In Vivo Chemical Footprinting of the *Escherichia coli* Ribosome†

Michael Laughrea* and John Tam

Lady Davis Institute for Medical Research, Sir Mortimer B. Davis—Jewish General Hospital and Division of Experimental Medicine, McGill University, Montreal, Quebec, Canada H3T 1E2

Received June 15, 1992; Revised Manuscript Received August 5, 1992

ABSTRACT: We have studied the in vivo chemical accessibility of 16S rRNA residues A349–G1505 in the small subunit of the *Escherichia coli* ribosome. Exponentially growing *E. coli* cultures were reacted with dimethyl sulfate, and the reactive sites on the 16S ribosomal RNA were analyzed by reverse transcription, an assay which detects reactions at N1-A and N3-C. In agreement with previous in vitro results, three regions of 16S RNA appeared particularly reactive to dimethyl sulfate: hairpin 27 (residues A892–A915) of the central domain, and hairpin 33-33A (residues A994–C1037) and the tip of hairpin 41 (residues A1256–A1275), both from the 3′ major domain. These three regions contained 52% of the reactive residues but only 8% of the residues scanned. In contrast to previous in vitro results, three small sections of 16S RNA appeared protected: the tip of hairpins 26 (residue A845) and 31 (residues A968–A969), and residues A1418, A1441, and A1483 of the middle body of hairpin 44. Four of the dimethyl sulfate reactive sites (A831, C948, A1019, and C1192) are located in positions usually assumed to be double-stranded (helices 26, 30, 33-33A, and 34), which suggests alternative structures for these helices at least during part of the translation process, as if the residues in question belonged to "conformational switches". The addition of chloramphenicol protected residues A831, A1035–A1036, and A1503, which suggests that they belong to the mobile regions of the elongating ribosome, and become exposed during some transition(s) from one ribosomal state to the other during the elongation cycle.

Escherichia coli ribosomes, by far the best known ribosomes, contain 3 RNA molecules (5S rRNA, 16S rRNA, and 23S rRNA) and 57 proteins distributed between 2 subunits: the 30S subunit (1 16S rRNA and 21 proteins) and the 50S subunit. The chemical and enzymatic reactivity of the 30S subunit has been extensively probed with reagents such as RNase V1, dimethyl sulfate, kethoxal, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT), and DNA oligomers. The obtainment of a general consensus from the in vitro 16S rRNA structure probings of several laboratories [e.g., see Stern et al. (1988), Wakao et al. (1990), Douthwaite et al. (1983), Meier and Wagner (1985), Ericson et al. (1989), Muralikrishna and Wickstrom (1989), Laughrea and Tam (1991), Lasater et al. (1988), and Weller and Hill (1992)] is comforting but does not hide the existence of a number of discrepant results between these groups.

The ribosome is functional in vitro, and reconstitution of the various steps of the ribosome cycle can be accomplished under controlled conditions. This permits one to probe, with chemicals, RNases, or DNA oligomers, the effect on the 30S subunit (or on 16S rRNA) of the binding of such components as individual ribosomal proteins (Stern et al., 1988; Laughrea & Tam, 1991), the 50S subunit (Chapman & Noller, 1977; Vassilenko et al., 1981; Meier & Wagner, 1985; Baudin et al., 1987, 1989; Gornicki et al., 1989; Laughrea & Tam, 1991; Tapprich & Hill, 1986), initiation factors (Wakao et al., 1990; Laughrea & Tam, 1991), A-site- and P-site-bound tRNAs (Moazed & Noller, 1990; Gornicki et al., 1989; Marconi et al., 1990), or antibiotics (Moazed & Noller, 1987a; Woodcock et al., 1991; Egebjerg & Garrett, 1991; Laughrea & Tam, 1991; Marconi et al., 1990). These experiments and many others (e.g., site-directed 16S rRNA mutagenesis) have added to our understanding of the structure and function of the ribosome. In vivo footprinting has never been attempted on *E. coli* ribosomes. We felt that such studies would represent a useful reality test from which three types of results might follow: (i) confirmation of previous in vitro footprinting data, a gratifying outcome which would strengthen the value of the corroborated work [similarly, several in vitro photochemical cross-links have been confirmed in vivo (Stiege et al., 1986)]; (ii) identification of novel reactive sites, as if the translating in vivo ribosomes had localized flexibilities not revealed in vitro; (iii) identification of sites reactive in vitro but protected in vivo, indicating that there were further molecular contacts in vivo than those so far identified in vitro.

Dimethyl sulfate, a commonly used reagent in footprinting analysis, readily penetrates growing bacterial cells and methylates and N7-G positions of DNA (Nick & Gilbert, 1985) and the N1-A and N3-C positions of single-stranded RNAs (Climie et al., 1988; Nielsen, 1990). Methylated adenosines and cytidines are detected by primer extension because the methylation blocks the movement of reverse transcriptase (Ehresmann et al., 1987). In this paper, we used dimethyl sulfate to probe the in vivo accessibility of about 470 adenosines and cytidines of the 16S rRNA of the *E. coli* ribosome.

MATERIALS AND METHODS

Buffers. TSEI–SDS buffer consisted of 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 40 mM EDTA, and 1% sodium dodecyl sulfate, while DNase I buffer was 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$.

Chemicals and Enzymes. AMV reverse transcriptase was from Pharmacia. [α-^{35}S]dATP (specific activity greater than 1000 Ci/mmol) was from Amersham. Chloramphenicol, tRNA (*E. coli* MRE600), and DNase I were from Boehringer Mannheim. Phenol and acrylamide were from Sigma, and

† This work was funded by a grant from the Medical Research Council of Canada to M.L.

* To whom correspondence should be addressed at the Lady Davis Institute for Medical Research, 3755 Cote-Ste-Catherine Rd., Montreal, Quebec, Canada H3T 1E2. Telephone: (514) 340-8260. Fax: (514) 340-7502.

0006-2960/92/0431-12035$03.00/0

N,N'-methylenebis(acrylamide) was from Bio-Rad. Dimethyl sulfate was from Aldrich.

Cell Growth and RNA Preparation. The methods were derived from Climie et al. (1988) and Zengel et al. (1990). Total cellular RNA was purified from log-phase cultures of *E. coli* MRE 600 grown at 37 °C in L-broth (Lennox, 1955). One hundred fifty milliliter cultures were grown to an optical density of 0.3 (OD 600 nm). Ten-milliliter portions were incubated (with shaking) for 60 s in the absence or presence of 1.5 mg of chloramphenicol, followed by the addition of 18 μL of dimethyl sulfate. The cultures were shaken vigorously for 2 min, and poured into 2.5 mL of ice-cold DMS stop buffer (1.0 M Tris–acetate, pH 7.5, 1.5 M NaOAc, 1.0 M β-mercaptoethanol, and 0.1 mM EDTA). The samples were cooled (but not frozen) in ethanol/dry ice, and the cells were immediately collected by centrifugation in a Sorvall SS-34 rotor (8000 rpm, 3 min, 3 °C). The pellets were dissolved in 0.25 mL of water preheated to 65 °C, followed by 0.75 mL of TSEI–SDS preheated to 65 °C and 4 μL of tRNA (50 μg/μL). After 2 min at 65 °C, the samples were deproteinized, ethanol-precipitated, washed, and dried as in Laughrea and Tam (1991). The pellets were resuspended in 100 μL of DNase I buffer containing 15 units of DNase I and incubated 10 min at 37 °C and 15 min at 68 °C to inactivate the DNase I. The samples were ethanol-precipitated overnight. The final pellets were washed with 75% ethanol, dried, and dissolved in H_2O. Control cultures (with and without chloramphenicol) were tested in the same manner except that dimethyl sulfate was not added. In general, all growths were done in duplicate in each experiment.

Primer Extension and Gel Analysis. The following oligodeoxynucleotide primers, named according to the position of the first transcribed nucleotide, were used as templates for reverse transcription: 480, 683, 837, 906, 985, 1046, 1199, 1329, 1391, 1490, and 1508 (Moazed et al., 1986; Baudin et al., 1987; Laughrea & Tam, 1991). Hybridization to 16S RNA, primer extension, and gel electrophoresis were as in Laughrea and Tam (1991). Extension reactions proceeded at 55 °C, and the gels contained 6% acrylamide. Residues probed extended from A349 to G1505, with three stretches not probed to our satisfaction (from U480 to A560, from G682 to A716, and from U1040 to U1086). In sum, 996 residues (or rather 468 adenosines and cytidines) were effectively inspected, covering two-thirds of the length of 16S rRNA. In comparison of in vitro to in vivo data, only autoradiograms exposed to similar levels of radiation were compared. All relevant autoradiograms were independently read and quantitated by each of us. Whenever a difference in interpretation was noted, the more conservative interpretation was accepted. If anything, we may have erred on the side of underreporting differences.

RESULTS

Four growth experiments were performed, and all samples were probed with all primers. The dimethyl sulfate reactive residues were divided into three groups: (i) nucleotide residues reactive both in vivo and on 70S ribosomes in vitro (Table I); (ii) residues reactive in vivo but unreactive on 30S subunits or 70S ribosomes in vitro (Table II); (iii) residues reactive on 70S ribosomes in vitro but unreactive in vivo (Table III). For in vitro results, we typically refer to the work of Laughrea and Tam (1991), performed under conditions closest to the present ones, but we shall also refer to the results of others [e.g., see Moazed et al., (1986), Meier and Wagner (1985), and Baudin et al. (1987)] when these results complete ours or differ significantly from them.

Table I: 16S rRNA Residues Reactive both in Vivo and in Vitro[a]

nucleotide position	in vivo	in vivo (with chloramphenicol)	in vitro[b] [70S(–S1) + S1]
A1503	+	↓	+
A1456	(±)		+
A1408	(±)		+
C1378	±		±
A1275	(±)		(±)
A1261	++		++
A1256	±		±
C1192	+		+[c]
A1014	±	(↓)	±
A1004	+	(↓)	(+)
A915	+		+
A901	(±)		(±)
A892	+		±
A831	+	↓↓	(±)[d]
A782	+		+
A759	+		++
A665	+		+
A432	++		++[e]

[a] Data summarize visual estimates of relative band intensities. Reactivities are summarized qualitatively by the symbols 0, ±, +, and ++ in order of ascending reactivity. Data reported in parentheses have been seen in 50–75% of the experiments. All other data have been seen in >75% of the experiments. The effect of chloramphenicol is summarized by the symbols ↓ and ↓↓ to indicate, respectively, that the reactivity has been reduced by 30–60% or ≥60%. A blank indicates that the reactivity has been left unchanged (±25%) by chloramphenicol. [b] Data taken from Laughrea and Tam (1991). [c] C1192 is reactive in 30S and 70S ribosomes at 37 °C (Baudin et al., 1987) and somewhat reactive in 30S subunits at 4 °C (Powers et al., 1988). [d] Though unreactive in 4 °C (Moazed et al., 1986a,b), A831 is reactive at 37 °C in 70S ribosomes (Moazed & Noller, 1986) and in 30S subunits (Moazed & Noller, 1986, 1987a; Muralikrishna & Wickström, 1989). [e] Laughrea and Tam (unpublished results). Primer 480 was used once to probe the reaction sites of dimethyl sulfate. A strong reaction was seen at A432 and a moderate one at A431. No other reaction was seen between G384 and A478.

Table II: 16S rRNA Residues Reactive in Vivo but Not Reactive in Vitro[a]

nucleotide position	in vivo	in vivo (with chloramphenicol)	in vitro [30S(–S1) and 70S(–S1) + S1
A1502	(+)		0
A1036	±	↓↓	0
A1035	(±)	↓	0
A1019	±	(↓)	0
A996	±		0
A994	±		0
C948	±		?[b]

[a] Data presented as in Table I. [b] C948 is unreactive in 70S ribosomes at 37 °C (Moazed & Noller, 1986, 1990; Baudin et al., 1987) and unreactive in 30S subunits at 4 °C (Moazed et al., 1986b; Powers et al.; 1988) but reactive at 37 °C (Baudin et al., 1987). The reactivity of C948 is unknown in Laughrea and Tam (1991), due to a control band at this position.

Table I shows that 18 residues were reactive both in vivo and on 70S ribosomes in vitro; 90% of the residues which were highly reactive in vitro were reactive in vivo. Table I reveals that two regions of 16S rRNA were particularly reactive: hairpin 27 (residues A892–A915 of the central domain) displays three reactive residues, and the tip of hairpin 41 (residues A1256–A1275 of the 3′ major domain) also has three reactive sites. These two regions contain 33% of the residues in Table I and include only 4% of the residues scanned (Figure 1).

Six residues were moderately or weakly reactive in vivo but unreactive in vitro (Table II). A1502 is adjacent to A1503, a residue highly reactive in vitro and in vivo (Table I, Figure 2C). This, and the fact that A1502 was not reactive in all

Table III: 16S rRNA Residues Reactive in Vitro but Not Reactive in Vivo[a]

nucleotide position	in vivo	in vitro [70S(-S1) + S1]
A1483	0	±
A1468	0	(±)
A1441	0	±
A1418	0	+
C1400	0	(±)
A1375	0	±[c]
A1306	0	(±)[d]
A1188	0	(±)
C1137	0	(±)[d]
A1110	0	(±)[d]
C1031	0	±
A969	0	±
A968	0	±
A845	0	(+)
G817	0[b]	(±)
A816	0[b]	(±)
A814	0	(±)
C795	0	(±)[d]
A794	0	(±)[d]
A790	0	(±)[d]

[a] Data presented as in Table I. [b] These were weakly reactive in one experiment. The others were never seen to react in any experiment. [c] Not reactive in 70S ribosomes according to the data of Moazed and Noller (1986, 1990) and Baudin et al. (1987). [d] These residues were more reactive in 30S subunits, suggesting that they are near the subunit interface.

experiments, suggests that there may not be substantial differences between the in vivo and in vitro reactivity of the C1501–G1504 region of 16S rRNA. The five following residues, A994, A996, A1019, and A1035–1036, are located in hairpin 33-33A. Two other residents of this hairpin, A1004 and A1014, have already been described in Table I. Hairpin 33-33A is therefore considerably accessible in vivo: it contains 28% of all the dimethyl sulfate reactive sites but only 4% of the residues scanned (Figures 1 and 2A). However, this should not mislead us into concluding that this hairpin is buried in vitro. First, residues A1004, C1031 (Table III), and A1014 are also reactive in vitro; second, many other residues of hairpin 33-33A have proven reactive to other probes (Laughrea & Tam, 1991). Hairpin 33-33A is in fact one of the most accessible hairpins in vitro, together with hairpins 24 and 26 and the tip of hairpin 41, and more so than hairpin 27 (Laughrea & Tam, 1991). The detailed conformation and accessibility of hairpin 33-33A may differ somewhat in vivo vs in vitro, but in both cases, it remains considerably reactive, as if it was at the surface of the ribosome. Figure 1 highlights the three regions of 16S rRNA which appear highly exposed both in vivo and in vitro. These three regions contain 52% of the reactive residues but only 8% of the residues scanned. Such results should, however, be put in context: in this paper, a "highly exposed" region is not one with a majority of residues accessible to dimethyl sulfate, but simply a region which, relative to other regions, is much more accessible to dimethyl sulfate. In fact, the three "highly exposed" regions of Figure 1 have more protected than reactive residues. They possess 49 A's and C's that could be probed on our gels, 27 of them in presumably single-stranded areas. Yet, only 13 A's and C's have reacted. In sum, the highly exposed hairpins 27, 33-33A, and 41 have a substantial minority of bases facing the cytoplasm, but probably a majority in contact with other nucleotide or amino acid residues.

One residue not assessed by Laughrea and Tam (1991) was C948 (Table II). It was unreactive in 70S ribosomes at 37 °C (Moazed & Noller, 1986, 1990; Baudin et al. 1987) and usually unreactive in 30S subunits at 4 °C (Moazed et al., 1986b; Powers et al., 1988), but reactive in 30S subunits at 37 °C (Baudin et al., 1987) and sometimes at 4 °C (Moazed et al., 1986a). Strictly speaking, C948 does not belong to Table I or Table II, but somewhere in between. C948 is located in helix 30, a region classically identified as double-stranded and rather protected from enzymatic and chemical probes: helix 30 was unreactive to dimethyl sulfate, RNase V1, kethoxal, and CMCT (Laughrea & Tam, 1991). Our results raise the possibility that this helix may be more flexible and available in vivo than in vitro. This will become clearer later, when it will be realized that among residues reactive in 30S subunits and protected in 70S ribosomes, none were detected by our in vivo approach, except C948.

Table III shows 20 residues that were reactive on 70S ribosomes in vitro but unreactive in vivo. The reactivity of 12 of these residues was rather weak and therefore of unknown significance. Of the remaining eight residues, one, A1375, is controversial: it was unreactive in the hands of two other groups. Another, C1031, belongs to hairpin 33-33A. C1031 reinforces an observation made in the context of Table II: hairpin 33-33A is, by and large, quite accessible both in vivo and in vitro, but its detailed conformation differs, C1031 being reactive in vitro, but not in vivo, and A994, A996, A1019, and A1035-A1036 being reactive in vivo, but not in vitro. The remaining six residues define three regions which appear more protected in vivo than in vitro. (i) The tip of hairpin 26 (residue A845) has one highly reactive site in vitro and none in vivo. Residues C839–C840, U842–U843, and G844 of this hairpin were highly reactive to RNase V1, CMCT, and kethoxal (Laughrea & Tam, 1991). (ii) The tip of hairpin 31 (residues A968–A969) has two dimethyl sulfate reactive sites in vitro and none in vivo. Residue G971 of this hairpin was reactive to kethoxal (Laughrea & Tam, 1991). G966, not probed by Laughrea and Tam (1991), was reactive in 70S ribosomes but, interestingly, unreactive in polysomes (Brow & Noller, 1983). (iii) The middle body of hairpin 44 has three dimethyl sulfate reactive sites in vitro (A1418, A1441, A1483) and none in vivo. [It is interesting to note that A1456, not far from A1441, was considerably more reactive in vitro than in vivo (Table I).] These three regions are highlighted in Figure 1. In the in vitro experiments, hairpins 24 and 38–39 were partially protected by the 50S subunit (Laughrea & Tam, 1991). Other footprinting or site-directed mutagenesis experiments suggest that the tip of hairpin 26 (Herr et al., 1979), the tip of hairpin 31 (Baudin et al., 1989), and some residues in the middle of helix 44 (Meier & Wagner, 1985; Rottmann et al., 1988; Gornicki et al., 1989; Baudin et al., 1988) are protected by the 50S subunit or implicated in subunit association. One may conclude that subunit association is perhaps tighter or more complete in vivo.

The effect of chloramphenicol was also studied. Chloramphenicol interacts with the ribosome on the 50S subunit, at the interface of the A- and P-sites. It blocks peptide bond formation without displacing mRNA or peptidyl-tRNA from the ribosome, and without binding at the same site as puromycin (Vazquez, 1979; Pongs, 1979; Gale et al., 1981). Among the 23S rRNA bases protected by chloramphenicol, one finds A2451, a base protected by A-site-bound aminoacyl-tRNA, and G2505, a base protected by P-site-bound peptidyl-tRNA. This is a weak overlap: less than 20% of the bases protected by the 3′ end of P-site-bound or A-site-bound tRNA are protected by chloramphenicol (Moazed & Noller, 1987b, 1989). After addition of chloramphenicol to the growth medium, the reactivity of 16S rRNA residues A831, A1004, A1014, and A1503, which were reactive in vivo and in vitro,

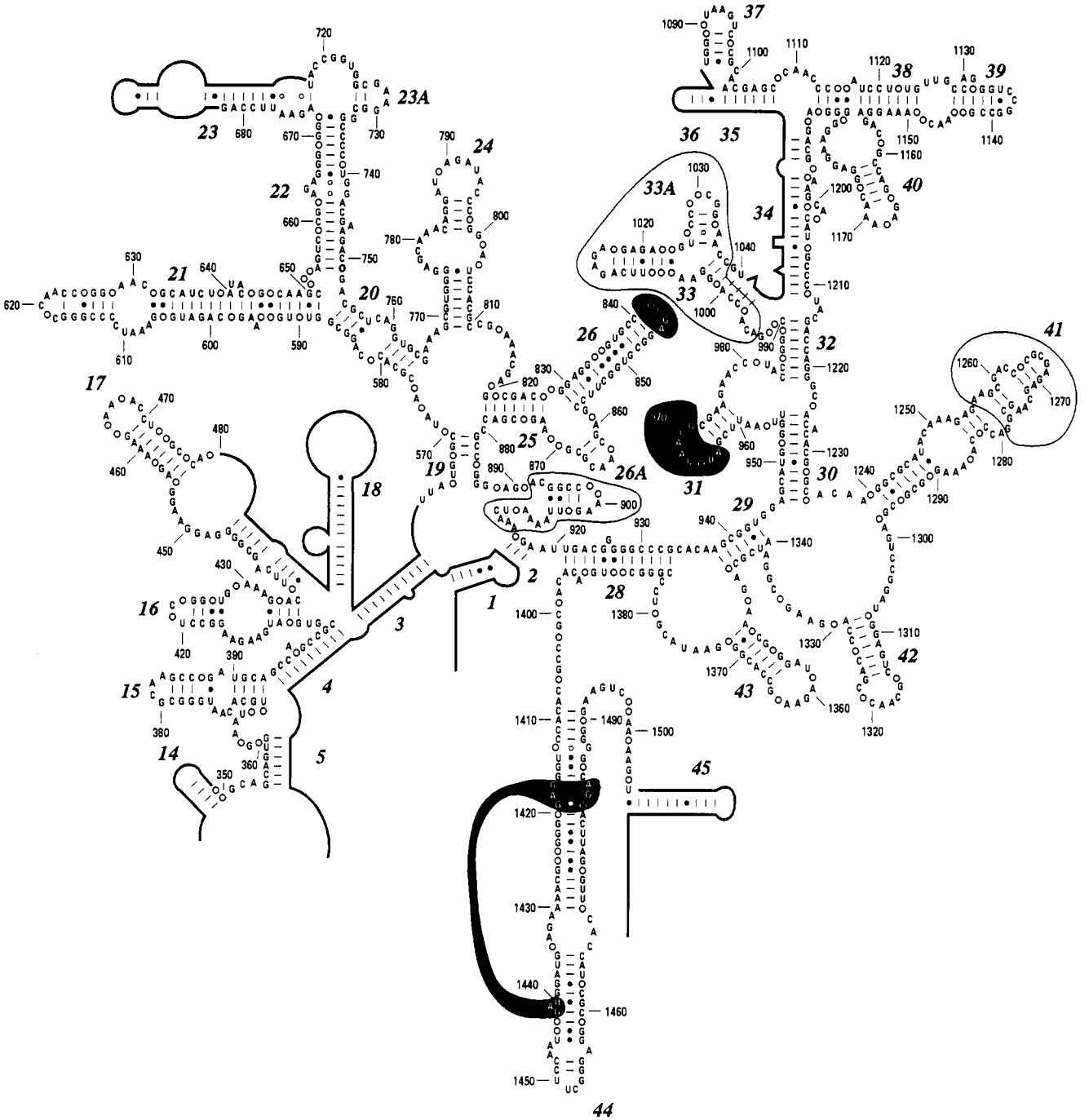

FIGURE 1: Schematic diagram of the secondary structure of *E. coli* 16S rRNA, emphasizing three regions highly reactive both in vivo and in vitro (enclosed by a line) and three regions unreactive in vivo but reactive in vitro (dark areas). The secondary structure model is from Stern et al. (1988) with the 5′ domain mostly omitted; helices are numbered (from 14 to 45) according to Brimacombe et al. (1988) and have been incorporated into Noller's model by Raué et al. (1988). (–) Bases outside the scope of this paper; (O) bases whose reactivity could not be probed due to stop of reverse transcriptase. For clarity, the diameter of the stems and the length of the single-stranded stretches are reduced by about half relative to what would be reasonable given the base-pair spacing.

and of A1019 and A1035–A1036, which were reactive only in vivo (Figure 2, Tables I and II), was decreased. Chloramphenicol did not significantly affect 16S rRNA sites influenced by the binding of peptidyl-tRNA and aminoacyl-tRNA, indicating that chloramphenicol does not seriously destabilize tRNA binding to the A-site and the P-site, consistent with previous results of Tai and Davis (1972).

DISCUSSION

The present experiments should give us a snapshot of *E. coli* ribosomes mostly in the elongating phase because in exponentially growing *E. coli*, 90–95% of the ribosomes sediment as polysomes and 70S monosomes (Van Knippenberg & Duijts, 1971; Flessel et al., 1967; Hapke & Noll, 1976). Our working hypothesis, that we are only able to detect the labeling of the predominant (i.e., polysomal) form of 16S rRNA in exponentially growing *E. coli*, is underlined by the fact that only 5% of the probed adenosines and cytidines were reactive and the fact that chloramphenicol did not increase the reactivity of any residue. It seems that if our technique was sensitive enough to record all of the dimethyl sulfate reactive sites during the life cycle of 16S rRNA from synthesis to degradation, many more residues would have been recorded as methylated. This theme is further developed in the section

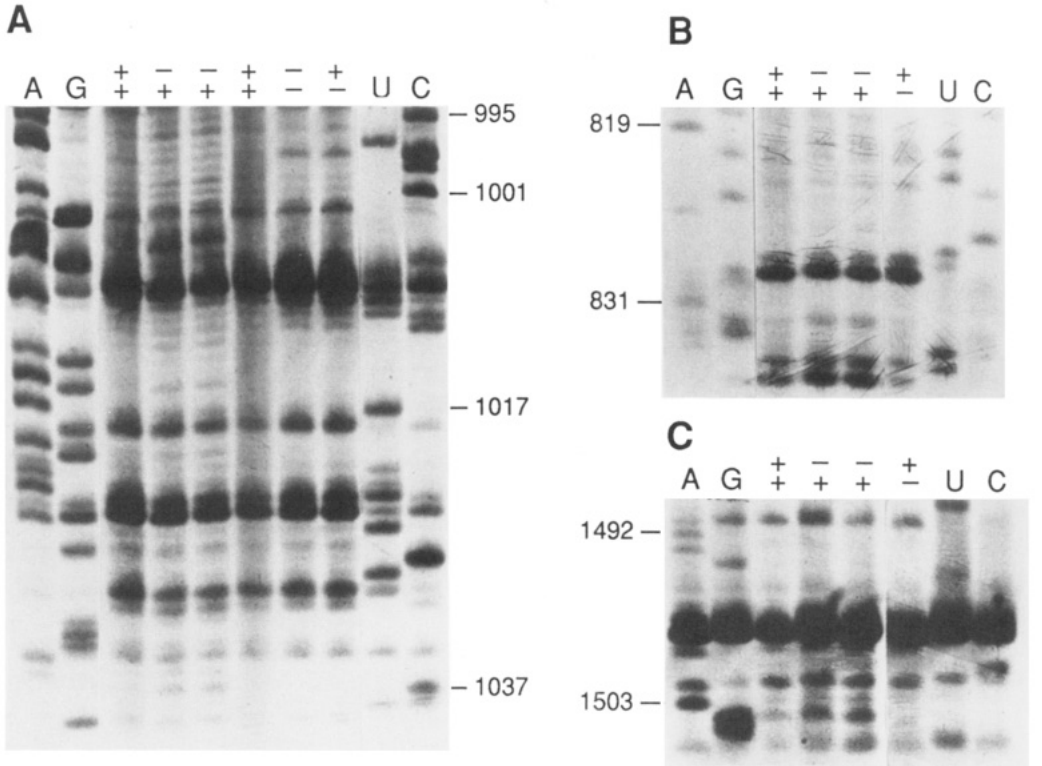

FIGURE 2: Typical autoradiograms showing the results of dimethyl sulfate probing of growing cells. The purified RNA was reverse-transcribed using primers 1046 (A), 837 (B), and 1506 (C). For each sample, the presence or absence of chloramphenicol and dimethyl sulfate are depicted by (+) or (−), respectively, the upper symbol corresponding to chloramphenicol; ++ means that 150 μg of chloramphenicol/mL was added, followed 60 s later by 1.8 μL of dimethyl sulfate/mL. Lanes A, G, U, and C are sequencing products generated in the presence of ddTTP, ddCTP, ddATP, and ddGTP, respectively.

on chloramphenicol. In a footprinting experiment, chemical modifications or RNase cuts are introduced at a statistical and low level on each macromolecule, e.g., about 1 hit per 300 nucleotides (Christiansen et al., 1990). This does not favor the detection of DMS reactive sites within a small subpopulation of 16S rRNA.

Conformational Switches at A831, C948, A1019, and C1192? The seemingly anomalous reactivities of A831, C948, A1019, and C1192 merit discussion. There is quite compelling phylogenetic evidence that C948 and A1019 must exist in rigid double-stranded structures during at least part of the protein synthesis cycle (Neefs et al., 1990; Woese et al., 1983). This evidence is, however, less compelling for A831 and C1192. Helix 26 has a large number of U-G base pairs—5 in the 6 base pairs following the A831-U855 pair. This pattern is typical of all eubacteria, and half of them even have a G-U base pair at the position corresponding to the A831-U855 pair of *E. coli* (Neefs et al., 1990). As for C1192, the sequence GACGUC1195 is completely conserved among eubacteria, removing the opportunity of checking for compensatory base changes during evolution (Woese et al., 1983; Neefs et al., 1990). This information, and the fact that A831 and C1192 are dimethyl sulfate reactive both in vivo and in vitro (Table I), suggests that parts of helices 26 and 34 have a single-stranded character during at least part of the translation cycle and that this is not inconsistent with the phylogenetic data.

Our finding that C948 and A1019 are dimethyl sulfate reactive in vivo is, however, inconsistent with phylogenetic data. The simplest way to reconcile the data is to infer that C948 and A1019 belong to molecular switches: they must belong to a double-strand during part of the protein synthesis cycle and belong to a single-strand during another part of this cycle. In sum, our in vivo results, together with our in vitro results and those of others, would suggest that ribosomal structure is dynamic at A831, C948, A1019, and C1192 (hairpins 26, 30, and 33 and at the top of helix 34). An alternative explanation is that exceptionally these happen to be residues which got labeled during rRNA synthesis, processing, or degradation. Brimacombe et al. (1986) had previously found experimental and phylogenetic evidence suggesting that G1064, the hydrogen-bonded partner of C1192 in Figure 1, was part of a "conformational switch": it would be hydrogen-bonded to C1192 or U390 (helix 15) depending on the state of the ribosome. In the second state, C1192 would be free to react with dimethyl sulfate. Our finding that C1192 is dimethyl sulfate reactive in vivo supports this idea. One may note that helices 15 and 34 are not dramatically far apart in two common models for the three-dimensional folding of 16S rRNA (Brimacombe et al., 1988; Stern et al., 1988).

Effect of Chloramphenicol. Three main steps take place during elongation: binding of aminoacyl-tRNA to the A-site, peptide bond formation, and translocation (Liljas, 1990; Moazed & Noller, 1989). A 2-min incubation in the presence of chloramphenicol should not change the predominantly polysomal status of the ribosomes (see last paragraph for details) but is expected to have two consequences. (i) The distribution of the various polysomal states will be changed, most ribosomes finding themselves in a P/PA/T-like or a P/PA/A-like state and hardly any in the P/E state [these states are defined by Moazed and Noller (1989)]. This has little relevance here because the above states do not have any differential impact on the reactivity of the 30S subunit, except for small differences at the level of A702 and G1338 (Moazed & Noller, 1989). (ii) The transitions from one ribosomal state to the other will be abolished within 0.07 s following chloramphenicol binding (Pedersen, 1984). This, the change of the polysome from a dynamic to an immobile object, seems highly relevant. It would be surprising if the ribosome, in the

process of moving from one state to the other during the elongation cycle, would not transiently expose otherwise buried residues or transiently hide otherwise available residues. We call such residues "transients". If transients exist during the elongation cycle, the "freezing" action of chloramphenicol should reveal them by protecting (making more reactive) the transiently exposed (buried) residues.

We shall assume that the ribosomal binding of chloramphenicol does not alter the conformation of the 30S subunit but simply alters the dynamics of elongation and the structure of the 50S subunit, on the grounds that chloramphenicol binds only to the 50S subunit and has a very punctual influence on the reactivity of the 23S rRNA (Moazed & Noller, 1987b; Marconi et al., 1990). This assumption is scrutinized in the last paragraph. From the observed effects of chloramphenicol at A831, A1035–A1036, and A1503 of 16S rRNA (we do not discuss the weaker or less reproducible effects of chloramphenicol), we are led to two conclusions. (i) The ribosomal transitions of elongation mainly involve "exposures" rather than "burials": chloramphenicol had only protective effects on the reactivity of 16S rRNA. This should be qualified, however, by noting an assymmetry inherent to in vivo footprinting: burials lasting less than 20% of an elongation cycle cannot be safely detected, but any transient exposure can be detected as long as the base, once exposed, is sufficiently reactive. A highly reactive base could be exposed during only 5% or so of an elongation cycle and still be detected. (ii) A831 of hairpin 26 and A1035–A1036 of hairpin 33A are involved in such transitions: residues A831 and A1035–A1036 are much more reactive in vivo than in vitro (Tables I and II), as if they would belong to the "moving parts" of the elongating ribosome. We note that hairpin 26 is influenced by S8 and S6+S18 and that hairpin 33A is influenced by S19 and perhaps S14 (Stern et al., 1988). All of these proteins have been cross-linked to the 50S subunit (Cover et al., 1981; Skold, 1981), which may move relative to the 30S subunit during the elongation cycle (Moazed & Noller, 1989). However, it seems disconcerting that hairpin 33A is not well conserved through evolution (Raué et al., 1988).

A1503 was also protected by chloramphenicol but was quite reactive both in vivo and in vitro (Table I; Figure 2C). Thus, if it is a "moving part" of the ribosome, we must assume that, already in vitro, it is able to experience all the movements that it has to go through in vivo. This is plausible because the 3′ end of 16S RNA is highly flexible in vitro: it has been cross-linked to ribosomal proteins S1, S7, S18, and S21, initiation factor IF3, 16S rRNA residues 0–20, 440–460, 500–520, 920–940, and 1393–1401, and domain IV of 23S rRNA (Czernilofsky et al., 1975; Van Duin et al., 1975; Brimacombe et al., 1988; Wollenzien & Cantor, 1982; Mitchell et al., 1992; Döring et al., 1992). Evidently, chloramphenicol reduces the freedom of motion of the 3′ end of 16S rRNA to a level lower than what is seen in vitro.

If C948 and C1192 belong to molecular switches, why was the reactivity of these residues unaffected by chloramphenicol? We suggest that C948 bulges and is highly reactive in 30S subunits, but becomes hydrogen-bonded to G1233 of helix 30 during the binding of the 50S subunit or soon after, i.e., before tRNA binding to the A-site. As for C1192, we suggest that it is part of a single-strand in 30S subunits, 70S ribosomes, and chloramphenicol-treated polysomes and becomes a double-stranded member of helix 34 for less than 20% of a typical elongation cycle. Alternatively, C1192 may become "double-stranded" for part of the initiation or the termination process. In sum, the in vivo experiments in the presence of chloramphenicol are compatible with the idea that C948 and C1192 belong to conformational switches, and strengthen the idea that A831 and A1019 belong to such switches.

We wish to end with a small caveat. Chloramphenicol inhibits protein synthesis but initially leaves rRNA synthesis unaffected. This results in the formation of "chloramphenicol particles", heterogeneous ribonucleoproteins containing 16S RNA, 23S RNA, and precursors of these two RNAs (Sykes et al., 1977). After addition of chloramphenicol to an exponentially growing culture of *E. coli*, a linear accumulation of rRNA (in the form of chloramphenicol particles) is seen for a period of 45–120 min after addition of the drug (Wisseman et al., 1954; Pardee & Prestidge, 1956; Nomura & Watson, 1959; Kurland & Maaloe, 1962; Hosokawa & Nomura, 1965). At this point, 30–50% of the cellular rRNA is in the form of chloramphenicol particles (Pardee & Prestidge, 1956; Nomura & Watson, 1959; Kono & Osawa, 1964; Hosokawa & Nomura, 1965; Sykes et al., 1977). These particles behave as loosely coiled polyelectrolytes with only half the protein content of normal ribosomes (Kurland et al., 1962; Hosokawa & Nomura, 1965). In sum, our chloramphenicol-treated cells most probably have 1–3% of their rRNA in the form of chloramphenicol particles, particles not present in the control experiments. We seriously doubt that this "contaminant" could affect the interpretation of the results. For example, if our technique had been sensitive enough to sample these chloramphenicol particles, it should have revealed new reactive sites and residues with increased reactivity, because chloramphenicol particles are unstructured and protein-poor. In fact, the contrary was seen: chloramphenicol had no or inhibitory effects on the dimethyl sulfate reactivity of 16S RNA.

ACKNOWLEDGMENT

We thank Dr. Lea Brakier-Gingras for her critical reading of the manuscript, Rhona Rosenzweig for the preparation of the typed text, and Luc Beauchemin for the illustrations.

REFERENCES

Baudin, F., Ehresmann, C., Romby, P., Mougel, M., Colin, J., Lempereur, L., Bachellerie, J. P., & Ebel, J. P. (1987) *Biochimie 69*, 1081–1096.

Baudin, F., Mougel, M., Romby, P., Eyermann, F., Ebel, J.-P., Ehresmann, B., & Ehresmann, C. (1989) *Biochemistry 28*, 5847–5855.

Brimacombe, R., Atmadja, J., Kyriatsoulis, A., & Stiege, W. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds) pp 184–202, Springer-Verlag, New York.

Brimacombe, R., Atmadja, J., Stiege, W., & Schüler, D. (1988) *J. Mol. Biol. 199*, 115–136.

Brow, D. A., & Noller, H. F. (1983) *J. Mol. Biol. 163*, 27–46.

Chapman, N. M., & Noller, H. F. (1977) *J. Mol. Biol. 109*, 131–149.

Christiansen, J., Egebjerg, J., Larsen, N., & Garrett, R. A. (1990) in *Ribosomes and protein synthesis. A practical approach* (Spedding, G., Ed.) pp 229–252, Oxford University Press, New York.

Climie, S. C., & Friesen, J. D. (1988) *J. Biol. Chem. 263*, 15166–15175.

Cover, J. A., Lambert, J. M., Norman, C. M., & Traut, R. R. (1981) *Biochemistry 20*, 2843–2852.

Czernilofsky, A. D., Kurland, C. G., & Stoffler, G. (1975) *FEBS Lett. 58*, 281–289.

Döring, T., Greuer, B., & Brimacombe, R. (1992) *Nucleic Acids Res. 20*, 1593–1597.

Douthwaite, S., Christensen, A., & Garrett, R. A. (1983) *J. Mol. Biol. 169*, 249–279.
Egebjerg, J., & Garrett, R. A. (1991) *Biochimie 73*, 1145–1149.
Ehresmann, C., Baudin, F., Mougel, M., Romby, P., Ebel, J. P., & Ehresmann, B. (1987) *Nucleic Acids Res. 15*, 9109–9128.
Flessel, C. P., Ralph, P., & Rich, A. (1967) *Science 158*, 658–660.
Fraenkel, D. G., & Neidhardt, F. C. (1961) *Biochim. Biophys. Acta 53*, 96–110.
Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., & Waring, M. J. (1981) *The Molecular Basis of Antibiotics Action*, John Wiley & Sons, London.
Gornicki, P., Baudin, F., Romby, P., Wiewiorowski, M., Kryzosiak, W., Ebel, J. P., Ehresmann, C., & Ehresmann, B. (1989) *J. Biomol. Struct. Dyn. 6*, 971–984.
Hapke, B., & Noll, H. (1976) *J. Mol. Biol. 105*, 97–109.
Herr, W., Chapman, N. M., & Noller, H. F. (1979) *J. Mol. Biol. 130*, 433–449.
Hosokawa, K., & Nomura, M. (1965) *J. Mol. Biol. 12*, 225–241.
Kono, M., & Osawa, S. (1964) *Biochim. Biophys. Acta 87*, 326–334.
Kurland, C. G., & Maaloe, O. (1962) *J. Mol. Biol. 4*, 193–210.
Kurland, C. G., Nomura, M., & Watson, J. D. (1962) *J. Mol. Biol. 4*, 388–394.
Lasater, L. S., McKuskie Olson, H., Cann, P. A., & Glitz, D. G. (1988) *Biochemistry 27*, 4687–4695.
Laughrea, M., & Tam, J. (1991) *Biochemistry 30*, 11412–11420.
Lennox, E. S. (1955) *Virology 1*, 190–206.
Liljas, A. (1990) in *The Ribosome Structure, Function and Evolution* (Hill, W. E., Moore, P. B., Dahlberg, A., Schlessinger, D., Garrett, R. A., & Warner, J. R., Eds.) pp 309–317, American Society for Microbiology, Washington, D.C.
Marconi, R. T., & Hill, W. E. (1989) *Biochemistry 28*, 893–899.
Marconi, R. T., Lodmell, J. S., & Hill, W. E. (1990) *J. Biol. Chem. 265*, 7894–7899.
Meier, N., & Wagner, R. (1985) *Eur. J. Biochem. 146*, 83–87.
Mitchell, P., Osswald, M., & Brimacombe, R. (1992) *Biochemistry 31*, 3004–3011.
Moazed, D., & Noller, H. F. (1986) *Cell 47*, 985–989.
Moazed, D., & Noller, H. F. (1987a) *Nature 327*, 389–394.
Moazed, D., & Noller, H. F. (1987b) *Biochimie 69*, 879–884.
Moazed, D., & Noller, H. F. (1989) *Nature 342*, 142–148.
Moazed, D., & Noller, H. F. (1990) *J. Mol. Biol. 211*, 135–145.
Moazed, D., Stern, S., & Noller, H. F. (1986a) *J. Mol. Biol. 187*, 399–416.
Moazed, D., Van Stolk, B. J., Douthwaite, S., & Noller, H. F. (1986b) *J. Mol. Biol. 191*, 483–493.
Muralikrishna, P., & Wickström, E. (1989) *Biochemistry 28*, 7505–7510.
Neefs, J.-M., Van de Peer, Y., Hendriks, L., & De Wachter, R. (1990) *Nucleic Acids Res. 18*, 2237–2317.
Nick, H., & Gilbert, W. (1985) *Nature 313*, 795–798.
Nielsen, P. E. (1990) *J. Mol. Recognit. 3*, 1–25.
Nomura, M., & Watson, J. D. (1959) *J. Mol. Biol. 1*, 204–217.
Pardee, A. B., & Prestidge, L. (1956) *J. Bacteriol. 71*, 677–683.
Pedersen, S. (1984) *EMBO J. 3*, 2895–2898.
Pongs, O. (1979) in *Antibiotics V/1 Mechanism of Action of Antibacterial Agents* (Hahn, F. E., Ed.) pp 26–42, Springer-Verlag, Berlin.
Powers, T., Stern, S., Changchien, L.-M., & Noller, H. F. (1988) *J. Mol. Biol. 201*, 697–716.
Raué, H. A., Klootwuk, J., & Musters, W. (1988) *Prog. Biophys. Mol. Biol. 51*, 77–129.
Rottmann, N., Kleuvers, B., Atmadja, J., & Wagner, R. (1988) *Eur. J. Biochem. 177*, 81–90.
Skold, S.-E. (1981) *Biochimie 63*, 53–60.
Stern, S., Weiser, B., & Noller, B. F. (1988) *J. Mol. Biol. 204*, 447–481.
Stiege, W., Atmadja, J., Zobawa, M., & Brimacombe, R. (1986) *J. Mol. Biol. 191*, 135–138.
Sykes, J., Metcalf, E., & Pickering, J. D. (1977) *J. Gen. Microbiol. 98*, 1–16.
Tai, P.-C., & Davis, B. D. (1972) *J. Mol. Biol. 67*, 219–229.
Tapprich, W. E., & Hill, W. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 556–560.
Van Duin, J., Kurland, C. G., Dondon, J., & Grunberg-Manago, M. (1975) *FEBS Lett. 59*, 287–290.
Van Knippenberg, P. H., & Duijts, G. A. (1971) *Mol. Gen. Genet. 112*, 208–220.
Vassilenko, S. K., Carbon, P., Ebel, J. P., & Ehresmann, C. (1981) *J. Mol. Biol. 152*, 699–721.
Vazquez, D. (1979) *Mol. Biol. Biochem. Biophys. 30*, 1–312.
Wakao, M., Romby, P., Laalami, S., Ebel, J. P., Ehresmann, C., & Ehresmann, B. (1990) *Biochemistry 29*, 8144–8151.
Weller, J. W., & Hill, W. E. (1992) *Biochemistry 31*, 2748–2757.
Wisseman, C. L., Smadel, J. E., Hahn, F. E., & Hopps, M. E. (1954) *J. Bacteriol. 67*, 662–673.
Wollenzien, P. L., & Cantor, C. R. (1982) *J. Mol. Biol. 159*, 151–166.
Woodcock, J., Moazed, D., Cannon, M., Davies, J., & Noller, H. F. (1991) *EMBO J. 10*, 3099–3103.
Zengel, J. M., McCormick, J. R., Archer, R. M., & Lindahl, L. (1990) in *Ribosomes and Protein Synthesis, A Practical Approach* (Spedding, G., Ed.) pp 213–228, Oxford University Press, New York.