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Secondary Structure of 16S Ribosomal RNA

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Summary. A secondary structure model for 16*S* ribosomal RNA which is based on available chemical, enzymatic, and comparative sequence data shows good agreement between constraints dictated by the model and a wide variety of experimental observations. The four major structural domains created by the base-pairing scheme correspond closely to RNA fragments isolated after nuclease digestion in the presence of bound ribosomal proteins. Functionally important sites appear to be located in unpaired regions and are phylogenetically highly conserved.

Ribosomes are responsible for translation of the genetic code in all organisms. They are large and complex macromolecular structures; for example, the *Escherichia coli* ribosome has a molecular weight of 2.6×10^6 comprising about 52 different proteins as well as the 5*S*, 16*S*, and 23*S* ribosomal RNA's (rRNA's) (*1*, *2*). This particle, called the 70*S* ribosome, can be dissociated into a 30*S* and 50*S* subunit, both of which contain RNA and protein in noncovalent association. The 30*S* subunit contains the 16*S* rRNA (1542 nucleotides) and a single copy of each of 21 different proteins. The 50*S* subunit contains the 5*S* rRNA (120 nucleotides) and the 23*S* rRNA (2904 nucleotides); it also contains a single copy of 31 different proteins and four copies of one protein (called L7-L12).

This complexity implies an extraordinarily high degree of asymmetry, and for this reason straightforward solutions to the structure of the ribosome are not likely to be forthcoming. At present, ribosome crystals suitable for x-ray diffraction analysis are not available, but if and when they become so, crystallographers are likely to be challenged with a formidable problem. In the absence of conventional physical approaches to ribosome structure, various ingenious approaches have emerged, including studies concerned with determining the spatial location of the ribosomal proteins by chemical cross-linking (*3*), neutron diffraction (*4*), singlet-singlet energy transfer (*5*), and immuno electron microscopy (*6*, *7*). The results of these studies appear to be converging on a mutually consistent set of coordinates for the location of several of the proteins in the 30*S* ribosomal subunit.

Although in one sense we have learned a lot about ribosomal structure during the past two decades, we nevertheless can still give no more than a superficial description of the translation mechanism. Our failure to apprehend the essence of this mechanism might, of course, reflect the inherent complexity of the ribosome; but it could equally mean that we have yet to focus on its essence. For technical reasons, if not conceptual prejudice, almost all effort up to now has gone into characterizing ribosomal proteins and various protein factors associated with translation. However, the rapid methods for sequencing nucleic acids now make it feasible to characterize the large rRNA's in detail, and so to begin inquiry into the functional significance, if any, of these molecules. We shall shortly see then whether a detailed understanding of rRNA's reveals some essence of ribosome function.

Nucleotide sequences of the *E. coli* 16*S* and 23*S* RNA's are now known (*8*–*10*). In addition, the 16*S* rRNA sequence for another bacterium, *Bacillus brevis*, is nearly complete (*11*), and extensive sequence information on 16*S* rRNA's exists in the form of ribonuclease T1 oligonucleotide catalogs from more than 150 species of bacteria (*12*). This then has permitted us to undertake a comparative approach to the molecule's secondary structure. Recently, sequences for a chloroplast 16*S* RNA, mitochondrial 12*S* and 16*S* RNA's, and part of a yeast 18*S* RNA have also become available (*13*). Such an analysis in conjunction with direct biochemical tests of secondary structure has now been completed (*14*). In this article, we summarize that structure and discuss its implications with regard to overall ribosome structure, function, and evolution.

Derivation of the Model

The nucleotide sequence of 16*S* RNA, as determined by DNA sequencing of the *rrn*B rRNA operon of *E. coli* (*8*), is the basis for derivation of the secondary structure model. A catalog of potential base-pairing regions is generated by a computer program, in which only helices with four or more base pairs and with predicted stabilities of at least the order of those seen in transfer RNA (tRNA) stems survive screening (*15*). These criteria are somewhat arbitrary, and are chosen as a compromise between having to consider both an unmanageable number of potential helices and the danger of missing an important structure. This results in a starting field of about 10^4 possibilities, of which fewer than 100 can exist. Predicted thermodynamic stability

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is used as a rough guide to the selection of helices but, as we have shown, is not an absolutely reliable criterion.

Single-strand-specific chemical modification of nucleotides in active or inactive ribosomes (*16–18*), ribonucleoprotein fragments (*19, 20*), or naked 16*S* RNA (*21*) is taken as strong evidence for absence of secondary structure at a given site. In such studies, we have used kethoxal and glyoxal, which are guanine (G)-specific, *m*-chloroperbenzoate, which is adenine (A)-specific, and bisulfite which is cytosine (C)- or uracil (U)-specific. Resistance to modification at a given site is also an important clue, but need not necessarily imply the existence of secondary structure there, since tertiary structure or protein binding could as easily shield the RNA. The reliability of chemical modification as a test of secondary structure is supported by studies with phenylalanyl-transfer RNA from yeast ($tRNA^{Phe}_{yeast}$ (*22*) where there is complete agreement with the structure obtained by x-ray crystallographic analysis.

Nuclease susceptibility, also of value in discerning single-stranded areas, suffers from the potential danger that an initial cut may lead to some unfolding of the RNA structure. This is especially hazardous in molecules such as 16*S* RNA, where the number of potential nuclease cleavage sites is very large. We therefore consider strong nuclease cleavage sites mainly as supportive to the other criteria.

Direct isolation of complexes of RNA fragments may be interpreted as evidence for base pairing, when the respective fragments contain complementary sequences. We have invoked this criterion in establishing features of the structure in the regions involving residues 585 to 750 (*19*) and 1410 to 1490 (*11*). Since completion of our model, Ross and Brimacombe (*23*) have reported several additional examples of RNA-RNA complexes that are presumably base paired.

Finally, and most significantly, we invoke comparative sequence evidence as a powerful criterion for secondary structure. Two of the most firmly established RNA secondary structure models are those of tRNA (*24*) and 5*S* RNA (*25*); both were derived mainly from comparative evidence. In a broad sense, the Watson-Crick model for base pairing of DNA itself was derived originally from comparative evidence (Chargaff's rules) (*26*). Our underlying assumption is that the secondary structures of 16*S* rRNA from different prokaryotes are likely to be very similar. There is as yet little direct support for this idea, but the interchangeability of 16*S* RNA from different bacteria in reconstitution experiments in vitro (*27*) is consistent with our assumption. In any case, mutational alteration of the structure of a major component of the translation apparatus is an event that should not be easily tolerated by an organism, and so we anticipate that base pairing in bacterial rRNA's should be highly conserved. Thus, conservation of a base-paired element in 16*S* rRNA in two organisms in spite of variation in the paired sequences provides a convincing argument for the true existence of a duplex structure at that position.

Description of the Model

Our secondary structure model for the *E. coli* 16*S* rRNA (*14*) is shown in Fig. 1. Overall structural organization appears to be dictated by three sets of long-range interactions, which partition the 16*S* RNA into four rather well-defined domains. The helices 27–37 ∩ 547–556 (this notation means that nucleotide residues 27 through 37 are base paired in the Watson-Crick sense with nucleotides 547 through 556) and 39–44 ∩ 398–403 organize residues 1–556 into a 5′-terminal domain. Helices 564–570 ∩ 880–886 and 576–580 ∩ 761–765 organize the central domain (residues 564–912). The 3′-terminal region is organized by the helix 926–933 ∩ 1384–1391 into a major (residues 926–1391) and a minor domain (residues 1392–1542). The 3′ major domain is further organized by two additional long-range interactions: 946–955 ∩ 1225–1235 and 984–990 ∩ 1215–1221. These latter interactions divide the 3′ major domain approximately into two halves.

The 5′ and 3′ major domains contain regions depicted as large unpaired loops. The available evidence suggests that these regions are at least partially structured, but we do not find base-pairing possibilities within them that can so far be supported by our criteria. There must certainly be tertiary structure in 16*S* RNA, and so these unpaired regions may be mainly involved in non–Watson-Crick interactions. The total fraction of nucleotides involved in base pairing in our model is 46 percent. Although this is significantly below the figure predicted by physical studies (about 60 to 70 percent) (*28*), it compares favorably with the extent of base pairing in the Fox and Woese model for 5*S* RNA (45 percent) (*25*), and is only slightly below that of the cloverleaf model for tRNA (about 50 percent) (*24*).

The structure shown in Fig. 1 has no knots, that is, there are no regions where a single strand of a base-paired region lies between the two strands of another helix (*29*). This may be significant for ribosome assembly, in that knotting would often present the opportunity for formation of two nonequivalent conformers as a result of a single base-pairing event, which could then lead to nonproductive intermediates in ribosome assembly. It could be that the strategy of ribosome assembly avoids possible knots, and that the secondary structures of the rRNA's are organized accordingly. (This appears to be true for both 5*S* RNA and tRNA.)

Another surprising finding is the lack of long, uninterrupted base-paired stems; the longest helical region containing only A·U and G·C pairs is only eight base pairs in length. This is to be contrasted with the ribonuclease III processing stem formed by the precursor sequences flanking mature 16*S* RNA, where 17 contiguous standard base pairs can be made (*30*). This observation may again reflect the need to avoid trapping nonproductive conformers during the folding of rRNA during assembly in vivo; smaller, less stable helices can more easily be disrupted and reformed during attempts to attain the correct final conformation. In addition, this should provide increased structural flexibility.

Besides conventional hairpin loops of the kind present, for example, in tRNA, there are various "compound" helices. As many as five or six short helical regions occur in a single compound helix, separated by interior loops or bulge loops. The majority of the base-paired regions are in fact part of more complex structures of this kind. Many helices contain G·U pairs, sometimes several of these in a single stem (for example, residues 830 to 860). There are four single-base bulge loops at G^{31}, A^{746}, A^{1227}, and A^{1441}. At least two of these (G^{31} and A^{746}) may be involved in ribosomal protein recognition (see below).

We may consider how the 16*S* RNA must be condensed to be packed within the confines of the observed dimensions of a 30*S* ribosomal subunit. The approximate dimensions of a 30*S* particle from low-angle x-ray scattering and electron microscopy are about 55 by 220 by 220 angstroms (*31*) and 80 by 190 by 250 Å (*6, 7*), respectively. Assuming an average internucleotide distance of 3 Å, the dimensions of the structure as drawn in Fig. 1 are about 300 by 450 Å. Thus, the RNA as displayed in Fig. 1 must be further condensed only about twofold in each dimension in order to be contained within the dimensions of a 30*S* subunit.

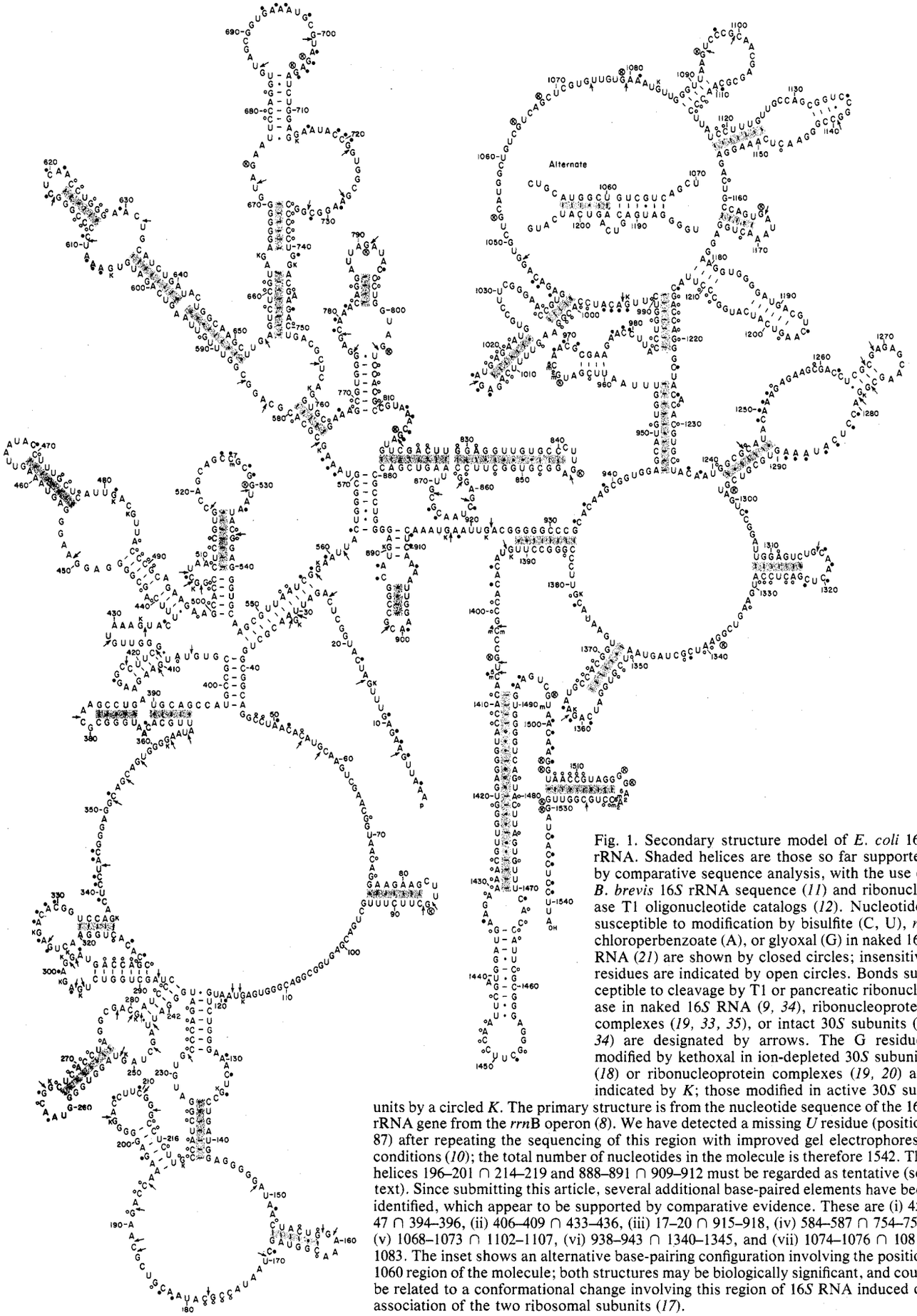

Fig. 1. Secondary structure model of *E. coli* 16*S* rRNA. Shaded helices are those so far supported by comparative sequence analysis, with the use of *B. brevis* 16*S* rRNA sequence (*11*) and ribonuclease T1 oligonucleotide catalogs (*12*). Nucleotides susceptible to modification by bisulfite (C, U), *m*-chloroperbenzoate (A), or glyoxal (G) in naked 16*S* RNA (*21*) are shown by closed circles; insensitive residues are indicated by open circles. Bonds susceptible to cleavage by T1 or pancreatic ribonuclease in naked 16*S* RNA (*9, 34*), ribonucleoprotein complexes (*19, 33, 35*), or intact 30*S* subunits (*9, 34*) are designated by arrows. The G residues modified by kethoxal in ion-depleted 30*S* subunits (*18*) or ribonucleoprotein complexes (*19, 20*) are indicated by *K*; those modified in active 30*S* subunits by a circled *K*. The primary structure is from the nucleotide sequence of the 16*S* rRNA gene from the *rrn*B operon (*8*). We have detected a missing *U* residue (position 87) after repeating the sequencing of this region with improved gel electrophoresis conditions (*10*); the total number of nucleotides in the molecule is therefore 1542. The helices 196–201 ∩ 214–219 and 888–891 ∩ 909–912 must be regarded as tentative (see text). Since submitting this article, several additional base-paired elements have been identified, which appear to be supported by comparative evidence. These are (i) 45–47 ∩ 394–396, (ii) 406–409 ∩ 433–436, (iii) 17–20 ∩ 915–918, (iv) 584–587 ∩ 754–757, (v) 1068–1073 ∩ 1102–1107, (vi) 938–943 ∩ 1340–1345, and (vii) 1074–1076 ∩ 1081–1083. The inset shows an alternative base-pairing configuration involving the position 1060 region of the molecule; both structures may be biologically significant, and could be related to a conformational change involving this region of 16*S* RNA induced on association of the two ribosomal subunits (*17*).

Agreement of the Model with Experimental Evidence

The data used to test the model have been described (*14*) and are summarized in Fig. 1. Shaded helices are those that we consider to have been proved by our comparative sequence evidence. All of the helices in the model that can be tested in this way so far do not violate the comparative criteria. With the availability of 16*S* RNA sequences from other organisms, it should eventually be possible to obtain definitive evidence to test each of them. Sites of kethoxal modification in active ribosomes (*16, 17*), shown by a circled *K* in Fig. 1, are found in single-stranded regions, without exception. Kethoxal-reactive sites (*18*) in ion-depleted inactive 30*S* subunits prepared according to Zamir and co-workers (*32*) are mainly in single-stranded regions of the model, but three sites occur in potential G·U pairs at the ends of helices. Nearly all of the more than 400 sites in free 16*S* RNA that can be monitored for reactivity toward bisulfite, glyoxal, and *m*-chloroperbenzoate (*21*) are in agreement with the proposed secondary structure. Exceptions are at positions G^{198}, C^{556}, C^{910}, C^{912}, C^{1011}, and A^{1012}. The secondary structures involving residues 196–201 ∩ 214–219 and 888–891 ∩ 909–912 must be regarded as tentative for this reason. In the other two cases, independent evidence lends support to the correctness of the proposed structure in spite of some contradictory chemical evidence. Possibly, the conformation of the naked 16*S* RNA differs from that found in the ribosome at these locations. Of the more than 130 sites of nuclease attack (*19, 33–35*) indicated in Fig. 1, only 11 fall in double-stranded regions. In nearly every case, there is strong independent evidence for base pairing at these 11 positions. In summary, there is close agreement between the model and the extensive available evidence with which it can be tested. It should also be kept in mind that there may be more than one biologically significant conformation for 16*S* RNA.

In some cases calculated free energies of potential helices (*36*) lead to incorrect predictions. As an example, in the region between residues 150 and 180, two competing stable hairpins can be drawn (*14*). Both hairpins are in agreement with the known sites of chemical and enzymatic attack in this region of the 16*S* RNA. Suprisingly, the one with the lower predicted free energy is decisively ruled out by comparative evidence (*14*). Another example involves the potential for pairing between sequences 732–738 ∩ 925–931, which has the lowest predicted free energy of all the complementary sequences in *E. coli* 16*S* RNA, −30 kilocalories per mole (25°C); pairing of these sequences is ruled out by comparative evidence (*14*).

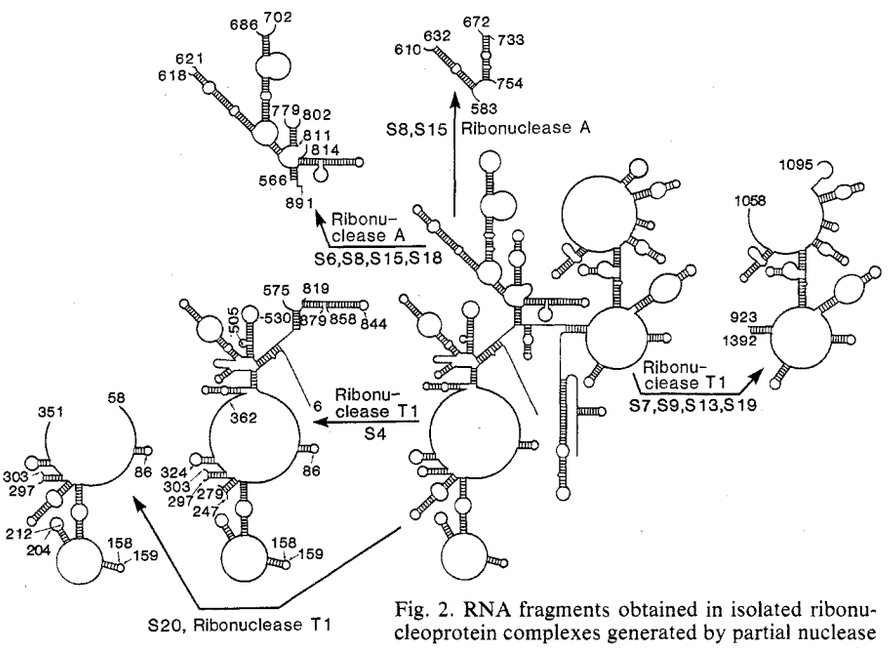

Fig. 2. RNA fragments obtained in isolated ribonucleoprotein complexes generated by partial nuclease digestion of 16*S* rRNA-protein complexes, or 30*S* ribosomal subunits. Representative examples of work (from several laboratories) show that the fragment is bound to the indicated ribosomal protein after ribonuclease T1 digestion of the 16*S* RNA-S4 complex or the 16*S* RNA-S20 complex (*33*), ribonuclease A digestion of the 16*S* RNA-S8, S15 complex (*19*) or the 16*S* RNA-S6, -S8, -S15, -S18 complex (*46*), and ribonuclease T1 digestion of gently unfolded 30*S* ribosomal subunits (generating the S7, S9, S13, S19 complex) (*35*). Nucleotide positions of the fragment ends and hidden breaks are shown.

Patterns of Sequence Conservation

The rRNA genes are among the most conserved in sequence of all genes in the cell (*37*). Ribosomal RNA sequence homology is easily detected among the three major genealogical categories—that is, the true bacteria, the archaebacteria, and the so-called cytoplasmic aspect of the eukaryote (*38*). The existence of such extensive conservation implies strong functional constraint on the rRNA's. In assigning functional significance to the rRNA molecules, the pattern of sequence constraints should provide important clues.

In that T1 ribonuclease oligonucleotide catalogs exist for the 16*S* rRNA's of more than 150 organisms, a considerable amount is known about some of the sequence constraints in the molecule. The main general conclusion is that sequence conservation tends to occur in nonhelical regions, regions that, moreover, tend to be exposed in the isolated RNA (accessible to chemical substitution). In tRNA's, conservation of sequence tends to imply involvement in tertiary structure. Perhaps sequence conservation in the rRNA's then implies quaternary interactions.

On the basis of the extent of phylogenetic lability of sequence the duplexes in 16*S* rRNA appear to exhibit a spectrum of types. In some, sequence is highly conserved or highly constrained; little or no variation from the pattern is seen across the entire kingdom of true bacteria (for example, the 960–963 ∩ 972–975 helix). Several cases exist where variation in sequence exists for a particular helix but only in a constrained way; some of the positions show no variation whatsoever, while others may vary quite often but, for example, only from one pyrimidine to another. These demonstrate the subtlety of the constraints affecting rRNA sequences. A duplex is not simply a collection of bases that pair and have some overall energy requirement; in some instances, at least, actual sequence in the duplex is important. In general, the terminal base pairs of helices are more likely to be conserved in sequence than are interior pairs.

At the other extreme of the spectrum are duplexes that show sequence variation even within a single genus. Examples are the structures in the 585–750, 820–880, and 1410–1490 regions. Such helices have other special characteristics as well; they tend to contain a relatively high proportion of non-normal base pairs (U·G and even A·G); they tend phylogenetically to be somewhat variable in length among different bacteria; and at

least some of them appear to bind protein. Their sequence variability might be accounted for in terms of this last characteristic: if helices are in part stabilized by an external interaction such as protein binding, then the instability that would result from a mutation creating a nonpair or weak pair should be less than otherwise—allowing the organism time to compensate by a subsequent suppressor mutation (that, for example, restored the base pair). Such helices then could drift in sequence more readily than others. Going a step further, one can see that the possible structures into which such a helix could evolve (that is, the structure's feasible evolutionary phase space) is far greater than would otherwise be the case.

Ribosomal Protein Binding Sites

Considerable progress has been made toward the identification of those features of the 16*S* RNA which are recognized or bound by ribosomal proteins (*39*). Most of these studies have involved the proteins that bind to 16*S* RNA independently of the others (*40*). For purposes of comparison with our secondary structure model, we restrict our attention to a few examples in which the RNA moiety has been precisely characterized. Here it will be apparent that the structural domains of 16*S* RNA, so far defined only by constraints created by the proposed secondary structure, are readily isolated as intact structures after partial nuclease digestion of 16*S* RNA-protein complexes. This provides strong direct evidence for the structural organization of 16*S* RNA implicit in our model.

Protein S4 has long been known to bind the 5′ region of 16*S* RNA (*41*). When S4 forms a complex with 16*S* RNA under reconstitution conditions, and digested gently with either ribonuclease T1 or ribonuclease A, it becomes bound to fragments of 16*S* RNA encompassing several hundred nucleotides. Two such fragments have been designated S4 RNA-I and S4 RNA-II (*33, 42*). The S4 RNA-II complex contains all of the sequence from position 6 to position 557, with excisions at 248–278, 298–301, 325–362, and 518–530, and five additional "hidden breaks." The S4 RNA-I complex (Fig. 2) contains S4 RNA-II plus fragments 558–575, 819–858, and 870–887. All of the sites of ribonuclease cleavage are in single-stranded sites. The S4 RNA-II complex includes almost precisely (except for excisions) the 5′ domain of 16*S* RNA. The complex S4 RNA-I contains also the lower part of the central domain, including the long-range interaction 564–570 ∩ 880–886. It is particularly significant that a fragment almost identical to S4 RNA-II can be obtained by digestion of 16*S* RNA with carrier-bound ribonuclease A in the absence of S4 (*33*). This result is a striking demonstration of the structural integrity of the 5′ domain, even in the absence of ribosomal proteins.

Cole *et al.* (*43*) have used electron microscopy of the S4-16*S* RNA complexes to localize the binding site of S4. After denaturation and spreading of fixed complexes, they visualized double-loop structures containing, respectively, 371 ± 45 and 464 ± 40 nucleotides in the small and large loops, and 704 ± 49 nucleotides in the free (presumably 3′) tail. Because of the above-mentioned structural integrity of the S4 region of 16*S* RNA even in the absence of S4, we would argue that one function of S4 is to stabilize features of the RNA structure that exist under optimum ionic conditions in the absence of protein. The two long-range interactions that together organize the 5′ and central domains are likely possibilities. These are 27–37 ∩ 547–556 and 564–570 ∩ 880–886, both of which are included in S4 RNA-I. The predicted sizes of the two loops and free end, respectively, are 329, 555, and 655 nucleotides. The sizes of the small loop and free end are in excellent agreement with the observed electron microscopy structures. The predicted size of the large loop is at least 40 nucleotides larger than the observed size. However, Cole *et al.* noted a discrepancy of about 68 nucleotides in the loop sizes between the average and fully extended loops, which could account for this difference. They attribute this discrepancy to structures that are particularly resistant to denaturation under their spreading conditions. Our interpretation allows the prediction that protein S4 binds to and stabilizes the two long-range helices at 27–37 ∩ 547–556 and 564–570 ∩ 880–886. The smaller of the two loops visualized by Cole *et al.* would thus correspond to the S4-stabilized long-range interaction observed by Ehresmann *et al.* (*33, 42*), which we attribute to the 564–570 ∩ 880–886 helix. The way in which these two helices appear to organize the 5′ and central domains is consistent with the well-documented pivotal role of protein S4 in ribosome assembly (*40*).

The RNA fragment protected by protein S20 (*33*) is wholly contained in the 5′ domain (Fig. 2). It partially overlaps that of S4 but, most obviously, contains in addition the stem around positions 248–278 which is excised in S4 RNA's. The likelihood that this stem is involved in binding of S20 is supported by ultraviolet-induced cross-linking of S20 to this region of 16*S* RNA (*44*).

Proteins S8 and S15 protect relatively small regions of the central domain (Fig. 2). Protein S8 binds to a site formed within the sequences 583–610 ∩ 632–653, and S15 binds to 654–672 ∩ 733–756 (*19, 45*). Kethoxal-reactive guanines at positions 664, 733, 734, and 742 are strongly protected by binding of S15 (*19*), implying that these single-stranded sequences flanking helical regions form part of the recognition site for protein S15. In electron microscopic studies of protein S8-16*S* RNA complexes, Cole *et al.* (*43*) visualize an S8-dependent hairpin structure which (their calculation) should contain 65 nucleotides. This is in remarkable agreement with the size of the S8-stabilized stem predicted from the secondary structure in conjunction with S8 binding-site sequence data. These studies (*19, 45*) predict that S8 should sequester the nucleotides between positions 587 and 652 into a modified hairpin structure containing precisely 65 nucleotides.

When proteins S6 and S18 are bound along with S8 and S15, additional features of the central domain are protected (Fig. 2) (*46*). Almost the entire central domain is contained in the complex, in spite of the excision of four small sections. This observation provides evidence for the existence of the central domain as a self-contained structural unit of 16*S* RNA.

Brimacombe and co-workers have isolated a ribonucleoprotein fragment by ribonuclease T1 digestion of mildly unfolded 30*S* subunits, and have extensively characterized the RNA and protein components of the complex (*35, 47*). It contains stoichiometric amounts of proteins S7, S9, S13, and S19, along with RNA fragments deriving exclusively from the 3′ major domain (Fig. 2). The two main RNA fragments found in the complex together comprise the entire 3′ major domain, except for the excision of residues 1059 to 1094. Ribonucleoproteins containing RNA from the 3′ major domain have also been found in nuclease protection experiments using proteins S7 (*39*) and S13 plus S19 (*48*). Brimacombe and co-workers have localized the site of binding of S7 to this region by ultraviolet-induced cross-linking of Met^{112} of this protein to U^{1240} of 16*S* RNA (*49*). In contrast, Ehresmann *et al.* (*50*), using a similar approach find that S7 is cross-linked instead to C^{1265}. In any case, isolation of a well-defined complex containing precisely the 3′ major domain (with one excision) provides good argu-

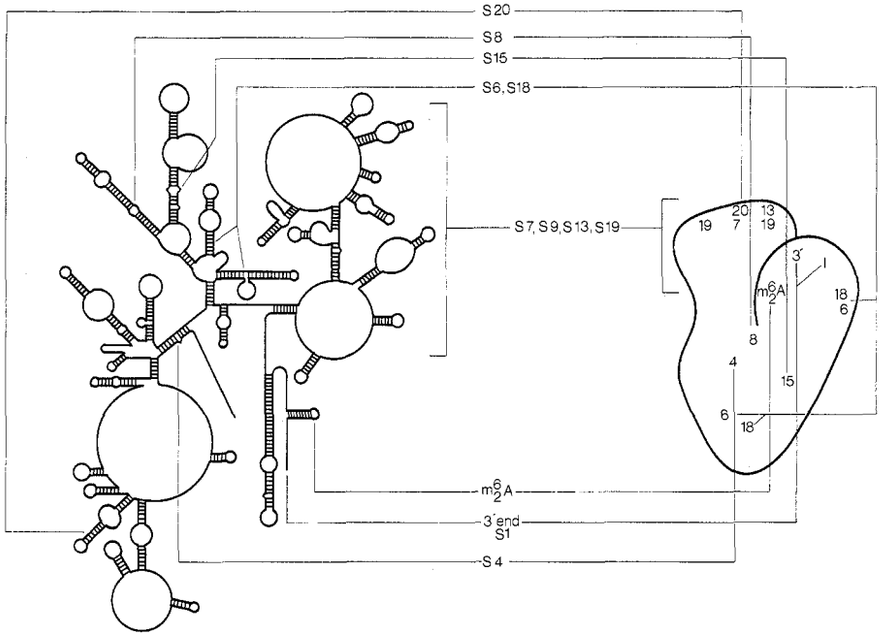

Fig. 3. Tentative location of secondary structure features of 16*S* rRNA in one of the electron microscope models for the 30*S* ribosomal subunit (*7*). Positions of ribosomal proteins, indicated by number, are our estimate of the "consensus" locations based on immuno electron microscopy (*6, 7*) and neutron diffraction (*4*) results. Positions of the dimethyladenosine (m^6_2A) residues and the 3′ terminus are also from immuno electron microscopy studies (*55–57*). Protein binding sites in the 16*S* RNA are from studies described in Fig. 2.

ment for the structural integrity of this region of 16*S* RNA.

Nuclease protection patterns revealed by these protein-binding studies agree extremely well with the secondary structure scheme. Studies with protein S4 (*51*) in particular suggest that the fragments produced are perhaps more a reflection of the RNA structure itself than of protein binding, although the proteins clearly help to stabilize the RNA structure.

We may now begin to search for features of rRNA structure that account for site-specific recognition by ribosomal proteins. So far, only three protein binding regions have been localized to RNA fragments small enough to be considered as actual protein-RNA contact regions. The S8 site has been localized by nuclease protection studies (*19, 45*), the site for L18 by protection of 5S rRNA from chemical modification (*52*), and the S15 site by both approaches (*19*). Among the structural features common to the three binding sites are well-established helical elements. [In 5*S* rRNA the 18–23 ∩ 60–65 helix is likely to make contact with L18 (*52*).] The helices are, however, irregular, and contain G·U pairs (S8 and L18) or single base bulges (S15) and interior loops (all three proteins). Bases in the interior loops are protected from kethoxal attack by proteins S15 and L18. In the case of L18, modification of these bases has been correlated with loss of protein binding (*52*).

As was noted above, comparative analysis shows that nucleotide sequences in these protein binding sites are phylogenetically variable. Structural irregularities such as the positions of single base bulges and G·U pairs appear to vary also.

Tracing the Path of the 16*S* RNA Chain in the Ribosome

Several groups of investigators have attempted to discern structural features of the 30*S* ribosomal subunit by electron microscopy (*6, 7, 53, 54*). There is, however, some disagreement among investigators about the detailed appearance of the 30*S* subunit, owing to lack of resolution as well as differing interpretations of the various views of the particle. One class of model, typified by that of Lake (*7*), represents the 30*S* subunit as in Fig. 3 with approximate dimensions 80 by 190 by 250 Å, in which the mass is partitioned between a "head," "body," and "platform." Other groups offer models with less prominent platforms (*53, 54*) or symmetric models with small platforms on either side (*6*). These differences in interpretation do not seriously affect our discussion.

Using antibodies to N^6-dimethyladenosine combined with electron microscopy, Politz and Glitz (*55*) have located one region of the 16*S* RNA, the 3′ minor domain. They located the dimethyladenosine sequence (positions 1518 and 1519) in the platform region of the electron microscopy model (Fig. 3). This assignment has been confirmed by use of antibodies to haptens covalently linked to the 3′ terminus of 16*S* RNA (*6, 56, 57*).

Further clues to the location of the 16*S* RNA chain can be deduced indirectly from the positions of proteins whose RNA binding sites are known. With the use of antibodies to specific ribosomal proteins, the approximate location of their antigenic determinants has been localized by electron microscopy (*6, 7*). Positions of many of the 30*S* subunit proteins have also been determined by neutron scattering (*4*). There is substantial agreement between the results of these two approaches; furthermore, they are supported by data obtained from singlet-singlet energy transfer (*5*), and protein-protein cross-linking (*3*). A "consensus" placement, based on our interpretation of available data, of the proteins relevant to our discussion is shown in Fig. 3. Widely spaced multiple determinants reported for some proteins have been omitted in most cases because of the present controversy over their authenticity [see (*58*)].

Figure 3 summarizes our knowledge of the location of specific structural features of 16*S* RNA in the electron microscope model of the 30*S* ribosomal subunit. Some general conclusions can be made. The 3′ major domain, known to bind proteins S7, S9, S13, and S19, can almost certainly be assigned to the "head" of the model. Features very near the 3′ terminus can be placed in the "platform." Placement of the central domain is less certain, mainly because of the ambiguity concerning the location of proteins S6 and S18. The 5′ domain binds proteins found in the central and "head" regions of the model. These considerations show that a rough picture of the packing of 16*S* RNA into the ribosome is beginning to emerge.

Comparison with Other Studies on 16*S* RNA Secondary Structure

Since completion of this work, the results of three other studies on 16*S* RNA secondary structure have become available. Ross and Brimacombe (*23*) have used two-dimensional gel electrophoresis, in combination with S1 nuclease digestion, to identify regions of the RNA that interact with each other. Their data are consistent with our model, and so offer independent support for our

base-pairing scheme. However, they present a number of helices not shown in Fig. 1, some of which conflict with available comparative evidence. Schwarz and Kössel (*13*), on the basis of comparative sequence analysis of *Zea mays* chloroplast 16*S* rRNA and that of *E. coli*, have also proposed a number of helical elements. Again, some of their proposed structures differ from ours (but these are not supported by comparative evidence). Also the chloroplast sequence provides comparative evidence for several of the helices we have proposed but were not able to prove on the basis of our data alone (for example the 769–775 ∩ 804–810 helix).

Wollenzien *et al.* (*59*) have used photoactivated cross-linking in the presence of hydroxymethyltrimethylpsoralen to study the folding of 16*S* RNA. This reagent is known to intercalate into double-helical regions of nucleic acids and there promote photochemically induced cross-linking of the base-paired strands (*60*). Molecules cross-linked either in the free state or in 30*S* subunits were examined by electron microscopy and found to contain covalently closed loops. Eleven classes of loops that occurred in high frequency (2 to 30 percent) were identified, and their respective sites of cross-linking were estimated. Surprisingly, none of their observed interactions appear to correspond directly to base-paired features of our model. We do not at present understand the basis of this disagreement; its resolution awaits more information on the nature of psoralen cross-links in complex RNA molecules. An obvious first step would be to chemically identify the sites of cross-linking to eliminate possible sources of uncertainty attributable to electron microscopy.

Functional Importance of 16*S* RNA

There is increasing evidence that the 3′ terminal region plays an important role in the selection of translational initiation sites in messenger RNA (mRNA) (*61*). This region of the molecule, included in the 3′ minor domain, has been placed on the platform or in the cleft of the 30*S* subunit, and it is possible that the cleft itself forms a site for mRNA binding and codon-anticodon recognition (*6, 7*). The two unpaired regions of the 3′ minor domain, flanking the large penultimate helix, have very highly conserved sequences. There is strong evidence for their involvement in the binding of 50*S* subunits (*17, 62, 63*). The extensive sequence conservation suggests that base pairing with 23*S* RNA may be involved (see also below).

The 5′ nucleotide of the anticodons of some tRNA's can be covalently cross-linked with high efficiency to 16*S* RNA by ultraviolet irradiation when the tRNA occupies the ribosomal P site (*64*). The region of attachment to 16*S* RNA has been localized to positions 1393 to 1497 (*65*). As noted above, the sequence 1390 to 1410 is very highly conserved, and a likely candidate for such a critical and universal function. Thus, the codon-anticodon interaction site can be placed near the junction of the 3′ major and 3′ minor domains, which we predict corresponds to the junction of the head and platform regions of the electron miroscope model, that is, possibly the cleft between them. This is in keeping with the location of ribosomal proteins which are most strongly implicated in the tRNA binding function (that is, S2, S3, S10, S14, S18, S19, S21) (*66*) in the region surrounding the cleft, as well as placement of the mRNA binding site in the cleft (*6, 7*).

Association of the 30*S* and 50*S* subunits has been shown to involve 16*S* RNA in experiments with limited nuclease digestion or chemical modification. Sites in the central, 3′ major, and 3′ minor domains have been shown to be strongly protected from nucleases (*63*) and from kethoxal modification (*17*) by binding of 50*S* subunits. In another study, these kethoxal-modified 30*S* subunits that retained competence in subunit association out of a population of partially inactivated subunits, were found not to be modified in these same positions (*62*). The critical guanine residues in these sites include positions 674, 703, 705, 791, 803, 818, 1166, 1405, 1497, 1505, 1516, and 1517. Sites in the central 3′ major and 3′ minor domains of 16*S* RNA all appear to be strongly implicated in ribosomal function. Thus far, they are found in single-stranded regions and show strong phylogenetic conservation.

Evolution

Translation is as much an evolutionary problem as it is a mechanistic one. The translation apparatus is sufficiently complex that it obviously did not evolve in a single step. Its accuracy, if not its essence, is the product of evolution. Therefore, the design of the translation apparatus is imprinted with and reflects this evolution, and to understand one is to understand the other. (The same could be said of computers, cars, and castles.)

We do not understand the mechanics of translation as yet; so we cannot say much in detail about its evolution. It may be useful, however, to point out those characteristics that are emerging. The extreme conservation of its sequence and (more so) secondary structure are indicators of the importance of rRNA to translation. The highly specific, strong constraints on the ways in which rRNA sequence can change, seen in the 16*S* rRNA oligonucleotide catalogs (*12*), are an even more convincing indicator of rRNA's importance. It is virtually impossible that rRNA is merely a structural element (positioning the proteins); rRNA's must be function-defining in the translation process.

A question that must now be asked is the extent to which ribosome function is defined by its RNA as opposed to its protein components. (A distinction should be made between *defining* a particular function and *facilitating* it—that is, making it precise, rapid, and so on.) Reconstitution experiments show that many ribosomal proteins can be eliminated without destroying translation (*67*); these cannot be function-defining. So-called nonenzymatic translocation occurs in the absence of guanosine triphosphate (GTP), initiation and elongation factors, and ribosomal protein L7-L12 (*68*). It may be that all ribosomal proteins have facilitating rather than function-defining roles in translation, and therefore the translation function is defined primarily by its (ribosomal and transfer) RNA components. This is an attractive view for it avoids the chicken-and-egg paradox implied in assuming proteins to be function-defining in the aboriginal translation mechanism.

We have seen above what could be a new role for ribosomal protein—facilitating the evolution of various structural elements in rRNA. In other words, when ribosomal proteins are associated with RNA structural elements, they can explore a host of evolutionary possibilities that are otherwise not feasibly explored. Perhaps a major function of some ribosomal proteins has been this evolutionary one.

In the conventional view, the ribosome is considered a mechanism; it is the source of the movement in translation. By the argument developed here, these dynamics could reside in the RNA components. In the absence of information on 23*S* rRNA and thus on 16*S* to 23*S* rRNA interaction, it is probably not useful to attempt to discuss the actual mechanism. The general principles involved are another matter. Movement is inherent in the coiling and uncoiling of nucleic acid duplexes. A more subtle form of

movement can be envisioned in terms of configurational shifts, and make-break interactions, involving the loops defined by the double-helical elements. The grosser type of movement (coiling and uncoiling) should be detectable through the proper chemical modification studies, comparative studies, and so on. So far, we have found little convincing evidence for such interactions. Some residues that are protected against chemical modification are only partly so, but this more likely reflects a partial denaturation of the RNA during preparation than a functional unfolding. There is, so far, no convincing evidence from comparative studies for any mutually exclusive duplexes in 16*S* rRNA; "switches" of this sort would be a strong indicator of conformational change.

The extent to which the evolution of translation can be approached by comparative study will be determined by the nature of the apparatus possessed by the (most recent) ancestor common to all living forms. Microfossil evidence combined with molecular phylogenetic measurements indicate that true bacteria existed perhaps 3½ billion years ago (*12, 69*). The universal common ancestor then existed some time during the preceding 1 billion years. Although the latter time interval is (perhaps very) short compared to the former, the amount of sequence difference between, for example, true bacterial and archaebacterial 16*S* rRNA's is remarkably large (*12*). It is as though the pace of evolution was somehow faster at times before the appearance of three major lines of descent than after their appearance. This concept is also inherent in the fact that posttranscriptionally modified bases in rRNA's and tRNA's, although they tend to be conserved within a given major line of descent, tend to vary in passing from one major line of descent to another. For example, tRNA's of archaebacteria contain no ribothymidine in the so-called TψC (T, thymine; ψ, pseudouracil; C, cytosine) loop (*70*). This indicates that the modifications may not all have been present in the translation apparatus of the common ancestor. In that these modifications probably represent an evolutionary "fine tuning" of the translation mechanism, it could be argued that the mechanism possessed by the universal common ancestor was more rudimentary than the extant versions thereof. In other words, that ancestor was not a prokaryote; it was a less complex form that has been termed a progenote (*38, 71*). If so, a comparative approach to the ribosome could yield a good deal of information about the mechanism's evolution.

Conclusions

Combined use of comparative sequence analysis, chemical modification, and enzymatic data has allowed us to construct a model for the secondary structure of 16*S* rRNA. The usefulness of this model for interpretation of structural and functional studies is already apparent. Several groups have isolated RNA fragments closely corresponding to structural domains predicted by the secondary structure model. Comparison of protein-binding site locations in the 16*S* RNA with the positions of their antigenic determinants in the electron microscope model of the 30*S* subunit begins to suggest how the RNA might be arranged in the ribosome. Finally, the comparative approach has revealed not only nucleotide sequences but structural features that are conserved in ribosomes from widely divergent species, and so are very likely to be essential for ribosome function. Most, if not all conserved sequences occur in unpaired regions and have been shown to be accessible in the ribosome. Many have already been implicated in specific functional roles.

Future experiments on 16*S* RNA will need to address yet more difficult aspects of ribosome biochemistry including the tertiary folding of the 16*S* RNA, the precise contacts between ribosomal proteins and 16*S* RNA, the nature of the interaction between 30*S* and 50*S* ribosomal subunits, the way in which this is related to ribosomal function, and the way in which ribosomal proteins affect rRNA structure and function.

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Indirect Costs of Federally Supported Research

Kenneth T. Brown

Summary. Indirect costs of federally supported research have increased steadily and dramatically since the current indirect cost policy became effective in 1966. The amount of research supported by any given level of federal funding has thus been markedly reduced, and this has become a critical factor limiting research support in the United States. The current policy has had multiple adverse effects that threaten the health of both the federal research program and the universities in which most of the research is conducted. This article examines the background and nature of the current policy, describes its consequences, and proposes simplifying modifications.

In federally supported research a distinction is drawn between direct costs, which cover the expenses of a specific research project, and indirect costs, which are allocated to research organizations to defray research expenses broadly described as overhead. This article concerns indirect costs at U.S. universities and colleges, which will be lumped for convenience under the term universities. As I will document in this article, indirect costs have increased steadily and markedly since 1966, when the current indirect cost policy became effective. This is the case even when indirect costs are expressed as a percentage of total research costs. The amount of research that can be supported with any total level of federal funding has thus been seriously reduced, and this has become a major factor limiting the support of research in this country.

The importance of this matter is incalculable but may be assessed partly by considering the groups affected. Of course, U.S. taxpayers must pay the higher indirect costs. Also affected are all beneficiaries of research, who constitute an even larger group that is worldwide in distribution, especially in research fields such as human health. But three groups are affected with special force in their daily work. One is the federal granting agencies, who have the duty of supporting as much high-quality research as they can. Another is administrators of universities where the research is performed, for whom a major concern must be the fiscal soundness of their institutions. A third group is the scientists who conduct the research, and who often depend largely on federal funds to pursue the research careers for which they have been trained. All three of these groups may be expected to favor the optimal support of research. But that goal is being compromised by the high indirect costs that now pertain at many universities, whose administrators are thus placed at odds with both the granting agencies and their own research faculties.

This situation cries out for all parties to pull together toward common goals. Research should be optimally supported, with smooth working relations between all of the main groups involved, and without imposing unnecessary financial or administrative burdens on the universities. Attainment of these goals requires that all parties have a clear and shared understanding of the problem. This is not currently the case, partly because many relevant facts are not readily available, and partly because partisan viewpoints have obscured some of the issues. Thus in the first part of this article I will describe the history of indirect costs, including a budgetary analysis of the extramural research program of the National Institutes of Health (NIH); this is one of the largest federally funded research programs and the one for which I have the best information (*1*). I will next attempt to identify the most critical aspects of this issue and the major problems that have arisen under the current indirect cost policy. Finally, I will propose modifications of the policy that I believe to be in the best interest of all parties, and suggest steps to expedite the desired modifications.

Background

The early history. Unless otherwise indicated, all indirect cost rates in this article have been calculated by expressing indirect costs as a percentage of total direct costs. Before 1955, an indirect cost rate of 8 percent was applied uniformly to NIH research grants at all educational institutions (*2*). From 1955 to 1963 the indirect cost rate became 15 percent; from 1963 to 1966 it was 20 percent of allowable direct costs, which amounted to about 16 percent of total direct costs (*3*). In 1966, apparently in response to strong representations by universities to the Bureau of the Budget, which is now the Office of Management

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