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Do ribosomal RNAs act merely as scaffold for ribosomal proteins?

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Abstract. Investigations that are being carried out in various laboratories including ours clearly provide the answer which is in the negative. Only the direct evidences obtained in this laboratory will be presented and discussed. It has been unequivocally shown that the interaction between 16S and 23S RNAs plays the primary role in the association of ribosomal subunits. Further, 23S RNA is responsible for the binding of 5S RNA to 16S.23S RNA complex with the help of three ribosomal proteins, L5, L18, L15/L25. The 16S.23S RNA complex is also capable of carrying out the following ribosomal functions, although to small but significant extents, with the help of a very limited number of ribosomal proteins and the factors involved in protein synthesis: (a) poly U-binding, (b) poly U-dependent binding of phenylalanyl tRNA, (c) EF-G-dependent GTPase activity, (d) initiation complex formation, (e) peptidyl transferase activity (puromycin reaction) and (f) polyphenylalanine synthesis. These results clearly indicate the direct involvement of rRNAs in the various steps of protein synthesis. Very recently it has been demonstrated that the conformational change of 23S RNA is responsible for the translocation of peptidyl tRNA from the aminoacyl (A) site to the peptidyl (P) site. A model has been proposed for translocation on the basis of direct experimental evidences. The new concept that ribosomal RNAs are the functional components in ribosomes and proteins act as control switches may eventually turn out to be noncontroversial.

Keywords. Ribosomes; rRNAs; protein synthesis.

In vitro protein synthesising system was developed initially by Zamecnik (for historical review see Zamecnik, 1969) with the microsomal preparations from rat liver, absolutely without any knowledge about the protein synthesising machinery. With the identify-cation of the ribonucleoprotein particles of microsomes (shortened as 'ribosomes' by Roberts, 1958) the so-called platform for protein synthesis was recognised. Ribosomes remained as 'platforms' for quite sometime till various groups of workers peeped into their structure in order to identify the numerous proteins that are present therein. After intensive studies those were unequivocally identified and up till now 53 proteins are known to be present in *Escherichia coli* and 80 or so in rat liver ribosomes (Wool, 1979; Wittmann, 1982). Due to the lesser number of proteins present in *E. coli* ribosomes and also various other reasons emphasis was initially laid on their proteins. Gradually various approaches were undertaken to understand the functions of the

Abbreviations used: GTP, Guanosine-5'-triphosphate; $GMPPCH_2P$, 5'-guanylyl methylene diphosphate; GMPPNHP, 5'-guanylyl imido diphosphate; IAEDANS, 5'-(iodoacetamidoethyl)-amino naphthalene-1-sulfonic acid.

proteins individually as well as in state of combination in which they occur in ribosomes. The fact slowly started to emerge that they are not inert structural proteins but functionally quite active. The exact functions of many of these in various steps of protein synthesis seemed to be well-understood and attempt is still being made, however, with limited success, to understand to structure-function relationship in ribosomal proteins (Noller and Lake, 1984). The existence of three RNA molecules (23S, 16S and 5S) in *E. coli* 70S ribosomes was established quite sometime ago, their base sequences have been known and their secondary structures built up by base pairing, and specially with the idea that the features of the secondary and tertiary structures will be conserved in the various organisms from the evolutionary point of view. Consensus models have thus been developed. Direct approaches with the help of crosslinking reagents and enzymic digestion have also been undertaken to establish the secondary and tertiary structures of rRNAs. The final answer would be X-ray crystallography but so far all attempts to crystallise even a small rRNA like 5S RNA have failed (Abdel-Meguid *et al*., 1983). Therefore this final approach has to wait for sometime.

The most basic question that is being raised in this paper is whether ribosomal RNAs act merely as scaffold for protein synthesis. So much effort was made in the past to study the structure and function of ribosomal proteins that very little attention had been paid to the ribosomal RNAs. Comparatively recently more attention is being focussed as more and more evidences are being obtained in various laboratories to indicate that ribosomal RNAs may be directly involved in several steps of protein synthesis. This has been reviewed by Burma (1984a). Some of these are: (i) recognition of mRNA by the 3'-end of 16S RNA, (ii) binding of tRNA, (iii) binding of 5S RNA by 23S RNA through proteins, (iv) antibiotic sensitivity and resistance, (v) binding of initiation factors, at least IF3, (vi) binding of EF-G and (vii) subunit association.

Very recently strong evidences have been obtained in this laboratory to indicate the deep involvement of rRNAs in various stages of protein synthesis. These studies were initiated with the demonstration that 16S and 23S RNAs form a bimolecular complex under two well-defined conditions (Burma *et al*., 1983; Burma, 1983; Nag and Burma, 1982; 1983b). Initiation factor IF 3 was found to partially dissociate such complex (Nag *et al*., 1983). 5S RNA could be incorporated into such complex in stoichiometric amount with the help of three ribosomal proteins, L5, L18, L15/L125 (Tewari and Burma, 1983). The various steps of protein synthesis (binding of poly U, phenylalanyl tRNA, N-acetyl phenylalanyl tRNA, EF-G-dependent GTPase activity, peptidyl transferase activity or puromycin reaction and polyphenylalanine synthesis) could be demonstrated with the 16S.23S RNA complex with the help of limited number of ribosomal proteins and requisite factors for protein synthesis (Burma *et al*., 1985a). Very recently it has also been shown that 23S RNAs in tight and loose couple 70S ribosomes have different conformations, and the conformational change of 23S RNA is responsible for translocation in protein synthesis (Burma *et al*., 1984, 1985b). A few examples will be chosen from our numerous data to show that rRNAs do not merely act as scaffold for ribosomal proteins but are directly involved in protein synthesis.

Ribosomal subunits undergo association and dissociation during protein synthesis and the initiation factor IF3 is known to regulate this process under in vivo condition

(review by Grunberg-Manago, 1980). Under *in vitro* condition, however, Mg^{2+} concentration along with the salt concentration can be utilised for the purpose. At low Mg^{2+} concentration (< 1 mM) the two dissociate while at high Mg^{2+} concentrations (4 mM or above) they associate. Since ribosomes are constituted of both RNAs and proteins there are various possibilities of interactions between ribosomal subunits, for example, RNA–RNA, protein-protein, RNA–protein and combinations of all the three. Although several workers indicated that RNA–RNA interaction may play primary role in the association this laboratory demonstrated unequivocally for the first time that 16S and 23S RNAs form specific bimolecular complex under two well-defined conditions (Nag and Burma, 1982; Burma, 1983; Burma *et al.,* 1983). Light scattering has been a very valuable tool in many of our studies (Nag and Burma, 1983a). The technique is not capable of yielding detailed information as X-ray scattering does. Still it has proved to be very useful in various types of investigations. We would have perhaps never been able to detect the bimolecular complex formation between 16S and 23S RNAs until and unless we were interested in using the tool in the studies of ribosomal subunit association with the help of an ordinary Aminco Bowman spectrofluorometer. Not only that we could demonstrate the complex formation but very quickly worked out the optimum condition of association by light scattering measurements. Eventually, however, we could use other methods like density gradient centrifugation, Polyacrylamide gel electrophoresis, nitrocellulose filter binding (Nag *et al.,* 1985) etc, for the same purpose. The use of light scattering led us to discover that tight couple 50S ribosomes behave quite differently from loose couple ones due to the difference(s) in the conformations of 23S RNAs in these.

In order to demonstrate that RNA–RNA interaction plays a major role in subunit association we thought of modifying the guanine bases of RNAs with kethoxal as had been done by Noller and his coworkers (Chapman and Noller, 1977; Herr and Noller, 1979; Herr *et al.,* 1979) to find out whether RNA–RNA interaction plays the major role in subunit association. The preliminary results obtained in this laboratory are shown in figure 1. Kethoxal-treated 30S subunits fail to associate even at high Mg^{2+} concentration (10 mM or so) as already demonstrated by Noller (Chapman and Noller, 1977). Kethoxal-treated 50S subunits, however, partially associate. This was not in agreement with the results of Noller (Herr and Noller, 1979) who observed complete loss of association capacity of 50S ribosomes on treatment with kethoxal. It took quite sometime to realise that this difference is due to the fact that we had used a mixture of tight and loose couple 50S ribosomes in our experiments, whereas Noller had used only tight couple ones. Tight couple ribosomes associate at low Mg^{2+} concentrations (4 mm or so) whereas loose couple ones require higher Mg^{2+} concentrations for association. Tight couple 50S ribosomes lost their association capacity on treatment with kethoxal whereas the association capacity of loose couple 50S ribosomes was not atall affected by such treatment (Burma *et al.,* 1984). Subsequently it has been shown that the interconversion of tight and loose couple 50S ribosomes can be effected by the translocating agents, EF-G and GTP (Burma *et al.,* 1985b).

Till now loose couple ribosomes are thought to be the damaged ones for the obvious reason that they are biologically much less active than the tight couples. It should be mentioned here that all attempts to detect any difference in the constituents of tight and loose couple ribosomes have so far failed (Van Diggelen *et al.,* 1973). The first

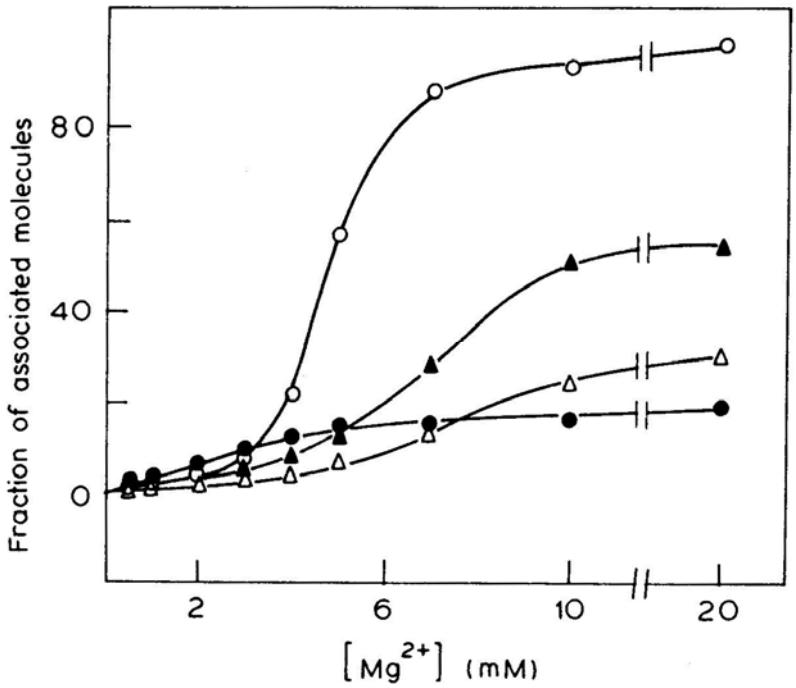

Figure 1. Demonstration of the existence of two populations of 50S ribosomes, by light scattering. The 50S and 30S ribosomes were individually treated with kethoxal in triethanol-amine buffer (in stead of cacodylate buffer) as described by Noller (1974). After treatment the ribosomes were precipitated with ethanol, dissolved in TMA (20 mM Tris-HCl, pH 7·6, 30 mM NH_4CI) buffer containing 10 mM magnesium acetate, 6 mM β-mercaptoethanol and dialysed against the same. In all cases 1·9 A_{260} units of 50S and 1 A_{260} unit of 30S ribosomes in 1 ml were used for light scattering measurements at different Mg^{2+} concentrations, as indicated. The scattering was measured at 90° at 400 nm in an Aminco Bowman Spectrofluorometer. Untreated 30S and 50S (O). Treated 30S and treated 50S (●). Untreated 30S and treated 50S (▲). Treated 30S and untreated 50S (Δ).

demonstration in this laboratory that loose couple 50S ribosomes can be converted to the tight couple ones and the converted populations are as active as tight couple 50S ribosomes clearly showed that the loose couple 50S ribosomes can not be the damaged ones and must have some important biological functions to play in translocation during protein synthesis. But one of the most important observations made during these studies is that the conformations of 23S RNAs in tight and loose couple 50S ribosomes are somewhat different.

Although detailed results have been published or are in the process of publication elsewhere (Burma *et al.,* 1984; 1985a, b) it may not be superfluous to summarise all the data so far obtained. As expected, tight couple 50S ribosomes associate at 4 mM Mg^{2+}, whereas loose couple ones need higher concentration (at least 10 mM) of Mg^{2+} to associate. It is also well established that loose couple 50S ribosomes are much less

biologically active than the tight couple ones and are thus thought to be damaged. But as mentioned already, the dramatic difference between the two is displayed on treatment with kethoxal. Tight couple 50S ribosomes completely lose their association capacity (even at 10 mM Mg^{2+}) on treatment with kethoxal, whereas loose couple ones are not at all affected on such treatment, so far as their association capacity is concerned. Another dramatic difference is the sensitivity to RNase I. Loose couple 50S ribosomes are quite resistant to RNase I whereas tight couple 50S ribosomes are highly susceptible.

Similar differences are also reflected at the ribosomal RNA level. The Mg^{2+} dependencies of the association capacities (with 16S RNA under reconstitution condition) of the isolated 23S RNAs from tight and loose couple 50S ribosomes (Burma *et al.,* 1984) are somewhat different. Although both of them fully associate at 20 mM Mg^{2+}, the association capacities are different at lower Mg^{2+} concentrations. For example, at 4 mM Mg^{2+} the association capacity of loose couple 23S RNA is practically negligible, whereas that of tight couple 23S RNA is about 40 %. Kethoxal treatment affects loose and tight couple 23S RNAs in the same way as the loose and tight couple 50S ribosomes. Tight couple 23S RNA completely loses its association capacity on such treatment whereas loose couple 23S RNA remains unaffected. This clearly indicates that the conformations of the two types of 23S RNAs in 50S ribosomes are quite different. The physical properties of isolated 23S RNAs are also quite different. For example, the binding sites of ethidium bromide to loose couple 23S RNA are more than tight couple 23S RNA. On thermal melting of equivalent A_{260} units of loose and tight couple 23S RNAs the former shows more hyperchromicity than the latter. The circular dichroic measurements also indicate that the former has somewhat more ellipticity than the latter. These data as well as the action of RNase I on loose and tight couple 50S ribosomes clearly indicate that loose couple 23S RNA has somewhat more ordered structure than tight couple 23S RNA. As already mentioned, the 16S.23S RNA complex is capable of mimicking the biological activities of the intact ribosomes (although very weakly) following the addition of requisite factors and a limited number of ribosomal proteins (Burma *et al.,* 1985a). Therefore it was of interest to compare the biological activities of 16S RNA in conjunction with 23S RNAs isolated from tight and loose couple 50S ribosomes. It is evident from the results presented in table 1 that tight couple 23S RNA (in association with 16S RNA) displays double as much activities in comparison to loose couple 23S RNA, except in case of EF-G-dependent GTPase activity. Thus the biological activities of tight and loose couple 50S ribosomes are reflected in the isolated 23S RNAs as well.

One of the most intriguing observations made quite early by Pestka (1969) and Gavrilova and Spirin (1971) is the nonenzymatic translocation as well as nonenzymatic protein synthesis. These processes are extremely slow in comparison to the enzymatic ones but the basic question arises whether the nonenzymatic processes reflect in any way the mechanism of protein biosynthesis? In the recent paper Bergemann and Nierhaus (1983) have argued that on binding of aminoacyl tRNA to the A site a significant portion of the ribosomes perform a complete round of the elongation cycle without the addition of elongation factor EF-G. We have recently observed that the conversion of tight couple to loose couple ribosomes which we believe represents

Table 1. Biological activities of 23S RNAs isolated from tight couple and loose couple 70S ribosomes.

Steps of protein synthesis	Types of 23S RNA	
	Tight couple	Loose couple
Binding of phenylalanyl tRNA (mol/mol of 16S.23S RNA complex)	0·018	0·009
Initiation complex formation (mol of N-acetylphenylalanyl tRNA bound per mol of 16S.23S RNA complex)	0·016	0·006
EF-G dependent GTPase activity (mol of GTP hydrolysed by one mol of 16S.23S RNA complex)	0·65	0·63
Peptidyl transferase activity (mol of phenylalanyl puromycin formed/mol 16S.23S RNA complex)	0·005	0·0025
Polyphenylalanine synthesis (mol of phenylalanine incorporated/mol of 16S.23S RNA complex)	0·025	0·016

The assay methods have been described earlier (Burma *et al.,* 1985a)

translocation, may be affected even in the absence of EF-G, the translocation factor. On addition of poly U, EF-T, guanosine-5'-triphosphate (GTP) and phenylalanyl tRNA to the tight couple 50S ribosomes about 70% of the tight couples are converted to loose couple population as determined by the density gradient centrifugation at 4mM Mg^{2+}. Phenylalanyl tRNA was distributed proportionately to the two populations, as determined by membrane filter binding assay as well as sucrose gradient centrifugation in the presence of 4 mM as well as 10 mM Mg^{2+} (table 2). A small amount of conversion (about 10%) is observed in the absence of phenylalanyl tRNA but its significance is not known. If the GTP hydrolysis is blocked in the former case by the addition of 5'-guanylyl imido phosphate (GMPPNHP) the conversion is comparatively less (only 30 %). Therefore the hydrolysis of GTP seems to be necessary for the

Table 2. Conversion of tight couple to loose couple ribosomes on enzymatic binding of phenylalanyl tRNA.

Addition	Puromycin reaction	Percentages* of	
		Tight couple	Loose couple
None	X	100	Nil
Poly U	X	100	Nil
Poly U, EF-T, GTP	X	90	10
Poly U, EF-T, GTP, PhetRNA	+	30	70
Poly U, EF-T, GMPPNHP, PhetRNA	X	70	30
Poly U, PhetRNA (nonenzymatic)	–	100	Nil
Poly U, N-acetyl phetRNA, IF1, 2 and 3, GTP	–	100	Nil

* Percentages of PhetRNA binding are proportionate in all cases.
X Not done; + Positive; – Negative.,

conformational change. However, when phenylalanyl tRNA is bound nonenzymatically to tight couple 50S ribosomes there is practically no conversion. These results are indicative of the roles of EF-T, phenylalanyl tRNA and GTP in the conversion process. It is well known from the earlier results (Rheinberger *et al.,* 1983) and also the puromycin reactions studied in this laboratory (results not presented) that phenylalanyl tRNA nonenzymatically binds to the A site whereas its enzymatic (EF-T-dependent binding involves initial attachment at the A site followed by switch over to the P site. If this is true (further experiments are in progress), the conformational change of 50S ribosome is most likely and this must be mediated by EF-T and GTP which results in the transfer of aminoacyl tRNA from the A site to the P site (as it happens in case of peptidyl tRNA in the presence of EF-G and GTP). No conformational change is observed on nonenzymatic binding of phenylalanyl tRNA but the subsequent addition of EF-G and GTP leads to the conversion (30–40%) of tight couple to loose couple 50S ribosomes. Further, the direct binding of N-acetylphenylalanyl tRNA to the P site does not result in any conversion. An interesting observation made in Cantor's laboratory (Lee *et al.,* 1981) should be mentioned in this connection. It was observed from the quenching of fluorescence of 5'-(iodoacetamidoethyl)-amino naphthalene-1-sulfonic acid (IAEDANS)-labelled L7/L12 that there is conformational change of 50S ribosome on binding with EF-Tu-phenylalanyl tRNA in the presence of poly U. Two basic questions, however, remain unsolved from these preliminary experiments: (i) does EF-T and GTP normally carry out the translocation as observed under *in vitro* condition, if so, what is its significance? and (ii) what do EF-G and GTP subsequently do? Experiments are in progress to answer these two questions.

Attempt will be made here to tackle another important question. Why do the tight couple and loose couple ribosomes have different association capacities? It appears from the kethoxal treatment data that they might be utilising different sites for the association of the subunits. This was experimentally established by prior kethoxal treatment of tight and loose couple 50S ribosomes, followed by interconversion by the translocating agents mentioned above. The results are summarised in figure 2. Loose couple 50S ribosomes on treatment with kethoxal do not lose their association capacity but on conversion to tight couple 50S ribosomes with the help of EF-G, GTP and fusidic acid and subsequent treatment with kethoxal lose this capacity. On removal of kethoxal by dialysis under slightly alkaline condition the association capacity is regained. Again, on pretreatment of loose couple 50S ribosomes with kethoxal followed by conversion to tight couple 50S ribosomes by EF-G, GTP and fusidic acid, the converted population do not retain any association capacity. This clearly indicates that the site for the association of tight couple 50S ribosomes is modifiable by kethoxal and not utilized by loose couple ones for the association with 30S ribosomes. Further, tight couple 50S ribosomes lose their association capacity on treatment with kethoxal but if they are at first converted to loose couple 50S ribosomes with EF-G, 5'-guanylyl methylene diphosphate ($GMPPCH_2P$) or GMPPNHP (70% conversion) and then treated with kethoxal they do not lose their association capacity, as expected. Subsequent removal of kethoxal has also no further effect. Further, if the tight couple 50S ribosomes are first treated with kethoxal and then converted to loose couple ones the latter population have full association capacity, again pointing out that the site utilised by loose couple 50S ribosomes for association with 30S ribosomes is different from that used by tight couple 50S ribosomes. On the basis of the above data a two site

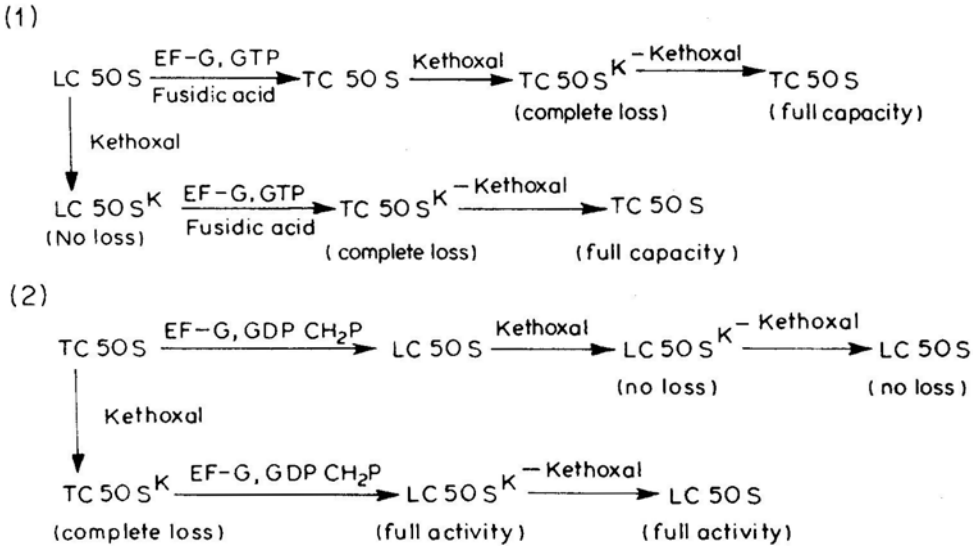

Figure 2. Summary of the efects of kethoxal treatment on tight couple (TC) and loose couple (LC) 50S ribosomes before and after their interconversion. The method of kethoxal treatment has been described in the legend to figure 1. Kethoxal was removed by dialysis against 0·013 M Tris.

model has been proposed for the association of 30S and 50S ribosomes through 16S and 23S RNAs (Burma *et al.,* 1985b). It has been assumed that 23S RNAs have two distinct conformations in the two types of 50S ribosomes due to the conformational change of 23S RNA in the L7/L12 stalk region induced by EF-G and GTP. In the G D P conformation (tight couple) site 1 (modifiable by kethoxal) is available for association with 16S RNA whereas in the GTP conformation (loose couple) site 2 (not modifiable by kethoxal) is utilised for the purpose.

On the basis of the data obtained in this laboratory and other laboratories a model for translocation involving the conformational change of 23S RNA was proposed (Burma, 1984b). Full justifications were given in that publication, so those won't be repeated here. Only a slight revision in the model will be suggested on the basis of the very recent data published by Gudkov and Gongadze (1984). They have shown that 70S.EF-G.GMPPCH$_2$P complex (ribosomes in the preGTP hydrolysis state) the L7/L12 proteins are digested by trypsin whereas in the 70S.EF-G.GDP.fusidic acid complex (ribosomes in the post GTP hydrolysis state) the L7/L12 proteins are trypsin resistant. In our model L7/L12 stalk was shown in extended and folded forms repsectively in GDP (tight couple) and GTP (loose couple) conformations. This was done arbitrarily in the absence of any data about the stalk position. In the revised model presented here (figure 3) the stalk positions have been altered according to the trypsin digestion data mentioned above. This is also in agreement with the crosslinking data of Traut *et al.* (1983) as they observed crosslinking of L7/L12 proteins with L5 (located in the crest of the 50S ribosomes) in the tight couple ones. Experiments are in progress in this laboratory to further substantiate the model by locating the L7/L12 stalk in the tight couple and loose couple 50S ribosomes (Burma *et al.,* 1985c).

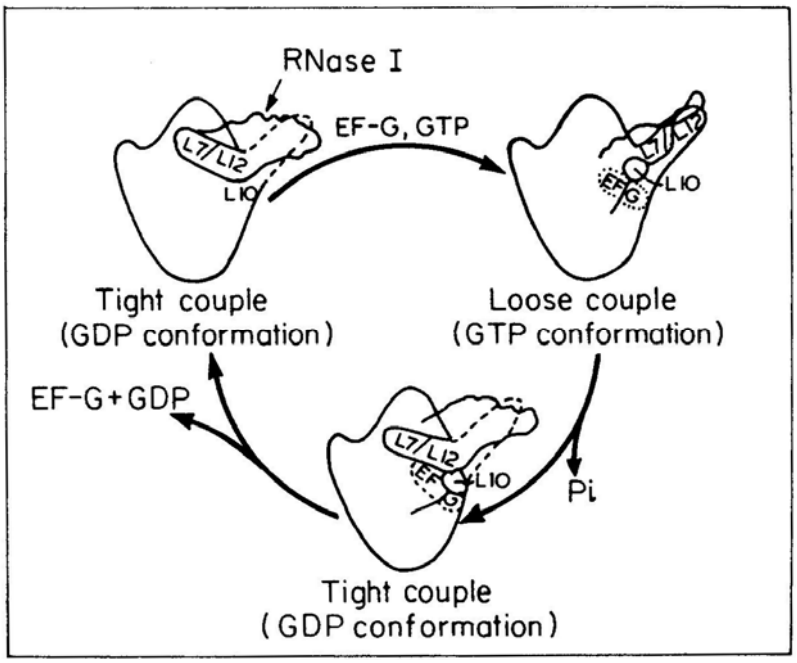

Figure 3. Revised model for translocation. The details have been described in the text.

Although the final solution is still not in sight yet it is abundantly clear from the work done in this laboratory that the processes of association of the ribosomal subunits, the creation of sites for the binding of aminoacyl tRNA and peptidyl tRNA and the translocation of peptidyl tRNA from the A site to the P site etc. are all linked up and operate through the conformational change of 23S RNA. Very recently it has been directly demonstrated in this laboratory that 23S RNA undergoes conformational change (as indicated below) during protein synthesis.

Tight couple 50S ribosomes (23S RNA) 100%	**EF-T, PhetRNA → Poly U, GTP**	**Loose couple 50S ribosomes (23S RNA) 70%**	**EF-G, GTP →**	**Tight couple 50S ribosomes (23S RNA) 90%**

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