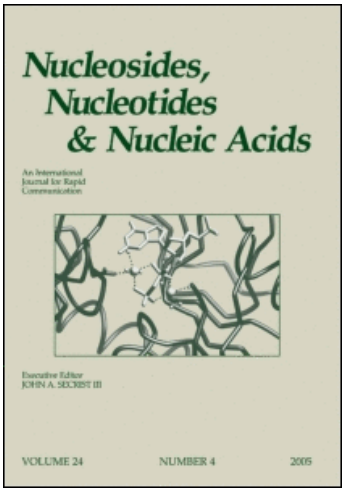

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NOVEL APPROACHES TO THE SYNTHESIS AND ANALYSIS OF BRANCHED RNA

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Abstract: This report describes a novel solid-phase synthesis of bRNA. The deprotected branched oligoribonucleotides were analysed by capillary electrophoresis and conveniently characterized by subjecting them to the yeast lariat debranching enzyme.

The structure of branched ribonucleic acid (bRNA) was first discussed in 1952.[1] However, it was not until 1983 that Wallace and Edmonds discovered it in mammalian cell nuclei.[2] Subsequent studies demonstrated that bRNA is the first biochemical intermediate in splicing of messenger RNA. This mRNA processing mechanism eventually results in the generation of a mature mRNA transcript and release of circular branched structures (RNA "lariats") which are later debranched and degraded (FIG. 1).[3]

bRNA occurs in two forms: as "lariat" or circular branched structures derived from cis-splicing reactions, and as non-circular forked or "Y" structures derived from trans-splicing reactions. A common structural feature of these bRNAs is the vicinal 2',5'- and 3',5'-phosphodiester linkages at their branchpoint. In budding yeast, excised intron lariats have the branchpoint sequence ...5'UACUA<u>A</u>(2'GU...)3'C..., however, in other eukaryotes, there is no strict consensus sequence surrounding the branched nucleotide [5'...YNYUR<u>A</u>(2'GU...)3'Y...; R = Pu and Y = Py]. But the adenosine as branchpoint acceptor nucleotide (<u>A</u>) appears to be conserved in almost all eukaryotes.

Inspired by the natural occurrence of bRNA and with the interest in the possible biological roles of bRNA, one of our laboratories developed methods for its chemical synthesis both in solution[5] and in the solid-phase.[6-8] The solid-phase method is characterized by the coupling of an adenosine 2',3'-O-bis(phosphoramidite) reagent[5] with two adjacent support-bound oligonucleotide chains yielding "V"-shape structures. Synthesis can then be continued in the normal 3'-to-5' direction from the "apex" of these bRNAs to yield forked or "Y"-shape structures. Because of the simultaneous introduction of both 2',5' and 3',5'-linkages internucleotide linkages, the 2' and 3' chains of the "Y" molecules are always identical in sequence. Given this limitation

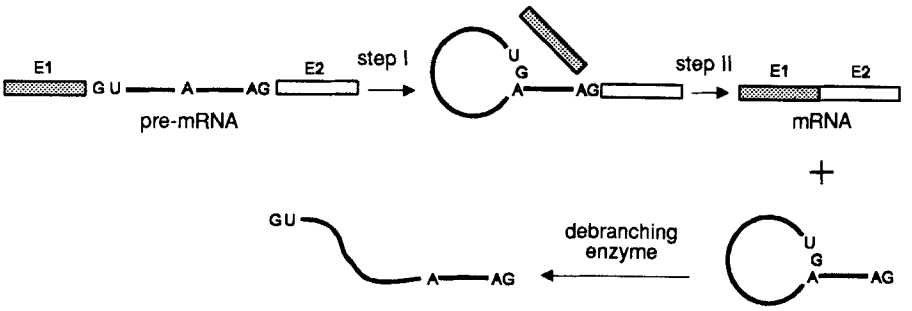
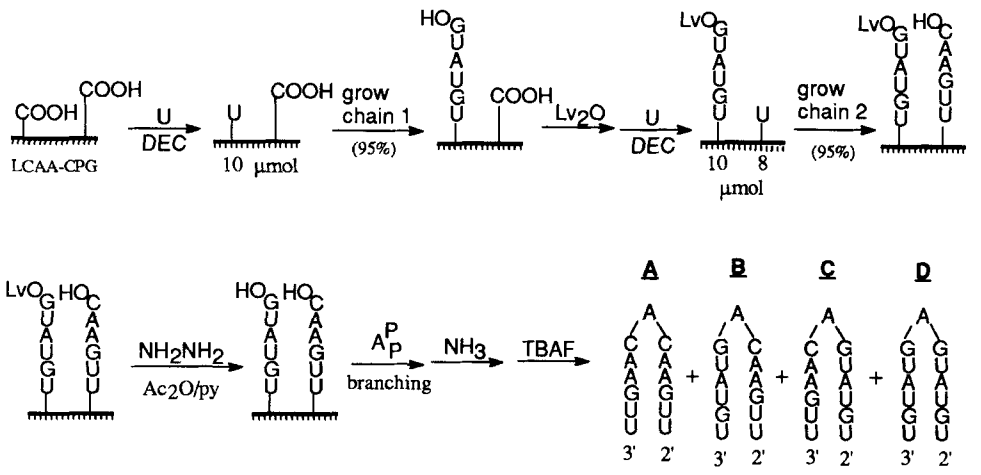


FIGURE 1. Illustration of cis splicing and debranching reactions. The debranching enzyme cleaves the lariat intron specifically at the single 2',5'-phosphodiester bond.[4]

and our continued interest in exploring the substrate specificity of the yeast debranching enzyme,[9,10] we have begun to investigate alternative strategies to the solid-phase synthesis of bRNA. Here we present some preliminary results from work based on our original "convergent" method that yields bRNA with 2' and 3' chains of different base sequence.[11,12]

Our route is illustrated by the synthesis of the tridecaribonucleotide A(2'GUAUGU)3'CAAGUU (SCHEME 1). The sequence of this "V"-13-mer corresponds to the sequence of the native yeast *rp51* intron branchpoint. The key feature of the method is the synthesis of *two different* RNA species on the surface of controlled-pore glass (LCAA-CPG). Theoretically, reaction of these two sequences with a suitably protected adenosine 2',3'-O-bis(phosphoramidite) would afford four isomeric bRNA species: two "symmetric" and two "asymmetric" V-structures. Thus, a mixture of succinyl-controlled-pore glass[13] (250 mg), 5'-MMT-uridine (0.1 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC, 1 mmol), triethylamine (40 μL), 4-dimethylaminopyridine (4-DMAP, 50 μmol) in anhydrous pyridine (4 mL) was shaken at room temperature for 45 min. The CPG was then filtered off and washed successively with pyridine, dichloromethane and ether. The resulting support had a loading of 10 μmol/g which corresponded to about half of its maximum loading capacity. Following a "capping" (Ac_2O) step, a small amount of the support (100 mg, 1 μmol) was placed in the column of an Applied Biosystems 381A synthesizer. Chain assembly in the normal fashion using 2'-O-(*tert*-butyldimethylsilyl)-5'-O-tritylated ribonucleoside (U, A^{Bz}, C^{Bz}, G^{iBu}) 3'-O-cyanoethyl phosphoramidites[5,14] afforded the first sequence GUAUGU (95% average coupling yield). The terminal 5'-hydroxyl was then protected with the levulinyl (4-oxopentanoyl) group[15] by reaction with levulinic anhydride (Lv_2O)[16] in the presence of 4-DMAP/pyridine. This step was conveniently carried out by replacing the "Cap A" reagent in the synthesizer with a solution of



SCHEME 1. Synthesis of Symmetric and Asymmetric bRNAs

0.5 M Lv_2O/THF. After a brief treatment with aqueous pyridine and thorough washing with dry MeCN, the remaining carboxyl groups were esterified manually by coupling with 5'-O-MMT-uridine and DEC. The column was reinstalled and after another washing (MeCN) step, the second hexanucleotide sequence CAAGUU was assembled with a 95% average coupling yield. The levulinyl protecting groups were then removed manually with a solution of 0.5 M hydrazine hydrate in pyridine/acetic acid (5 min), conditions which did not cause the cleavage of the related succinyl linker or base protecting groups.[17] Quantitation of the trityl cations released during chain assembly indicated that GUAUGU and CAAUGU were present on the support surface in a ratio of 1.3 to 1 (*i.e.*, 10 and 8 μmol/g, respectively). In the final step, the solid support was treated with N^6-benzoyl-5'-O-monomethoxytrityladenosine 2',3'-O-bis(β-cyanoethylphosphoramidite) (0.03M in MeCN) and tetrazole to join the 5'-ends of neighbouring hexanucleotide chains. At the end of the synthesis the standard treatments with aqueous ammonia/ethanol (3:1, r.t., 48 h) and tetra-*n*-butylammonium fluoride (1M in THF, r.t., 16h) removed the protecting groups and cleaved the oligomers from the support to afford 41 A_{260} units of the crude mixture. This material was then applied on a preparative 24% polyacrylamide/7M urea gel and subjected to electrophoresis. The slowest moving band excised and extracted with sterile water by incubation at 37 °C for 16 h. The sequences were further purified by reversed-phase chromatography using a C18 Sep-Pak cartridge to yield 4 A_{260} units (10 %) of the mixture of four V-RNA species (**A**, **B**, **C** and **D**, FIG. 1).

Nucleoside and branched nucleotide composition analysis[7] corroborated the structure of the isolated products (data not shown). Attempts to separate the mixture by reversed-phase and

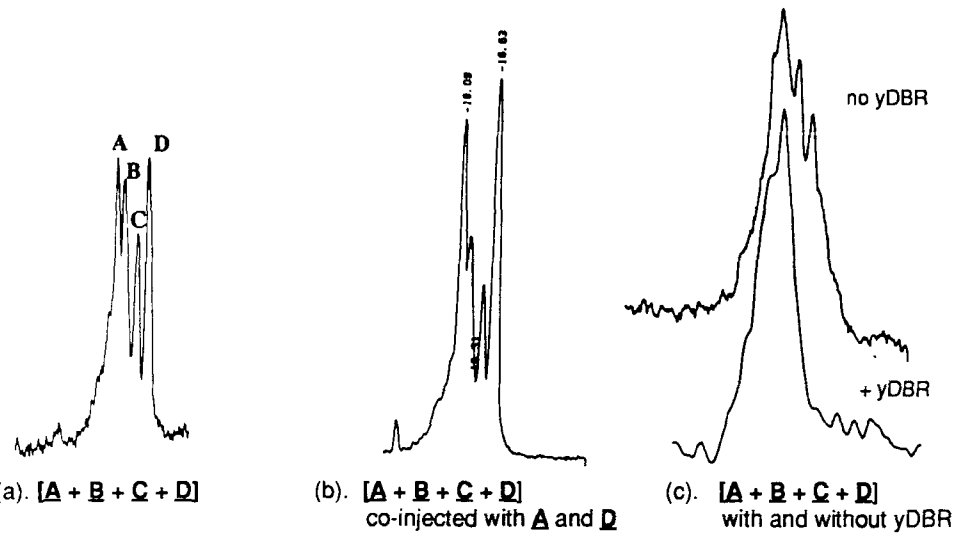


FIGURE 2. Capillary electrophoresis analysis of branched oligoribonucleotides. The CE system used was constructed at McGill and is described in ref. 18. Capillaries containing 9% linear polyacrylamide in TBE[19] were run with 0.9% polyacrylamide in TBE running buffer. Capillaries had a total length of 62 cm and a separation length of 42 cm. An applied voltage of 18 kV was used. Retention times of species are indicated in b. Oligomers (*ca.* 10 pmol) were incubated with purified yDBR (50 units, 30 min, 30 °C) as previously described.[10]

ion-exchange HPLC or polyacrylamide gel electrophoresis (24%/ 7M urea) were unsuccessful. However, capillary electrophoresis[18,19] proved to be very effective for resolution of the four closely related bRNA species (peaks **A**-**D**, FIG. 2a). Two of these sequences were identified as the "symmetric" sequences by co-injection with authentic samples[7] of A(2'CAAGUU)3'CAAGUU and A(2'GUAUGU)3'GUAUGU (peaks **A** and **D**, respectively, FIG. 2b). The identity of the regioisomeric or "asymmetric" bRNAs (peaks **B** and **C**) was determined by subjecting the mixture to the yeast debranching enzyme (yDBR). In vitro studies conducted by the Johns Hopkins group indicate that this enzyme is a specific 2',5'-phosphodiesterase that can cleave a variety of branched RNA including group II intron lariats and synthetic branched oligoribonucleotides.[9,10] In addition, the identity of the 2'-residue seems particularly important, with cleavage at 2'-purines preferred over 2'-pyrimidines.

CE analysis revealed that only two of the four "V"-RNA species (**C** and **D**) were substrates of the debranching enzyme (FIG 2c). This result was expected since only two of the species have a 2'-G. Since peak **D** had already been assigned to the "symmetric" sequence A(2'GUAUGU)3'GUAUGU, one can safely conclude that compound **C**, also a substrate of yDBR, correspond to the "asymmetric" species A(2'GUAUGU)3'CAAGUU. Thus, **B** corresponds to

A(2'CAAUGU)3'GUAGUU. These studies not only confirm that cleavage of bRNA by yDBR depends on the 2'-position nucleotide,[10] but they also indicate that the 3'-residue has little or no effect on the debranching rate (*i.e.*, both **C** and **D** were good substrates). Confirmation of this hypothesis will require complete analysis of additional branched oligonucleotides having all possible nucleotides at each position around the branch point. This work is currently in progress.

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