM-cDNA 2483−2475 (Figure 6).

Partial localization of photoincorporation into 23S rRNA was carried out by PAGE and autoradiographic analysis of the products of RNase H treatment, with or without added cDNA probes (Figure 7). In such analyses, the apparent sizes of labeled fragments are typically larger (10−20 nt) than would be expected for the corresponding unlabeled fragments generated with complete RNase H digestion (Muralikrishna & Cooperman, 1994). RNase H digestion of p*2483−2475HHABA-labeled 23S rRNA in the presence of cDNA probe 2116−2100 gives rise to a ^{32}P-labeled 370-nt fragment (lane 3). This result indicates that photoincorporation takes place into 23S rRNA between nucleotides 2100−2116 and 2475−2483, the latter corresponding to the hybridization site of p*2483−2475HHABA. The lack of a labeled 430-nt fragment in lane 2 indicates that no photoincorporation takes place between 2483 and 2904. Addition of cDNA probes progressively nearer the 2475−2483 target site produces progressively smaller labeled fragments (Figure 7, lanes 4−6), locating the labeling site between nucleotides 2438−2447 and 2475−2483. Similar experiments were also performed by adding 23S rRNA cDNA probes 360−351, 750−741, 1050−1041, 1400−1391, and 1750−1741 to p*2483−2475HHABA-labeled 23S rRNA, one at a time or in pairs. No evidence was found for a second major site of photoincorporation within 23S rRNA.

Specific sites of photoincorporation within the nucleotide region 2438−2483 were identified by reverse transcriptase-dependent primer extension (Muralikrishna & Cooperman, 1991) on 23S rRNA extracted from 50S subunits photolabeled by 2483−2475HHABA (Figure 8). Stops or pauses arising from the presence of covalently bound 2483−2475HHABA were found at positions A2471, G2472, and U2473, providing evidence for photoincorporation into the neighboring nucleotides G2470, A2471, and G2472.



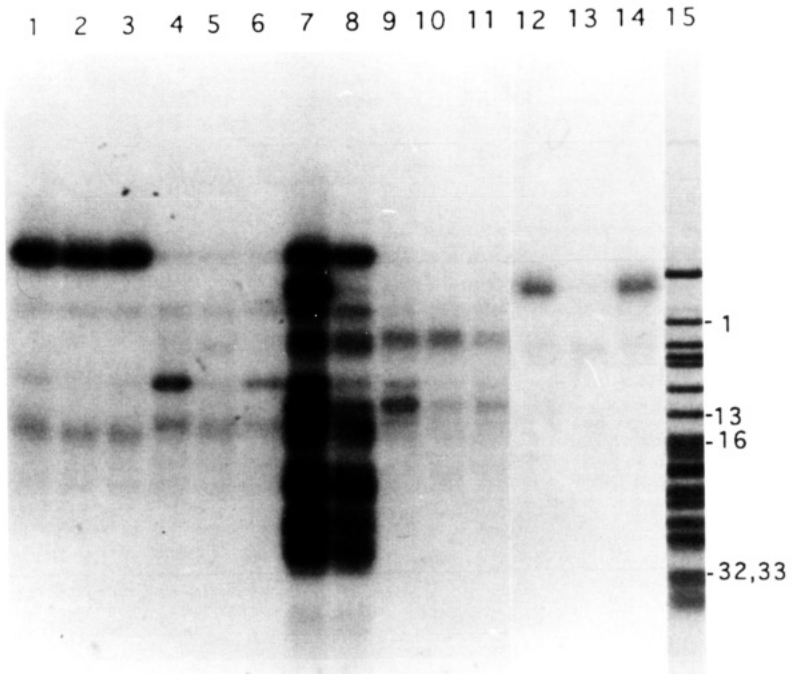


FIGURE 4: RP-HPLC analysis of proteins photolabeled with p*2483−2475HHABA. (A, top panel) Proteins prepared from 50S subunits photolabeled with p*2483−2475HHABA in the absence or presence of cDNA 2483−2475 (as in Figure 3) were applied to a Synchropak RP-P column and eluted with the following gradient: 15% acetonitrile for 10 min; 15−45% acetonitrile in 120 min (curve 0.2, convex gradient, Perkin-Elmer Series 4); 45−75% acetonitrile in 30 min (linear). Flow rate, 0.7 mL/min. The corresponding analysis of proteins labeled in the presence of MM-cDNA 2483−2475 is omitted for clarity. Peak X is not a 50S protein. (B, bottom panel) Autoradiogram of SDS−PAGE analysis of peaks A (lanes 1−3), B (lanes 4−6), C (lanes 9−11), and D (lanes 12−14). Lanes 1, 4, 9, and 12 are peaks labeled by p*2483−2475HHABA in the absence of added cDNA; lanes 2, 5, 10, and 13 are peaks labeled in the presence of added cDNA 2483−2475; lanes 3, 6,11, and 14 are peaks labeled in the presence of added MM-cDNA 2483−2475. Lanes 7 and 8 are for TP50 labeled in the presence and absence of cDNA 2483−2475, repeating lanes 1 and 2 in Figure 3, and are presented for ease of comparison. Lane 15 is TP50 stained with Coomassie Blue, with migration of unlabeled proteins indicated.

DISCUSSION

The results reported in this paper provide compelling evidence that proteins L1, L13, L16, L32, and L33 all fall within 21 Å of C2475. The validity of this conclusion depends upon the clear demonstration that these proteins are labeled by photolyzed p*2483−2475HHABA bound to its target site, as shown by three results presented above. First, RNase H cleavage of the heteroduplex formed between 2483−2475HHABA and 23S rRNA within 50S subunits produces the appropriate ~425-nt fragment. Second, nucleotides adjacent to the target site, G2470, A2471, and G2472, constitute the major site of p*2483−2475HHABA photoincorporation into 23S rRNA. Third, photoincorporation into proteins L1, L13, L16, L32, and L33 is reduced when photolysis is carried out in the presence of cDNA 2483−2475. MM-cDNA 2483−2475, which is designed not to bind the target site but to otherwise mimic cDNA 2483−2475, has little affect on photoincorporation. Proteins L1, L16, and L32 all form cross-links with 30S subunit proteins (Traut et al., 1986), consistent with the notion that the 2475

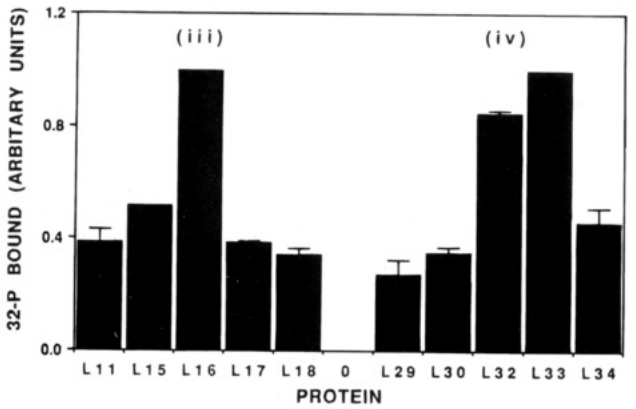


FIGURE 5: Agarose antibody affinity chromatography analysis of protein bands iii and iv. Bars denote radioactivity bound to antibody to each of the indicated ribosomal proteins, performed as described by Gulle et al. (1988). Labeled protein bands iii and iv were extracted from a gel (Figure 3, lane 1) and analyzed. Bound radioactivity is relative to L16 for band iii analysis and relative to L33 for band iv. Values shown are the average of two determinations, with the positive average deviation indicated. Background values were 0.3−0.4.

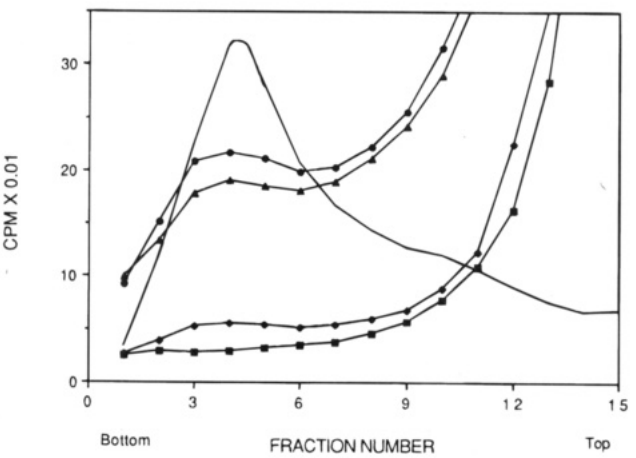


FIGURE 6: Photoincorporation of p*2483−2475HHABA into 23S rRNA: Sucrose gradient in the presence of urea and SDS. 50S subunits (150 pmol) were incubated with p*2483−2475HHABA (50 pmol) in a total volume of 75 μL (■) without photolysis, (●) with photolysis, (◆) with photolysis in the presence of cDNA 2483−2475 (750 pmol), or (▲) with photolysis in the presence of MM-cDNA 2483−2475 (750 pmol). The solid line without symbols is a trace of absorbance at 260 nm. Photoincorporation levels were as follows: with photolysis, 5.0 pmol, 10% of p*2483−2475HHABA; with photolysis in the presence of cDNA 2483−2475, 1.2 pmol, 2.4%; with photolysis in the presence of MM-cDNA 2483−2475, 4.1 pmol, 8.2%; without photolysis, 0.15 pmol, 0.3%.

loop is at the 30S−50S interface. Interestingly, we find no evidence of site-specific photoincorporation of p*2483−2475HHABA into two 50S components that have been previously found to cross-link to the 2475 loop, L6 (Wower et al., 1981) and U89 of 5S rRNA (Dontsova et al., 1994).

Electron microscopy and immunoelectron microscopy studies (Stöffler-Meilicke & Stöffler, 1990) have shown the 50S subunit to have three articulated features: a central protuberance containing 5S rRNA and, toward the bottom, L27; a projection off to one side containing L1 (the L1 projection); and the L7/L12 stalk (Figure 9). Several studies are consistent in placing the peptidyltransferase center between the L1 projection and the central protuberance (Stöffler & Stöffler-Meilicke, 1984; Olson et al., 1985). The cross-link we find to L1 and the U89(5S rRNA)−U2477

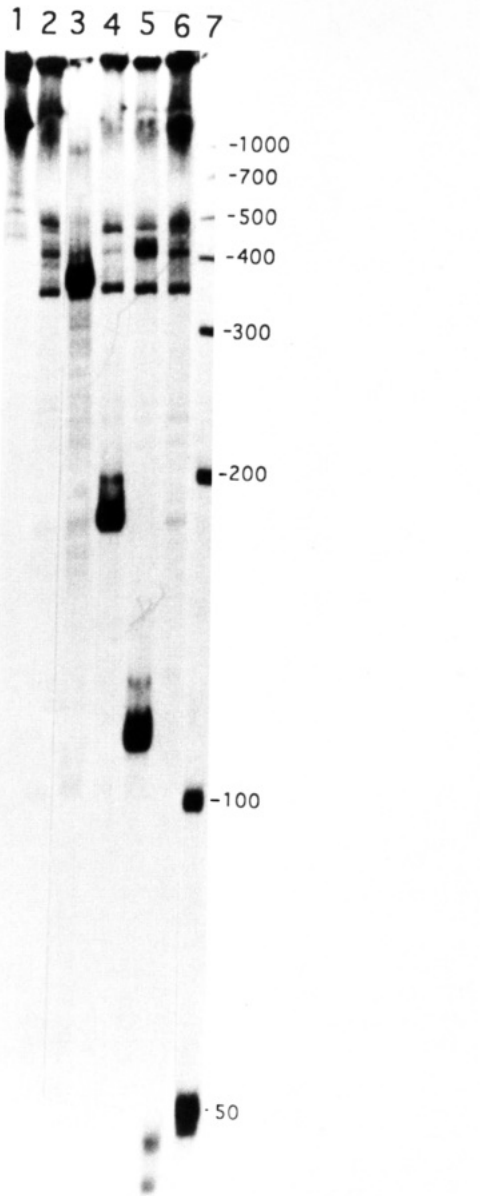


FIGURE 7: Autoradiogram of a PAGE analysis of RNAse H digested 23S rRNA photoaffinity labeled with p*2483−2475HHABA. In a typical experiment, labeled rRNA (8 pmol), purified by sucrose gradient centrifugation (Figure 6), was incubated with the indicated cDNA probe (16 pmol) in 10 μL of TND buffer [20 mM Tris-HCl (pH 7.9),100 mM NaCl, and 1 mM dithiothreitol] and digested with RNase H. Lane 1, labeled 23S rRNA in the absence of RNase H. Lanes 2−6 were all digested with RNase H. Lane 2, no added cDNA; lane 3, cDNA probe 2116−2100; lane 4, cDNA probe 2310−2301; lane 5, cDNA probe 2377−2368; lane 6, cDNA probe 2447−2438; lane 7, DNA size markers (in bases).

cross-link found by Dontsova et al. (1994) places C2475, by inference, in this same general area. Consistent with this placement, both L16 and L33, two other proteins that cross-link to p*2483−2475HHABA, have also been localized to this area, L16 by direct immunoelectron microscopy studies (Nag et al., 1991) and L33 by virtue of its forming cross-links to both L27 and L1 (Walleczek et al., 1988; Podkowinski & Gornicki, 1991). Furthermore, proteins L1, L16, and L33 each form cross-links to P-site-bound tRNAs (Wower et al., 1993), and L1 can be cross-linked to L33 (Redl et al., 1989; Walleczek et al., 1989a,b).

Our placement of the 2475 loop disagrees with the tentative location proposed by Mitchell et al. (1990), below

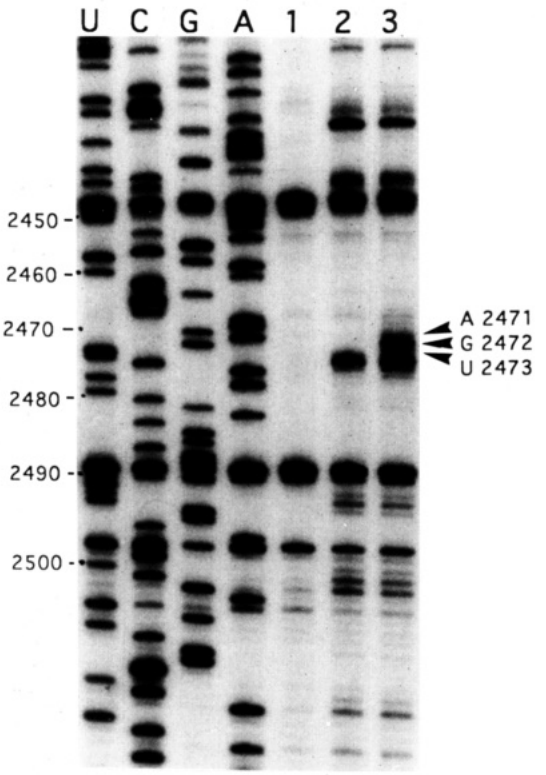


FIGURE 8: Autoradiogram of primer extension on 23S rRNA photolabeled with 2483−2475HHABA. Photoincorporation was carried out in a reaction mixture containing 50 pmol of 50S subunits and 500 pmol of 2483−2475HHABA in a total volume of 50 μL. Reverse transcriptase primer extension, using cDNA 2576−2560 as the primer, was carried out on extracted rRNA. Lane 1, not photoyzed; lane 2, photolyzed, no added 2483−2475HHABA; lane 3, photolyzed in the presence of 2483−2475HHABA. Lanes U, C, G, and A are sequencing products generated in the presence of ddATP, ddGTP, ddCTP, and ddTTP, respectively. Nucleotides at which pauses or stops are observed are indicated.

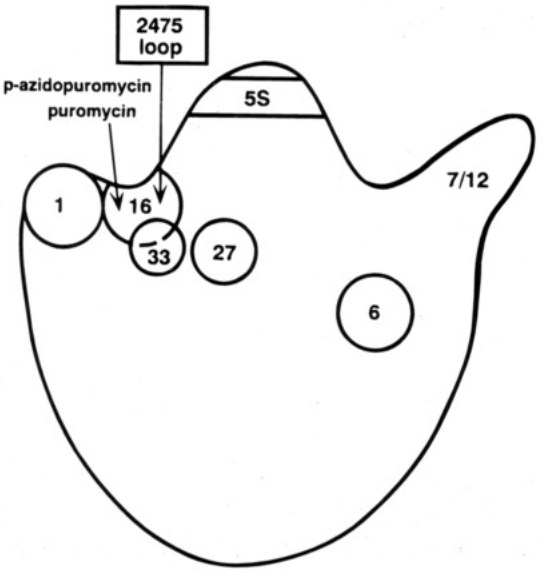


FIGURE 9: Inferred placement of the 2475 loop within the 50S subunit. The model for the 50S subunit and the placement of proteins L1, L6, L7/12, L27, and L33 follow Walleczek et al. (1988). Sources for other placements: L16, Nag et al. (1991); puromycin and *p*-azidopuromycin, Olson et al. (1982, 1985); 5S rRNA, Shatsky et al. (1980), Stöffler-Meilicke et al. (1981), and Evstafieva et al. (1985).

the base of the L7/L12 stalk (Figure 9), on the basis of cross-links found by Wower et al. (1981) between L6 and nucleotides 2473−2481. Dontsova et al. (1994) suggested that an elongated L6 could account for the cross-linking of the 2475 loop to both 5S rRNA and L6. Our results are even more difficult to reconcile with a 2473−2481 to L6 cross-link, unless L6 is a very elongated protein indeed.

In addition to L1, L16, and L33, p*2483−2475HHABA also photoincorporates into proteins L13 and L32, consistent with cross-links reported by Traut et al. (1986) between both of these proteins and L1. The L1−L32 and L1−L13 cross-links were ignored by Walleczek et al. (1988) in constructing their model for protein placement within the 50S subunit and, not surprisingly, are inconsistent with it. On the basis of our results, any updated version of the Walleczek et al. model should place L13 and L32 in closer proximity to L1. Finally, according to the scheme proposed by Leffers et al. (1987) to describe the secondary structure of 23S rRNA, the C2475 loop is at the end of helix 89. Our results can be taken as providing some indication of proximity between helix 89 and helices constituting the interaction sites for L1 (helices 75−79), L32 (helices 100 and 101), and L33 (helices 74−88), all within the 3′-terminal region of 23S rRNA, and for L13 (helix 25), closer to the 5′-terminus (Brimacombe, 1991; Egjeberg et al., 1991; Mitchell et al., 1993).

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