Phosphoryl migration during the chemical synthesis of RNA

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ABSTRACT

By the use of high sensitivity assay systems, we have measured the occurrence of strand scission and phosphoryl migration that accompany the deblocking of chemically synthesized oligoribonucleotides. Substantial phosphoryl migration was observed for both enzymatically derived poly(uridylic acid) and synthetic uridine oligoribonucleotides 2'-O-protected with the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) group, when these species were subjected to the acidic conditions suggested for Fpmp deprotection. Strand scission occurred in parallel and could be demonstrated readily by 5'-³²P end labeling, but not by 3'-³²P end labeling, of the acid-treated oligoribonucleotides. Increasing the pH of the deprotection solution and decreasing the temperature at which the deprotection was accomplished diminished both phosphoryl migration and strand scission. A mechanism that can rationalize these results is discussed.

INTRODUCTION

Chemically synthesized oligoribonucleotides have been employed in many types of chemical, biochemical and biophysical investigations. These have included studies of RNA–RNA (1) and RNA–protein interactions (2), the aminoacylation of transfer RNAs (3,4), isolation and characterization of a human RNA debranching enzyme (5), characterization of ribozyme structure and function (6–8) and structural characterization of DNA–RNA heteroduplexes (9,10).

Central to the development of methods for the chemical synthesis of RNA is the choice of protecting groups for the 2' and 5'-OH groups of the ribonucleoside building blocks. The 5'-OH protecting group must be removed as part of every cycle of ribonucleotide addition; its removal must be facile and involve some reagent compatible with the stability of the growing RNA chain. In contrast, the 2'-OH protecting group must remain intact throughout oligonucleotide assembly, but be amenable to removal at the end of the synthesis without concomitant strand scission or phosphoryl migration. Because the choice of these protecting groups is critical to the overall success of RNA synthesis, this aspect of RNA synthesis has been studied intensively.

While the dimethoxytrityl (DMTr) protecting group has been used extensively for 5'-OH protection in both DNA and RNA

synthesis in recent years (11,12), the development of a suitable 2'-OH protecting group has proven to be considerably more challenging. Currently, the protecting groups of choice are the *t*-butyldimethylsilyl (TBDMS) (13,14) and [1-(2-fluorophenyl)-4-methoxypiperidin-1-yl] (Fpmp) groups (15). In the present report, we describe findings pertinent to the observation, quantification and minimization of strand scission and phosphoryl migration during RNA synthesis.

MATERIALS AND METHODS

Uridine, U^{2'}p^{5'}U, U^{3'}p^{5'}U and poly(uridylic acid) ($M_r > 10^5$; prepared using polynucleotide phosphorylase) were purchased from Sigma Chemicals; tetrabutylammonium fluoride in tetrahydrofuran was from Aldrich Chemicals. (Up)₂₀U, protected with 2'-O-*t*-butyldimethylsilyl groups, was obtained from the Keck Biotechnology Resource Laboratory (Yale University). 2'-O-Fpmp-protected ribonucleoside phosphoramidites were purchased from Cruachem Ltd. Fpmp-protected (Up)₂₀U was obtained both from Oligos Etc. Inc. and from Cruachem Ltd.

RNase A was purchased from Qiagen Inc. RNases T₁ and T₂ were obtained from BRL; calf intestinal alkaline phosphatase was from Boehringer-Mannheim. T4 RNA ligase and T4 polynucleotide kinase were purchased from United States Biochemical Corporation.

Deprotection and radiolabeling of a 22 nt RNA

The oligoribonucleotide CUUGAAAUAGCAAUUAUUUAUUG was synthesized as described (15) on a 0.2 μmol column, then treated with ammonia for (partial) deprotection and removal from the solid support. The derived 5'-O-DMTr-2'-O-Fpmp-protected RNA was treated with 600 μl of 15% (2.6 M) acetic acid and shaken at 22°C for 18 h. The reaction mixture was neutralized with 800 μl of 2 M Tris–HCl, pH 8.0, then treated with 100 μl of 5 M NH₄OAc containing 0.5% sodium dodecyl sulfate and 1 mM EDTA. The RNA solution was filtered (Millipore Ultra free MC filter), divided into portions and precipitated by the addition of 5 vol of cold ethanol.

A portion of the RNA was 3'-³²P end labeled in 15 μl (total volume) of 50 mM Hepes buffer, pH 8.3, containing 10 mM MgCl₂, 5 mM dithiothreitol, 50 μg/ml of bovine serum albumin, 6.7 μM ATP, 60 μCi of [5'-³²P]pCp (> 3000 Ci/mmol), 13 μM RNA and 6 U of T4 RNA ligase. The reaction mixture was incubated at 4°C for 6 h prior to analysis of the RNA by

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electrophoresis on a 15% denaturing polyacrylamide gel (0.8 mm $\times$ 20 cm $\times$ 40 cm).

A portion of the RNA was 5'-³²P end labeled in 16 μ l (total volume) of 50 mM Tris-HCl, pH 7.5, containing 10 mM MgCl₂, 5 mM dithiothreitol, 50 μ g/ml of bovine serum albumin, 166 μ Ci of [γ -³²P]ATP (> 7000 Ci/mmol), 13 μ M RNA and 30 U T4 polynucleotide kinase. The reaction mixture was incubated at 37°C for 30 min prior to analysis by 15% denaturing polyacrylamide gel electrophoresis.

Deprotection of silyl-protected (Up)₂₀U

Silyl-protected (Up)₂₀U (10–20 A₂₆₀ units) was deblocked using 300 μ l of 1 M tetrabutylammonium fluoride in tetrahydrofuran (16). The reaction mixture was maintained at room temperature for 18 h, then treated with 100 μ l of 50 mM NH₄OAc solution and incubated at 25°C for an additional 30 min. The solution was treated with 1.5 ml acetone containing 2% LiClO₄ to effect oligonucleotide precipitation; the solid product was washed successively with 1 ml of acetone and two 1 ml portions of absolute ethanol. The oligonucleotide product was then purified by electrophoresis on a 16% polyacrylamide gel, visualized by UV shadowing (17), excised from the gel and recovered by the 'crush and soak' method (18).

Deprotection of Fpmp-protected (Up)₂₀U

Typically, 1 A₂₆₀ unit of Fpmp-protected (Up)₂₀U was dissolved in 100 μ l of the acid deprotection solution and incubated at 25 or 4°C for the time indicated. The acidic solution was then treated with NH₄OAc, mixed thoroughly and treated with 3 vol of cold absolute ethanol to effect precipitation of the oligonucleotide product. The precipitated product was washed with cold ethanol and dried.

Enzymatic digestion of RNAs

Approximately 0.2 A₂₆₀ unit of poly(uridylic acid) or the deprotected oligonucleotide was dissolved in 65 μ l of 50 mM NaOAc, pH 4.5, containing 2 mM EDTA, 1.25 mg RNase A and 0.25 U each of RNase T₁ and RNase T₂. The incubation mixture was maintained at 37°C for 16 h, then concentrated to dryness. The residue was dissolved in 20 μ l (total volume) of 50 mM Tris-HCl, pH 8.5, containing 0.1 mM ZnCl₂, 10 mM MgCl₂ and 1 U calf intestinal alkaline phosphatase; the resulting solution was incubated at 37°C for 16 h prior to HPLC analysis. The use of multiple ribonucleases was employed to facilitate the application of the present method to more detailed studies involving oligonucleotides of mixed sequence.

HPLC analysis of enzymatically digested RNAs

All solutions were prepared using water that had been purified on a Millipore water purification system. Solution A was 0.25 M NH₄OAc, pH 6.0. Solution B was 40:60 CH₃CN-H₂O (v/v). The components of the RNA digest were separated by HPLC using a 5 μ m Lichrosorb C₁₈ reversed phase column (250 $\times$ 4.6 mm, Alltech). Separation was achieved using a step gradient involving mixtures of solutions A and B at a flow rate of 2 ml/min: 0% solution B initially; 5% solution B starting at 10 min; 25% solution B starting at 25 min; 50% solution B starting at 30 min. The absorbance was monitored at 260 nm. Uridine (~16 min

Figure 1. Analysis of 3'- and 5'-³²P end labeled RNA's by denaturing polyacrylamide gel electrophoresis. The 22 nt oligomer CUUGAAAUAGCAAUUAUUUAUUG, synthesized on controlled pore glass beads using 5'-O-DMTr-2'-O-Fpmp-protected monomers, was deprotected with 15% acetic acid. Portions of the deprotected RNA were either 3'-³²P end labeled using T4 RNA ligase + [³²P]pCp (A) or 5'-³²P end labeled via the agency of T4 polynucleotide kinase + [γ -³²P]ATP (B). Samples of each radiolabeled RNA were applied to a 15% denaturing polyacrylamide gel (0.8 mm $\times$ 20 cm $\times$ 40 cm) and analyzed by autoradiography following electrophoresis.

elution time), uridylyl(2'→5')uridine (~23.5 min) and uridylyl(3'→5')uridine (~25 min) were readily separable under these conditions, as demonstrated by co-injection of authentic standards (not shown).

RESULTS

A sample of fully protected CUUGAAAUAGCAAUUAUUUAUUG, prepared by solid phase RNA synthesis using 2'-O-Fpmp-protected ribonucleoside phosphoramidites, was deprotected using 15% acetic acid, a reagent initially thought to be compatible with removal of the Fpmp protecting group. However, when a portion of the acid-treated material was 5'-³²P end labeled, subsequent analysis on a polyacrylamide gel revealed a complex mixture of products (Fig. 1B). That the multiple bands were not due to failure sequences formed during the synthesis was suggested by the ostensibly more favorable result obtained when another aliquot of the same RNA was 3'-³²P end labeled and then analyzed in the same fashion on a polyacrylamide gel (Fig. 1A).

The contrasting results obtained with 5' and 3'-³²P end labeled samples of the RNA seemed most readily explained by acid-promoted hydrolysis of the synthetic RNA during the deblocking procedure (Fig. 2). As illustrated in the figure, acid-catalyzed hydrolysis would be expected to produce new 5'-OH termini, which would be amenable to radiolabeling. However, 3'-³²P end labeling at sites of hydrolysis would not be possible due to the presence of 2',3'-cyclic phosphate termini. While acid-catalyzed RNA hydrolysis would obviously reduce the yield of the desired RNA, a potentially more serious problem is that the same pentavalent intermediate (i) that likely results in RNA hydrolysis can plausibly also lead to phosphoryl migration, affording 2'→5' linkages in the RNA (Fig. 2).

Figure 2. Chemical scheme illustrating that the mechanism envisioned for RNA cleavage (via processes a and b) proceeds through an intermediate (i) that could also lead to phosphoryl migration (via processes a and c). The cleavage products formed via processes a and b could potentially be radiolabeled at the newly formed 5'-termini, but not at the newly formed 3'-termini.

In order to determine whether the acidic conditions used for RNA deblocking had, in fact, caused phosphoryl migration, we studied the effect of acid treatment on an authentic RNA, the enzymatically prepared homopolymer poly(uridylic acid). Poly(uridylic acid) was treated with 0.01 N HCl, pH 2.2, that is conditions similar to those utilized previously for Fpmp deblocking (15). As shown in Figure 3, when the acid-treated poly(uridylic acid) was degraded enzymatically and then analyzed by C₁₈ reversed phase HPLC, a peak having a retention time of ~23.5 min appeared in addition to uridine. This peak was shown to be identical to uridylyl(2'→5')uridine by direct comparison with an authentic sample, prepared by chemical synthesis. Authentic uridylyl(3'→5')uridine eluted at ~25 min under the same conditions, indicating that the new peak was not due to incomplete digestion of poly(uridylic acid).

The extent of phosphoryl migration under several different conditions is summarized in Table 1A. The extent of migration ranged from ~4–7.5% under the conditions employed. A control sample of poly(uridylic acid) that was degraded enzymatically without any acid treatment gave no detectable phosphoryl migration. The extent of phosphoryl migration was next measured

under a greater range of acidic conditions, especially those involving less forcing treatments; poly(uridylic acid) was again employed as the substrate. As shown in Table 1B and C, the extent of migration varied according to the conditions employed. While some of the differences in results are likely due to run-to-run variations, the overall trend indicated less migration than in Table 1A. It is probably worth noting that the percent U^{2'}p^{5'}U in the acid-treated samples was determined after recovery by ethanol precipitation, a process that would disfavor recovery of oligonucleotides that had undergone cleavage to smaller fragments (i.e. via pathway b in Fig. 2). It is conceivable, albeit unlikely, that the extent of phosphoryl migration within these fragments might differ from that in the recovered oligonucleotides.

Also measured was the extent of phosphoryl migration using samples of 2'-O-Fpmp-protected (Up)₂₀U obtained from two commercial suppliers. The use of conditions similar to those in Table 1B and C gave slightly more favorable results than those obtained with poly(uridylic acid), possibly due to the lesser length of the substrate. As shown in Table 2A, it was possible to deblock 2'-O-Fpmp-protected (Up)₂₀U under conditions that afforded <1% phosphoryl migration; this was reduced to <0.5% through

Figure 3. Analysis of poly(uridylic acid) for phosphoryl migration as a consequence of treatment with aqueous acid. A sample of poly(uridylic acid) that had been treated with 0.01 N HCl, pH 2.2, at 25°C for 20 h (cf Table 1) was degraded by incubation in the presence of RNases A, T₁ and T₂. Following treatment with calf intestinal alkaline phosphatase to hydrolyze all phosphate monoesters, the incubation mixture was analyzed by HPLC on an Alltech C₁₈ reversed phase column. The peaks at 16.15 and 23.49 min co-eluted with authentic samples of uridine and uridylyl(2'→5')uridine respectively.

further optimization (Table 2B). As noted in Table 2C, these values compared favorably with the phosphoryl migration observed upon deblocking of 2'-O-TBDMS protected (Up)₂₀U with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran. Although the conditions used for the latter process were typical (16), it should be noted that we have made no attempt to optimize the TBAF-promoted deprotection.

Table 1. Phosphoryl migration resulting from acid treatment of enzymatically synthesized poly(uridylic acid)

Acid treatment conditions	Phosphoryl migration (%) ^a
A ^b –	– ^c
0.01 N HCl, pH 2.2	6.0
15% Acetic acid, pH 2.1	7.6
4% Acetic acid, pH 2.3	5.2
1% Acetic acid, pH 2.5	3.9
B 4°C, 26.5 h	
0.01 N HCl, pH 2.25	1.9
15% Acetic acid, pH 2.5 ^d	1.3
C 4°C, 72 h	
0.01 N HCl, pH 2.25	2.1
15% Acetic acid, pH 2.5 ^d	2.9
4% Acetic acid, pH 2.6 ^d	2.7

^aDetermined as amount of U^{2'}p^{5'}U present in samples after enzymatic digestion, relative to all internucleotide linkages in the acid-treated sample.

^bAll samples were incubated under the acidic conditions indicated at 25°C for 20 h.

^c<0.25%.

^dpH adjusted to final value with aqueous NaOH.

DISCUSSION

As noted above, there have been increasing numbers of studies involving RNAs of defined composition. Further, many of these involve RNAs containing modified nucleosides, or employ amounts of RNAs most conveniently available via chemical synthesis.

Obviously, the validity of such studies depends critically on the purity of the synthetic RNAs prepared, especially where the studies involve direct physical measurements of the synthetic RNAs. It is known, for example, that RNAs containing 2'→5' phosphodiester linkages exhibit lower melting temperatures (1), suggesting that control of phosphoryl migration during RNA synthesis is particularly important.

Table 2. Phosphoryl migration accompanying acid deprotection of 2'-O-Fpmp protected (Up)₂₀U

Acid deprotection conditions	Phosphoryl migration (%)
A 0.25 M NaOAc, pH 3.1 ^b	0.60
0.5 M NaOAc, pH 3.6 ^b	0.72
0.25 M NaOAc, pH 4.4 ^b	0.50
0.5 M NaOAc, pH 4.4 ^b	0.59
B 2'-O-Fpmp (Up) ₂₀ U ^c	
25°C, 20 h	0.40
25°C, 38 h	0.46
4°C, 48 h	0.29
C 2'-O-silyl (Up) ₂₀ U ^d	
25°C, 20 h	1.2, 1.4

^aAll samples were deprotected under the acidic conditions indicated at 4°C for 48 h.

^bpH adjusted to final value by admixture of aqueous NaOH to acetic acid solution.

^cDeprotected with 0.5 M NaOAc, pH 3.25.

^dDeprotected with 1 M tetrabutylammonium fluoride in tetrahydrofuran.

At the outset of our study, it had been reported that no migration of internucleotide linkages occurred concomitant with acid-catalyzed deprotection of 2'-O-protected oligonucleotides (15,19,20). One of these studies employed ApA for up to 240 h at 23°C, but did not result in detectable phosphoryl migration (20). It may be noted, however, that this study employed dinucleoside monophosphates as substrates. The absence of phosphoryl migration noted by Norman *et al.* (20) parallels our finding that less phosphoryl migration was noted when we treated 2'-O-Fpmp (Up)₂₀U with acid, than when the much longer poly(uridylic acid) was employed under comparable conditions. A second study employed 2'-O-Fpmp protected oligonucleotides of mixed sequences that included oligonucleotides 10, 19 and 37 nt in length (15). These were analyzed following deprotection with 0.01 N HCl, pH 2.0–2.3, at room temperature for 20 h. The method of analysis involved a comparison of the relative proportions of the four ribonucleosides, obtained after enzymatic digestion of the oligonucleotides, with the theoretical ratio based on intended oligonucleotide composition. These ratios were in reasonable agreement, consistent with lack of significant phosphoryl migration since the resulting 2'→5' phosphoryl linkages would have been resistant to enzymatic degradation. However, this method of characterization was not specifically designed to measure phosphoryl migration and lacks the sensitivity of detection of the method employed in the present study. If phosphoryl migration had occurred randomly throughout the synthesized RNA molecules, the relative proportions of the four ribonucleosides

obtained following enzymatic digestion might still have approximated the calculated proportions.

A recent study from the same laboratory (21) employed conditions analogous to those described here, and reached similar conclusions about the need for careful pH control to preclude phosphoryl migration. The quality of the RNA produced under optimal deblocking conditions from 2'-O-Fmp-protected oligonucleotides seems entirely suitable for most applications.

We were surprised to find evidence for phosphoryl migration upon treatment of 2'-O-silyl-protected (Up)₂₀U with tetrabutylammonium fluoride (TBAF), since it has been reported that no migration occurs concomitant with desilylation (13). However, this conclusion was also based on the lack of material resistant to enzymatic degradation, when synthetic oligoribonucleotides were subjected to enzymes known to specifically hydrolyze 3'→5' phosphodiester linkages. As noted above, this type of assay may not be appropriate for measuring phosphoryl migration, especially where limited amounts of migration at any given position are involved.

The present study of phosphoryl migration has been limited to the use of poly(uridylic acid) and synthetic uridine oligoribonucleotides. It has been noted that acid-promoted strand cleavage is sequence-dependent and that cleavage and phosphoryl migration occur approximately in a 2:1 ratio (21); since cleavage and phosphoryl migration proceed via a common intermediate, it is not illogical to think that phosphoryl migration may also exhibit sequence dependence. The protocol for analysis of phosphoryl migration utilized in this study should be more generally applicable for substrates of mixed sequence.

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REFERENCES

- 1 Kierzek, R., He, L. and Turner, D.H. (1992) *Nucleic Acids Res.*, **20**, 1685–1690.
- 2 Talbot, S.J., Goodman, S., Bates, S.R.E., Fishwick, C.W.G. and Stockley, P.G. (1990) *Nucleic Acids Res.*, **18**, 3521–3528.
- 3 Ogilvie, K.K., Usman, N., Nicoghossian, K. and Cedergren, R.G. (1988) *Proc. Natl. Acad. Sci. USA.*, **85**, 5764–5768.
- 4 Gasparutto, D., Livache, T., Bazin, H., Duplaa, A.-M., Guy, A., Khorlin, A., Molko, D., Roget, A. and Teoule, R. (1992) *Nucleic Acids Res.*, **20**, 5159–5166.
- 5 Sproat, B.S., Beijer, B., Grotli, M., Ryder, U., Morand, K.L. and Lamond, A.I. (1994) *J. Chem. Soc., Perkin Trans.*, **1**, 419–431.
- 6 Thomson, J.B., Tuschl, T. and Eckstein, F. (1993) *Nucleic Acids Res.*, **21**, 5600–5603.
- 7 Komatsu, Y., Koizumi, M., Nakamura, H. and Ohtsuka, E. (1994) *J. Am. Chem. Soc.*, **116**, 3692–3696.
- 8 Fu, D.J., Benseler, F. and McLaughlin, L.W. (1994) *J. Am. Chem. Soc.*, **116**, 4591–4598.
- 9 Salazar, M., Champoux, J.J. and Reid, B.R. (1992) *Biochemistry*, **32**, 739–744.
- 10 Fedoroff, O.Y., Salazar, M. and Reid, B.R. (1993) *J. Mol. Biol.*, **233**, 509–523.
- 11 Brown, T. and Brown, D.J.S. (1991) In Eckstein, F. (ed.) *Oligonucleotides and Analogues*. Oxford University Press, pp. 4–9.
- 12 Gait, M.J., Pritchard, C. and Slim, G. (1991) In Eckstein, F. (ed.) *Oligonucleotides and Analogues*. Oxford University Press, pp. 25–32.
- 13 Usman, N., Ogilvie, K.K., Jiang, M.-Y. and Cedergren, R.J. (1987) *J. Am. Chem. Soc.*, **109**, 7845–7854.
- 14 Goodwin, J.T., Stanick, W.A. and Glick, G.D. (1994) *J. Org. Chem.*, **59**, 7941–7943.
- 15 Rao, M.V., Reese, C.B., Schehlmann, V. and Yu, P.S. (1993) *J. Chem. Soc., Perkin Trans.*, **1**, 43–45.
- 16 Hogrefe, R.I., McCaffrey, A.P., Borozdina, L.U., McCampbell, E.S. and Vaghefi, M.M. (1993) *Nucleic Acids Res.*, **21**, 4739–4741.
- 17 Hassur, S.M. and Whitlock, H.W. (1974) *Anal. Biochem.*, **59**, 162–164.
- 18 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor University Press, Cold Spring Harbor, NY.
- 19 Griffin, B.E., Jarman, M. and Reese, C.B. (1968) *Tetrahedron*, **24**, 639–662.
- 20 Norman, D.G., Reese, C.B. and Serafinowska, H.T. (1984) *Tetrahedron Lett.*, **25**, 3015–3018.
- 21 Capaldi, D.C. and Reese, C.B. (1994) *Nucleic Acids Res.*, **22**, 2209–2216.