# Initial analysis of 750 MHz NMR spectra of selectively $^{15}$N-G,U labelled *E. coli* 5S rRNA

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**Abstract The overall folding of an RNA molecule is reflected in its base pairing pattern. The identification of that pattern provides a first step towards the determination of the structure of an RNA molecule. We show that the application of heteronuclear NMR methods at 750 MHz to *E. coli* 5S rRNA (120 nucleotides) selectively labelled with $^{15}$N in guanine and uridine allows observation of base pairing patterns for a larger RNA molecule. We also present evidence that the fold of the E-domain of the 5S rRNA (nt 79–97) as a contiguous part of the 5S rRNA and as an isolated molecule is virtually the same.**

*Key words:* NMR; $^{15}$N labelling; Ribosome; 5S rRNA; E-domain; *E. coli*

## 1. Introduction

In recent years the variety of biological processes in which RNA is known to play an important role has steadily increased [1]. In some cases RNA is known to play a catalytic role [2,3] and it is possible that RNA catalysis is involved in even such central biological processes as protein synthesis [4]. These developments have led to an increased interest in the determination of three-dimensional tertiary structures of RNA molecules. The recently developed ability to produce large amounts of $^{13}$C-, $^{15}$N-labelled RNA molecules by in vitro synthesis [5,6] has led to the development of new NMR methods designed to determine RNA structures under solution conditions [7,8]. So far these methods have largely been applied to fairly small RNA molecules (up to 35 nucleotides) in which tertiary structure is limited. In the present paper we attempt to assess the possibility of using NMR to study structure for substantially larger RNA molecules. Technically there are several problems to overcome. Firstly, it is necessary to be able to produce large amounts (ca. 0.5–1.0 μmol) of a homogeneous, isotope-labelled RNA molecule. Secondly, homogeneous folding of RNA molecules, particularly at NMR concentrations, is not always easy to achieve [9]. Indeed, recognition of the RNA folding problem has led to the suggestion that during in vivo folding, RNA chaperones may be necessary [10]. Finally, it will be necessary to develop NMR experiments which are appropriate for the study of large RNA molecules. One aspect of this will be the development of NMR experiments to determine conformations for the phosphate-ribose backbone of larger RNA molecules. Equally important will be the development of NMR methods for observation of base pairing patterns utilizing resonances from base imino and amino hydrogens which in RNA can exchange rather rapidly with solvent water. In the present paper we assess to what extent base pairing patterns are detectable with modern NMR methods in *E. coli* 5S rRNA (120 nucleotides) selectively labelled with $^{15}$N in the guanine and uridine nucleotides.

## 2. Materials and methods

### 2.1. *Selectively $^{15}$N-G and $^{15}$N-U base labelled* E. coli *5S rRNA*

T7 RNA polymerase was purified from overexpressing *E. coli* cells according to [11,12] in the modification of [13]. $^{15}$N-labelled rNMPs were produced by nuclease P1 digestion of rRNA from ribosomes of *E. coli* grown on M9 media supplemented with [$^{15}$N]$NH_4Cl$ [5,14,15]. 13 000 $A_{260}$ units of NMPs were chromatographed on a 45 ml Dowex 1X8 column as described [16]. Individual rNMPs could be separated completely by eluting [$^{15}$N]CMP, [$^{15}$N]AMP, [$^{15}$N]UMP and [$^{15}$N]GMP in that order using 1 mM, 2 mM, 3 mM, and 6 mM HCl, respectively. Separated, reconcentrated NMPs were rephosphorylated, reconcentrated by ethanol precipitation and used for the in vitro transcription without further purification as described [17]. The separation and the rephosphorylation of the NMPs was monitored using TLC [18].

The transcription unit for the *E. coli* 5S rRNA from the plasmid pKK3535 [19] was fused to a T7 promoter using PCR and subcloned into pUC19 in such a way that the product could be linearized for transcription by *Nae*I. By this subcloning step the terminal bases of the 5S rRNA were changed from U-1 to G-1 and from A-119 to C-119, respectively. This modification yielded a favorable initiation sequence for the T7 polymerase yet left the terminal 5′:3′ stem base pairing intact. The 3′ terminal U-120, which does not seem to play a significant functional role [20,21], was deleted.

*Nae*I linearized plasmid was transcribed at 150 nM concentration in vitro with T7 RNA polymerase [22]. The optimized reaction conditions were 200 mM Tris-Glu, pH 8.1, 2 mM spermidine, 26 mM $Mg(OAc)_2$, 20 mM DTT, 80 μg/ml T7 RNA polymerase, for 8 h at 37°C in the presence of 24 mM NTPs of which rGTP and rUTP were $^{15}$N-labelled (see above) and rATP and rCTP were from Sigma, Munich, Germany. For quantification purposes a 50 μl aliquot of the transcription reaction was incubated separately in the presence of 10 μCi [$^{32}$P]GTP (Amersham, Braunschweig, Germany). In vitro transcription products were analyzed on 10% polyacrylamide gels containing 7 M urea and quantified in the gel using a Fuji phosphoimager. A 20 ml reaction produced 76 mg of full length product. Commercially available *E. coli* 5S rRNA (Boehringer Mannheim, Mannheim, Germany) was used as a size standard.

The RNA from a 20 ml reaction was diluted 4-fold with 0.1 M sodium acetate, pH 5.3, and chromatographed on a 25 ml DEAE sepharose FF column (Pharmacia, Freiburg, Germany). The 5S rRNA eluted at 1 M sodium acetate was precipitated with ethanol, resuspended in folding buffer (10 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 40 mM NaCl) at a concentration of 45 μM and folded into the A-conformation [23] according to [24]. The folded RNA was ethanol precipitated again and dissolved into NMR buffer without $D_2O$ (10 mM sodium phosphate, pH 6.3, 50 mM NaCl, 1 mM $MgCl_2$), subsequently exchanged into NMR buffer containing 10%

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$D_2O$ and concentrated to a final concentration of 1.5 mM RNA by ultrafiltration using a Centricon 50 device (Amicon, Witten, Germany). The final yield was 64 mg of 5S rRNA. The correct folding at each step of the preparation was ascertained using native 10% polyacrylamide gels [25]. The integrity of the sample was monitored during the entire period of experimentation using denaturing 10% polyacrylamide gels containing 7 M urea.

*2.2. Unlabelled E-domain of* E. coli *5S rRNA*

The E-domain of *E. coli* 5S rRNA (5′-rGUGUGGGGUCUCCCCAUGC-3′), corresponding to nucleotides 79–97, was synthesized chemically [41].

*2.3. NMR experiments*

For the NMR experiments on the E-domain fragment of the *E. coli* 5S rRNA, a 0.7 mM sample of this fragment in 90% $H_2O$/10% $D_2O$, pH 6.5 and 10 mM sodium phosphate buffer was folded by cooling it quickly from 75°C to 0°C. A 2D NOE spectrum was acquired on a Bruker AMX600 spectrometer at 2 C. The NMR experiments on the 5S rRNA were performed at 27°C on a 750 MHz Varian Unity INOVA spectrometer located at Oxford Instruments, Oxford, UK. Two-dimensional $^1H$-$^{15}N$ correlation spectra were acquired using a WATERGATE-HSQC sequence with water flip-back pulses [26]. A new pulse sequence used for the 3D $^{15}N$-edited NOESY experiment is given in Fig. 3 and discussed in more detail below.

## 3. Results and discussion

Optimization of the in vitro production of *E. coli* 5S rRNA using T7 polymerase and a linearized plasmid template has allowed us to obtain 80% incorporation of the input nucleotide triphosphates into transcription products. Quantification of radioactively labelled transcription products on denaturing polyacrylamide gels using a phosphoimager indicated that approximately 50% of the material from the transcription reaction was the full length 5S rRNA as compared to native purified *E. coli* 5S rRNA obtained commercially. Other products were almost exclusively a small amount of high molecular weight RNA, possibly arising from incomplete linearization of the plasmid template, abortive transcripts of about 8–10 nucleotides and unconsumed mononucleotides. The major differences in the sizes of these side products compared to 5S rRNA allowed for a simple and efficient purification of the desired product by ion-exchange chromatography (see section 2).

An important criterion in our decision to use *E. coli* 5S rRNA for assessing the feasibility of NMR structural studies of larger RNA molecules was the substantial body of previous work on the refolding of this molecule ([23–25,27] and references cited therein). In practice, we have been able to use a previously published folding protocol [24] at about 5-fold higher concentrations (45 μM) to obtain *E. coli* 5S rRNA homogeneously folded into the A-form, which is believed to be the native form [23,25,27–30]. On native gels [25] our in vitro synthesized and commercially obtained natural *E. coli* A-form 5S rRNA exhibited virtually identical electrophoretic mobility. A further important observation was that the in vitro produced 5S rRNA retained the A-form during the final steps of preparing the NMR sample by concentrating it 30-fold and exchanging it into NMR buffer using ethanol precipitation and ultrafiltration (data not shown). Overall, about 40% of the $^{15}N$-labelled nucleotides used in the T7 polymerase transcription reaction were contained in the final 1.5 mM NMR sample. One-dimensional $^1H$ NMR spectra (not shown) of the selectively $^{15}N$-G,U labelled 5S rRNA were virtually indistinguishable from NMR spectra reported earlier for this molecule isolated from *E. coli* ribosomes [31,32]. On the basis of electrophoretic behavior and NMR spectroscopy, the final NMR sample has been found to be stably folded over several months.

The currently accepted secondary structure model for *E. coli* 5S rRNA (A-form) is shown in Fig. 1. The alternative folding form of this molecule, the B-form, is thought to differ through the formation of a helical duplex between nucleotides G-33–C-42 and G-79–C-88 (indicated by thick lines in Fig. 1) in place of helix E of the A-form. In this model, the A (B) form contains 22 (23) GC, 5 (5) GU and 5 (6) AU base pairs within the proposed double helical regions. These numbers of base pairs are approximately consistent with a two-dimensional $^1H$-$^{15}N$ correlation spectrum (Fig. 2a) showing the region of the NMR spectrum which contains the imino resonances from the $^{15}N$-labelled G and U nucleotides. This experiment allows clear differentiation between guanine and uridine imino groups on the basis of the characteristic $^{15}N$ chemical shifts [33]. A further, less stringent, indication for the type of base pairs is given by the $^1H$ chemical shifts. On the basis of the observed $^1H$ and $^{15}N$ chemical shifts, a tentative assignment of the resonances to types of base pairs can be obtained (Fig. 2a). For G assigned to GU base pairs as well as U assigned to either GU or AU base pairs, many individual resonances can be observed. For G assigned to GC base pairs, substantial overlap of resonances occurs. Further evidence of

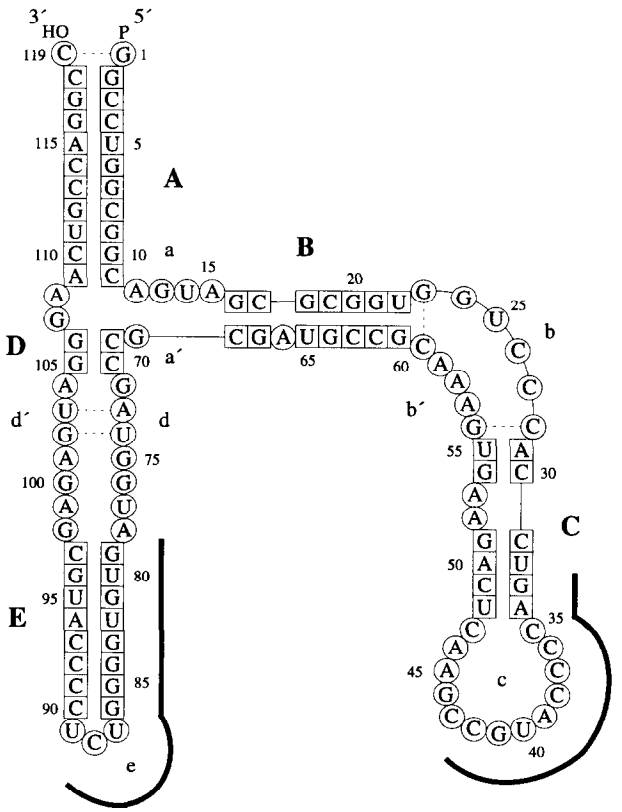


Fig. 1. *E. coli* 5S rRNA secondary structure model [24]. Capital letters A–E designate distinct duplex domains of the 5S rRNA and small letters a/a′ to d/d′ refer to bulged or looped regions between those domains. The alternative folding scheme, B-form, is proposed to differ from A-form by the formation of a duplex composed of the regions G-33–C-42 and G-79–C-88 (indicated by the thick lines) instead of duplex E of the A-form. The sequence shown corresponds to the molecule used for the NMR measurements and differs from native *E. coli* 5S rRNA in the substitutions U-1–G-1 and A-119–C-119 as well as deletion of U-120.

the number of observable imino hydrogens in each class has been obtained from a 3D $^{15}$N-edited NOESY spectrum (see below). On the basis of a preliminary analysis of these spectra, we conclude that the number of observable G and U imino hydrogens tentatively assigned to the different types of base pairs is at least 18 GC, 8 GU, 7 GU and 9 AU. With the exception of the heavily overlapped GC imino resonances, these numbers clearly exceed the number of base pairs expected in the helical regions of the current 5S rRNA secondary structure model. This suggests that imino hydrogens from both G and U which participate in hydrogen bonds in irregular secondary structure regions or in tertiary interactions are observable in these spectra. Further evidence for this is presented below. Here we note that the improved resolution and sensitivity of the 750 MHz spectrometer, as well as improved experimental techniques for observation of exchangeable hydrogens in water, has allowed us to observe structural features which were not seen in earlier experiments on *E. coli* 5S rRNA. A clear example of this is provided by the AU resonances labelled 1–3 in Fig. 2a, which were not observed in a previous $^{1}$H-$^{15}$N correlation spectrum of selectively $^{15}N_3$-U labelled *E. coli* 5S rRNA [34].

A full analysis of NMR spectra recorded for isotope-labelled 5S rRNA will clearly be a major task. To further assess the extent to which resonances corresponding to defined structural features may be observable in the spectrum of the full 5S rRNA molecule, we have undertaken a comparison of spectra recorded for the complete *E. coli* 5S rRNA with spectra re-

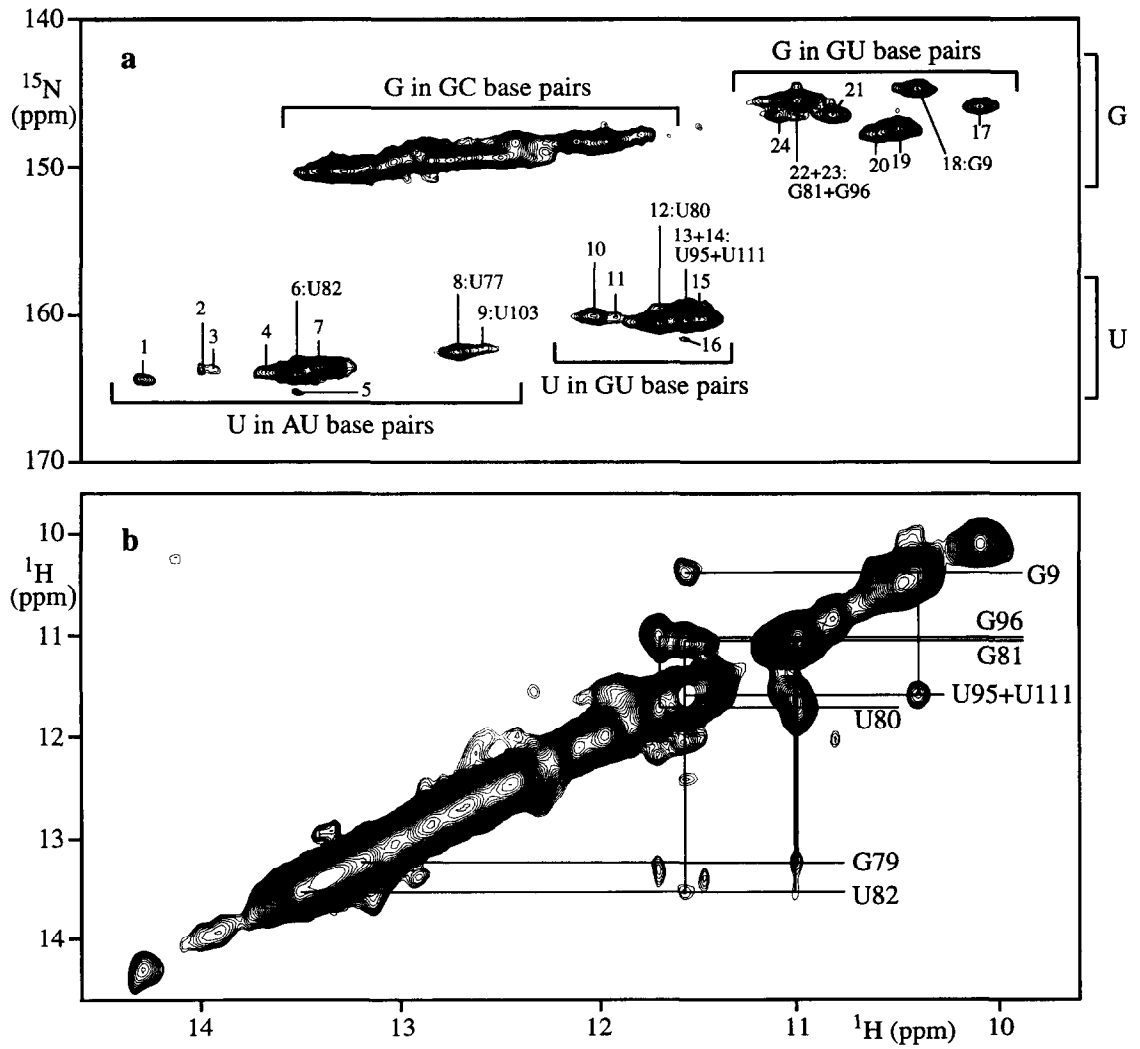


Fig. 2. (a) $^{1}$H-$^{15}$N one bond correlation spectrum of the selectively $^{15}$N-G,U labelled *E. coli* 5S rRNA. The typical chemical shift regions which are indicated are described in more detail in the text. The assignments given are based on comparison with the E-domain fragment, nucleotides 79–97, and partially on previously reported NMR results on *E. coli* 5S rRNA fragments [36–39]. The spectral widths were 3000 and 17000 Hz in F1 and F2, respectively. 49 complex points were acquired in $t_1$ with 64 scans per FID and a recycle time of 1 s resulting in a total experimental time of 2 h. (b) The region containing imino to imino NOEs from a 2D F1,F3 $^{1}$H-$^{1}$H version of the modified 3D $^{15}$N-edited NOESY experiment shown in Fig. 3. The indicated assignments are discussed in the text. The spectral widths in F1 and F3 were chosen to be 9750 and 17000 Hz. 40 complex points were collected in $t_1$ with 256 scans per FID, a mixing time of 27 ms and a recycle time of 1 s resulting in a total experimental time of 6.3 h. Selective 90° water flip-back pulses of 600 μs duration were used. The $^{15}$N carrier frequency was set to the center of the imino region (155 ppm). An rf field strength of 1.1 kHz was used for the GARP decoupling during $t_1$ and $t_3$. The delay $\Delta_1$ was optimized to 1.9 ms. Rectangular pulse field gradients with the following {duration, intensity} were applied: $G_1$ = {1.0 ms, 30 G/cm}, $G_2$ = {1.0 ms, 20 G/cm}, $G_3 = -G_2$, $G_4$ = {0.1 ms, 30 G/cm}. A corresponding 3D experiment was acquired with a $^{15}$N spectral width of 2100 Hz, 64 scans per FID and 40 and 15 complex points in $t_1$ and $t_2$, respectively.

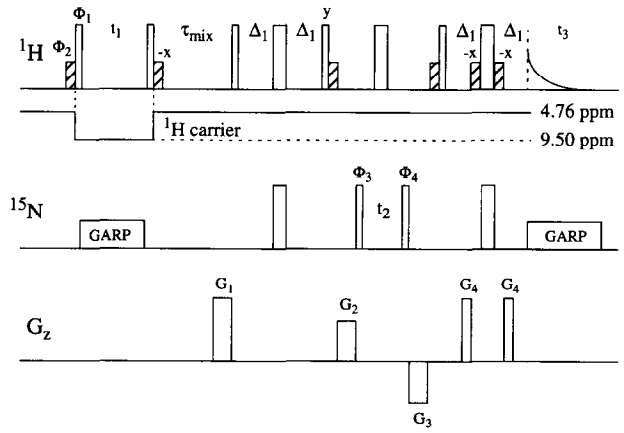


Fig. 3. Pulse sequence for the modified 3D $^{15}$N-edited NOESY experiment. The narrow and wide bars represent 90° and 180° pulses, respectively. If not otherwise specified, the pulses are applied along the x-axis. The hatched bars represent rectangular selective 90° water flip-back pulses used to align the water magnetization along the z-axis for most of the time covered by the experiment and to perform the final WATERGATE water suppression. Since the water relaxation induced by radiation damping may not be complete at the end of the short mixing time (27 ms) chosen for the spectrum of Fig. 2b, a water flip-back pulse was also applied at the beginning of $\tau_{mix}$. The following phase cycle was used: $\Phi_1 = 4[x-45],4[-x-45]$, $\Phi_2 = \Phi_1+180$, $\Phi_3 = [x,-x]$, $\Phi_4 = [x,x,-x,-x]$, $\Phi_{receiver} = [x,-x,-x,x,-x,x,x,-x]$. ($\Phi_1$, $\Phi_2$) and $\Phi_3$ are incremented according to the States procedure [40] to achieve quadrature detection in $t_1$ and $t_2$, respectively. The $^1$H carrier frequency was shifted from the water resonance at 4.76 ppm to 9.50 ppm and back as indicated to reduce the spectral width in $t_1$.

corded for a fragment corresponding to the E-domain stem-loop (Fig. 1, nucleotides 79–97). A detailed interpretation of the NMR spectrum of the E-domain fragment of *E. coli* 5S rRNA will be presented elsewhere. Here we note that for the nucleotides proposed to form the stem of the E-domain stem-loop, a large number of NOEs characteristic of an RNA duplex can be observed between exchangeable imino and amino hydrogens of nucleotides 79–86 and 90–97 (Fig. 4). As will be shown in detail elsewhere, the full stem, including the two successive G-U base pairs, is formed for the E-domain fragment 79–97 in solution.

Observation of NOEs involving the imino and amino hydrogens of the full 5S rRNA molecule was found to be a challenging task due to the line-widths of the $^1$H (approx. 50–60 Hz) and $^{15}$N (approx. 30–35 Hz) resonances as well as to the rather facile exchange of such labile hydrogens with solvent water [35]. For this purpose we have developed a modified 3D $^{15}$N-edited NOESY experiment (Fig. 3) in which we have incorporated WATERGATE water suppression, selective 90° water flip-back pulses, pulsed field gradients, switching of the $^1$H carrier frequency during $t_1$ and $^{15}$N broadband decoupling during $t_1$. The order of the NOE and HSQC parts of the sequence was chosen to minimize the number of delays. Because of the broad $^1$H and $^{15}$N line-widths observed for *E. coli* 5S rRNA, inclusion of $^{15}$N editing leads to appreciable loss of signal intensity compared to simple 2D NOE spectra. On the other hand, the $^{15}$N chemical shifts provide a decisive advantage in the structural interpretation of the NOE spectra. With the new pulse sequence we have been able to observe NOEs between labile hydrogens involved in base pairs as well as the more intense of the sequential and cross-strand NOEs with acquisition times as short as 6 h (Fig. 2b).

As an initial analysis of the full 5S rRNA molecule, special consideration has been given to whether similar NOE patterns can be detected amongst exchangeable imino hydrogens in the sequences G-79–U-82 and A-94–C-97. In the E-domain fragment 79–97, the six imino hydrogens contained in these nucleotides show five imino-imino NOEs (thick lines in Fig. 4). For the full 5S rRNA molecule, five NOE crosspeaks at very similar chemical shifts are seen (Fig. 2b) and from the 3D $^{15}$N-edited NOESY spectrum, it was possible to ascertain whether the NOE crosspeaks correspond to GU, GG or UU NOEs. For hydrogen bonded GU wobble base pairs, the imino-imino hydrogen distances are very short (<3 Å) and are expected to lead to strong NOEs. In accordance with this, two of the five NOE crosspeaks are very strong and are attributed to the G-96–U-80 and G-81–U-95 base pairs. The remaining three NOE crosspeaks are much weaker and are attributed to a sequential G-81–U-82 and to two cross-strand U-82–U-95 and G-79–G-96 NOEs (Fig. 4). In short, the full 5S rRNA molecule and the E-domain fragment show a very similar pattern of strong and weak NOEs at very similar chemical shifts and with the same assignments of the crosspeaks to G or U imino hydrogens. This high degree of similarity of the NOE pattern strongly supports the conclusion that very similar duplex conformations exist for nucleotides G-79–U-82 and A-94–C-97 in both the full 5S rRNA and the E-domain fragment.

The spectra recorded for *E. coli* 5S rRNA show a very large number of NOEs in other spectral regions (data not shown) which will require very considerable effort to fully interpret. At present we note only that a number of other NMR studies of fragments of 5S rRNA have previously been reported and that some evidence that these fragments also have similar structures in the full 5S rRNA is apparent in the present

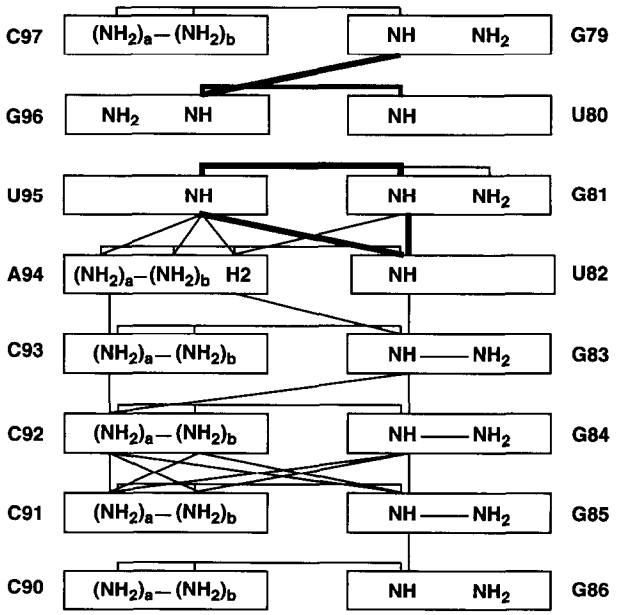


Fig. 4. NOE connectivities between exchangeable hydrogens observed in a 2D NOE spectrum of the E-domain fragment, corresponding to nucleotides 79–97 of *E. coli* 5S rRNA. The thick lines represent imino-imino NOEs which could also be observed in the full 5S rRNA (see text and Fig. 2b).

spectra. For example, a previous study of the A-domain duplex (nucleotides 1–11 and 108–119, Fig. 1) has identified the $^{1}H$ chemical shifts of the imino hydrogens of the G-9–U-111 base pair [36]. A very strong GU imino-imino NOE characteristic of a GU base pair is observed at very similar $^{1}H$ chemical shifts in the full 5S rRNA molecule (Fig. 2b) and has tentatively been assigned to the G-9–U-111 base pair. Similarly, a study of a fragment containing the irregular d,d′ region of 5S rRNA (nucleotides 72–78 and 98–104, Fig. 1) has identified $^{1}H$ chemical shifts for the U77 and U103 imino hydrogens [37-39]. Two uridine imino hydrogens with very similar chemical shifts are also observed in the full 5S rRNA (Fig. 2a).

## 4. Conclusions

It has recently become possible to obtain detailed NMR solution structures for $^{13}C$-, $^{15}N$-isotope labelled oligoribonucleotides of up to at least 35 nucleotides. The present study indicates that critical NOEs that define such structures are observable in molecules as large as 5S rRNA. This allows for an analysis of whether the conformations obtained for fragments of larger RNA molecules are retained in the full molecule. The observations in the present study also suggest that it may become possible to directly study the conformation of molecules as large as 5S rRNA. Exchangeable hydrogens which are stabilized by hydrogen bonds are easily observed in molecules of this size. Furthermore, the cross-strand and sequential NOEs necessary to define base pairing patterns are also observable, at least for the stronger NOEs, which opens the way to detection of secondary and possibly tertiary interactions. The biggest problems to be surmounted may well be the resolution of individual NOEs and the assignment of these to specific locations in the RNA covalent structure. Here it seems likely that it will be necessary to take full advantage of the possibilities for selective isotope labelling which are afforded by the in vitro synthesis of the RNA molecules.

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