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Production of double-stranded RNA during synthesis of bromouracil-substituted RNA by transcription with T7 RNA polymerase

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Abstract Using T7 RNA polymerase we synthesized a short oligoribonucleotide containing bromouracil by in vitro transcription of a synthetic DNA template. Whereas the major transcript obtained had the expected size and was apparently homogeneous on a sequencing gel, additional analysis revealed the presence of double-stranded RNA in this preparation. As this was not observed when the same template was transcribed in the presence of uracil, we hypothesize that bromouracil promoted the apparition of double-stranded 'parasitic' RNA presumably by favouring priming for the RNA-dependent RNA synthesis of the T7 RNA polymerase or by facilitating an end-to-end copy mechanism.

Key words: T7 RNA polymerase; Transcription (in vitro); RNA synthesis; Modified nucleotide; Bromouracil

1. Introduction

The synthesis of milligram amounts of short RNAs is required for many physico-chemical studies dealing with the structure and function of RNAs. The most popular procedure is the one described by Milligan et al. [1,2] which allows the production of multimilligram amounts of oligoribonucleotides and authorizes the incorporation of some modified nucleotides, an interesting property for many of the aforementioned studies. For example, incorporation of photosensitive analogs is very useful for elucidating tertiary interactions in RNAs by the possibility it offers to generate intrastrand crosslinks [3] or when studying protein–RNA interactions by creating protein–nucleic acid crosslinks under irradiation with UV light [4]. We have used this reaction to generate a short, 12 nucleotide long bromouridine-substituted RNA which, once annealed to its complementary DNA strand, was used to identify RNA–DNA hybrid binding proteins in whole cell extracts (unpublished results) by photoaffinity radiolabelling [5]. In the preparation of transcript purified according to its size by denaturing gel electrophoresis we have found significant amounts of double-stranded RNA (dsRNA). The presence of this contaminating dsRNA results very likely from elongation of abortive transcripts by the RNA-dependent RNA synthesis activity of the T7 RNA polymerase recently described [6]. This phenomenon is favoured by BrU, probably as a consequence of enhanced stability of putative primers of this reaction due to the increased stability of BrU–A compared to A–U base pairs, or alternatively, from a possible facilitated utilization of BrU as initiator nucleotide for an end-to-end copy mechanism.

2. Materials and methods

2.1. Enzymes and chemicals

Nucleoside triphosphates, including BrUTP were from Boehringer Mannheim. Stock solutions were made in autoclaved water and titrated to pH 8.1 with autoclaved Tris base 1 M. Concentrations (between 35 and 100 mM) were determined from their absorbance at maximum; T7 RNA polymerase was prepared from the overproducing strain BL21/pAR1219 [7] according to the procedure of Grodberg and Dunn [8].

2.2. DNA synthesis and purification

Oligodeoxyribonucleotides (listed in Table 1) were synthesized by solid phase synthesis using phosphoramidite chemistry on a Milligen 7500 DNA synthesizer. DNA was deprotected by overnight (≈14 h) incubation at 55°C in concentrated ammonium hydroxide. After removal of the ammonium hydroxide under vacuum, the DNA was purified on a denaturing 20% (w/v) polyacrylamide gel. The DNA band was localized by its UV shadow, cut out, eluted and ethanol precipitated according to standard procedures [9]. It was redissolved in autoclaved, high quality deionised water.

2.3. RNA synthesis

2.3.1. Preparation of the annealed DNA template. 4 nmol of the bottom strand were mixed with a slight excess of the top strand (4.4 nmol) in 497.5 μl TE buffer (Tris-HCl pH 8.0 10 mM, EDTA 1 mM), then heated for 1 min at 100°C. The solution was allowed to cool slowly to room temperature, then 2.5 μl $MgCl_2$ 1 M was added and the solution was incubated further at 37°C.

2.3.2. Transcription reaction. After optimisation in small (50 μl) reaction volumes, large scale (5 ml) production of RNAs were performed in buffer containing 40 mM Tris-HCl (pH 8.1 at 37°C), 25 mM MgCl2, 5 mM DTT, 1 mM spermidine, 0.01% (v/v) Triton X-100, with 4 mM each NTP, 0.8 μM DNA template and 0.03 mg/ml T7 RNA polymerase for 3 h at 37°C. In addition, a small amount (37 kBq [α-^{32}P]ATP, 111 TBq/mmol) was included in transcriptions with BrUTP to allow the visualisation of BrU-substituted transcripts by overnight autoradiography instead of UV shadowing. Transcription products were purified as described [10].

2.4. Concentrations of oligos

Concentrations of oligos were determined by their absorbance at 260 nm, using extinction coefficients calculated from their base composition and sequence as indicated in [11].

2.5. Identification of the 3'-terminal nucleotide

Identification of the 3'-terminal nucleotide of transcripts was done by complete RNase digestion of oligonucleotides 3'end-labelled by the addition of [^{32}P]Cp (NEN, 111 TBq/mmol) with T4 RNA ligase [12], and two-dimensional PEI-cellulose TLC with 3' phosphate nucleosides (Ap, Cp, Gp and Up) as standards [13]

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2.6. Melting curves

Oligonucleotides at the desired concentration were made in 10 mM sodium cacodylate pH 7, sodium chloride 0.1 M. They were placed in a 1-cm path-length quartz cuvette inserted in a cuvette-holder thermostatted by a 'Ministat' water bath from Huber, the temperature of which was externally controled by a Huber PD 410 programmer. A cuvette containing buffer alone was placed in the reference beam. Temperature of the solution contained in that cuvette was continuously recorded through a thermistance captor immerged in the solution outside of the path of the beam. Optical densities were recorded at 260, 280 and 330 nm during an integration time of 2 s, every 20 s from 25°C to 90°C. The rate of the temperature increase was 30°C/h.

3. Results and discussion

The sequence (GGGAUACAUACA) of the BrU-substituted RNA was choosen in such a way as to meet several criteria, some relevant to the planned photoirradiation experiments (Revers et al., unpublished results) and others derived from constraints imposed by the procedure of synthesis, such as the choice of the 3 consecutive Gs present at the 5′ end, as it corresponds to one of the preferred initiation sequence for the T7 RNA polymerase [1]. A time course of this reaction, performed over 3 h in optimal conditions (see section 2) is presented in Fig. 1. From the radioactivity present in the major transcript of the expected size we could calculate that 2 nmol of the RNA oligonucleotide was synthesized, indicating that each template had been used 50 times to produce full-length transcript and that, under these conditions, one should expect 0.2 μmol of this transcript in the large scale (5 ml reaction volume) synthesis. Instead we obtained 90 nmol of purified 12-mer only, presumably as the result of lower transcription efficiency but also, more likely, from cumulative losses all along the purification of the transcript.

In order to determine the stability of our photoreactive RNA–DNA hybrid, its melting temperature was measured as indicated in section 2. As shown in Fig. 2, the melting curve of the bromoU-substituted RNA–DNA hybrid displayed 2 transitions, one at ≈50°C and another one of smaller amplitude at ≈70°C. These transitions were also observed when the temperature was decreased after the hybrid has been melted, demonstrating that the fusion processes observed are perfectly reversible. The transition at 50°C was expected from thermodynamic calculations: if we assume that thermodynamic parameters for RNA–DNA hybrids are identical to those established for RNA helix initiation and propagation [14], then parameters calculated for the hybrid are: $\Delta H^0 = -106.7$ kcal/mol, $\Delta S^0 = -289.9$ entropy units, and $\Delta G^0 = -16.5$ kcal/mol. As $1/T_m = (R/\Delta H^0)\mathrm{Ln}C_t + (\Delta S^0 - R\mathrm{Ln}4)/\Delta H^0$ [11], where C_t represents the strand concentration, we could predict a T_m of 61.5°C in 1 M NaCl. As $\Delta T_m/\Delta\log[Na^+] \approx 17$°C [15,16], then the T_m under our experimental conditions (0.1 M NaCl) should be close to 44.5°C. This agrees with the experimental value, $T_m = 45.6$°C, determined for the unsubstituted hybrid, that is the hybrid where the RNA strand was obtained by transcription with UTP (Fig. 2, panel B). Consequently, the higher value ≈50°C obtained with the brominated hybrid is due to a contribution of BrU to the stability of the hybrid, as observed for other double helices [17] and ascribed to the better stacking properties of BrU, compared to U [16,18]. The second transi-

Table 1
Oligodeoxyribonucleotides used in this study

Top strand	5′ TAATACGACTCACTATA 3′
Bottom strand 1	3′ ATTATGCTGAGTGATATCCCTATGTATGT 5′
Bottom strand 2	3′ ATTATGCTGAGTGATATCCGTATGTATCT 5′
cDNA 1	3′ CCCTATGTATGT 5′
cDNA 2	3′ CCGTATGTATCT 5′

Differences in sequence are highlighted by bold face letters.

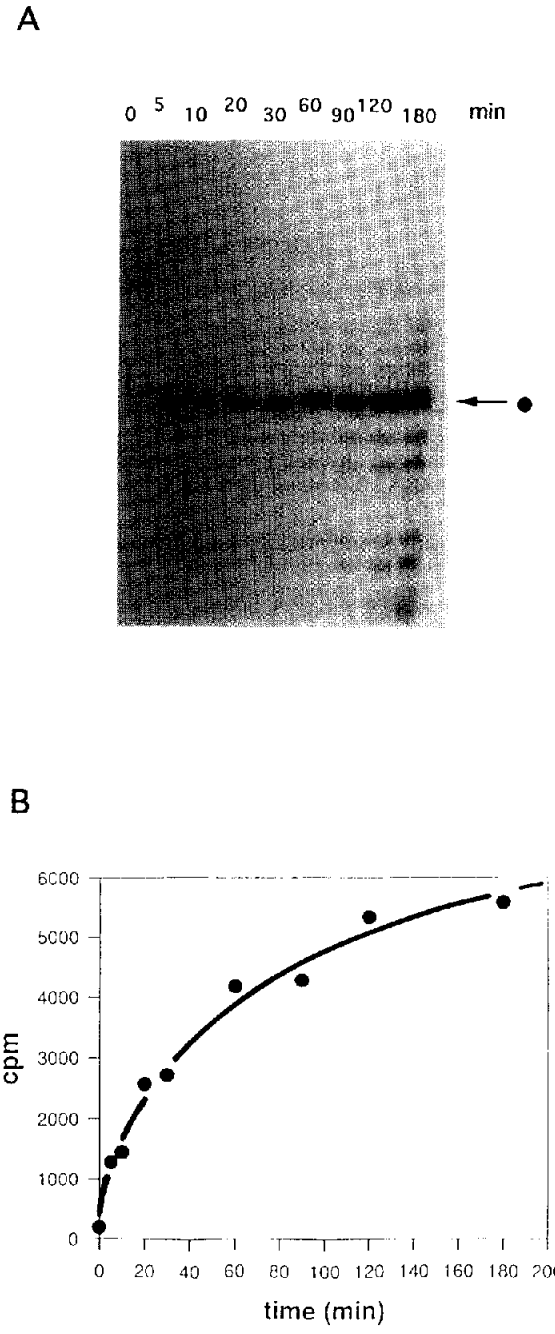

Fig. 1. Time course of the transcription reaction. DNA template (0.8 μM) was made of top strand annealed to bottom strand 1. Reaction conditions were those used for large scale syntheses (NTPs 4 mM with 10 μCi [α-^{32}P]ATP but in a small reaction volume (50 μl). Analysis of transcription products contained in 5 μl aliquotes by gel electrophoresis on a 20% acrylamide 8 M urea gel is shown in panel A. Electrophoresis was run until bromophenol blue reached the bottom of the gel. The bands corresponding to the major 12-mer transcript (●) were cut out and counted (panel B).

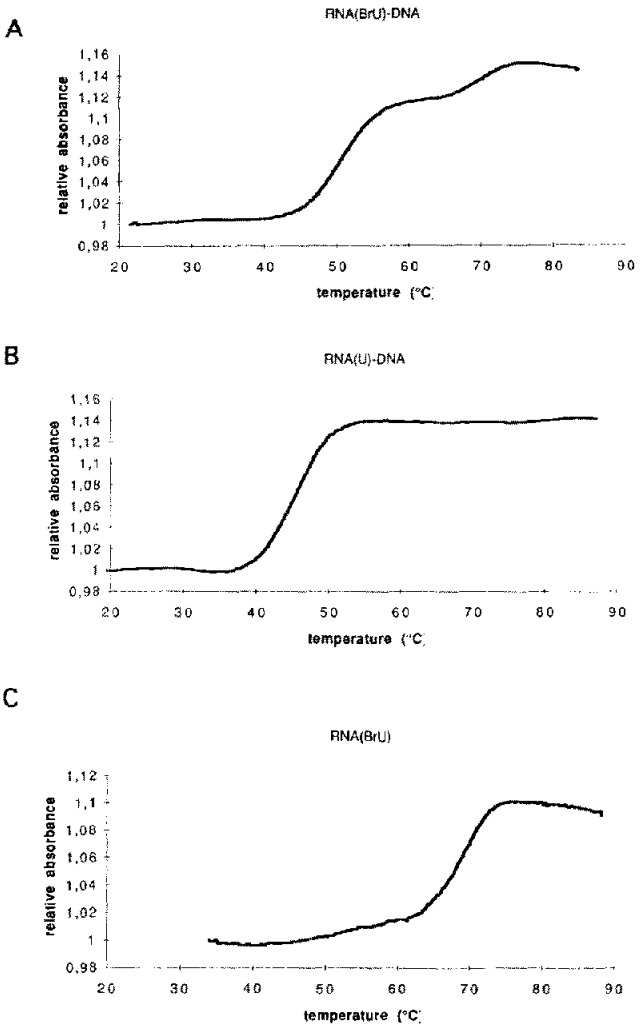

Fig. 2. Melting curves of 1 μM BromoU-substituted RNA–DNA hybrid (panel A), of 1 μM RNA DNA hybrid (panel B) and 1 μM RNA strand alone (panel C) in sodium cacodylate, pH 7.0, 10 mM, sodium chloride 100 mM. Absorbance was recorded at 260 nm. The RNA strand was obtained by transcription of the bottom strand 1 annealed to top strand, and the DNA strand was oligonucleotide cDNA1 (see Table 1).

tion at a higher temperature was unexpected. We suspected that it could be a property of the transcript itself as it has been found that, in some occasions, transcripts obtained with T7 RNA polymerase can be perfectly self-complementary due to RNA-dependent RNA polymerase activity of this enzyme [6]. As a matter of fact, the RNA itself underwent a well defined transition at ≈70°C (Fig. 2, panel C). This led us to suspect the RNA transcript to be heterogenous despite it moved as a perfectly defined species, 12 nucleotides long, on a sequencing gel. 3′-end analysis (see section 2) of this transcript has shown that a significant proportion (≈35%) ended with a C residue instead of the expected A residue (Fig. 3). Heterogeneities at the 3′ end are observed for transcripts at the end of which the polymerase has randomly added an extra nucleotide [1,2]. However, alkaline hydrolysis of 5′end-labeled transcripts indicated a size of 12 nucleotides and not 13 (data not shown), as expected when this mechanism had been responsible for the observed 3′-end heterogeneity. Therefore we concluded that part of the transcription products corresponded to double-stranded RNA with an apparent length of 12 nucleotides. This could originate from the production of the antisense strand of the DNA-encoded transcript (primary transcript) by an end-to-end copy mechanism of the primary transcript, as observed in some instances with the SP6 polymerase [19], or by primer extension of abortive products of the transcription, as proposed by Cazenave and Uhlenbeck [6] (see Fig. 4). Both mechanisms would lead to the production of double-stranded RNA consistent with a size of 12 nucleotides on a denaturing polyacrylamide-urea gel, and with a predicted high thermal stability, the primer extension leading to the obtention of 7 GC base pairs, and the end-to-end copy mechanism leading to 7 BrU–A base pairs of increased stability compared to normal A–U base pairs.

The production of this double-stranded RNA is absolutely dependent on the presence of BrU as homologous transcripts obtained with UTP did not display this melting transition (data not shown). In an attempt to lower the amount of these aberrant RNAs we have changed slightly the sequence of the transcript to GGCAUACAUAGA , by using bottom strand 2 for transcription (Table 1) with the idea that this alteration of the 3′ sequence could decrease the efficiency of the putative end-to-end copy mechanism or would destabilize the possible partial annealing of abortive product to the full-length product (see Fig. 4). This should not, however, prevent abortive products to pair to each other. Disappointingly, the resulting purified transcript displayed the same behavior as the former one, indicating that this reaction is not very sensitive to sequence effects.

The two schemes of primer extension proposed (Fig. 4) are both plausible. Their common feature is that the 2 terminal base pairs are BrU–A base pairs, and the last base is a bromouracil, which could explain the critical importance of using BrU instead of U to observe the production of dsRNA. Moreover, we have found during our optimisation reactions the presence of transcripts of ≈14 or ≈16 nt, already detectable (see Fig. 1) at times longer than 1 h, but much more intense in reactions performed with lower concentrations of NTPs, or higher amounts of enzyme; these longer products could be easily explained by a primer extension mechanism (Fig. 4, panel B)

The end-to-end copy mechanism would involve initiation at the 3′ end of the sense (DNA template-encoded transcript) transcript, the initiating nucleotide being BrU if initiation occurs in front of the ultimate nucleotide of the transcript, or G (the preferred nucleotide of T7 RNA polymerase for initiation of genuine transcription) if initiation takes place in front of the

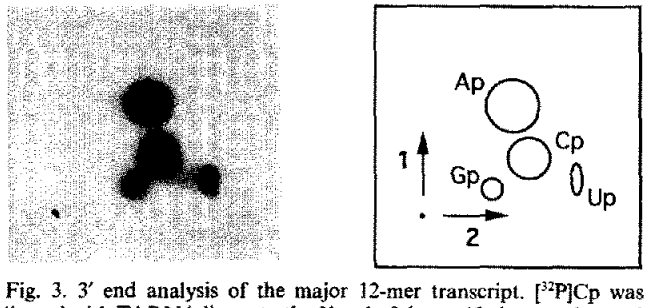

Fig. 3. 3′ end analysis of the major 12-mer transcript. [^{32}P]Cp was ligated with T4 RNA ligase to the 3′ end of the purified transcript, and the resulting oligonucleotide was digested with a cocktail of nucleases producing nucleosides 3′ phosphate which were then separated by two-dimensional thin-layer chromatography.

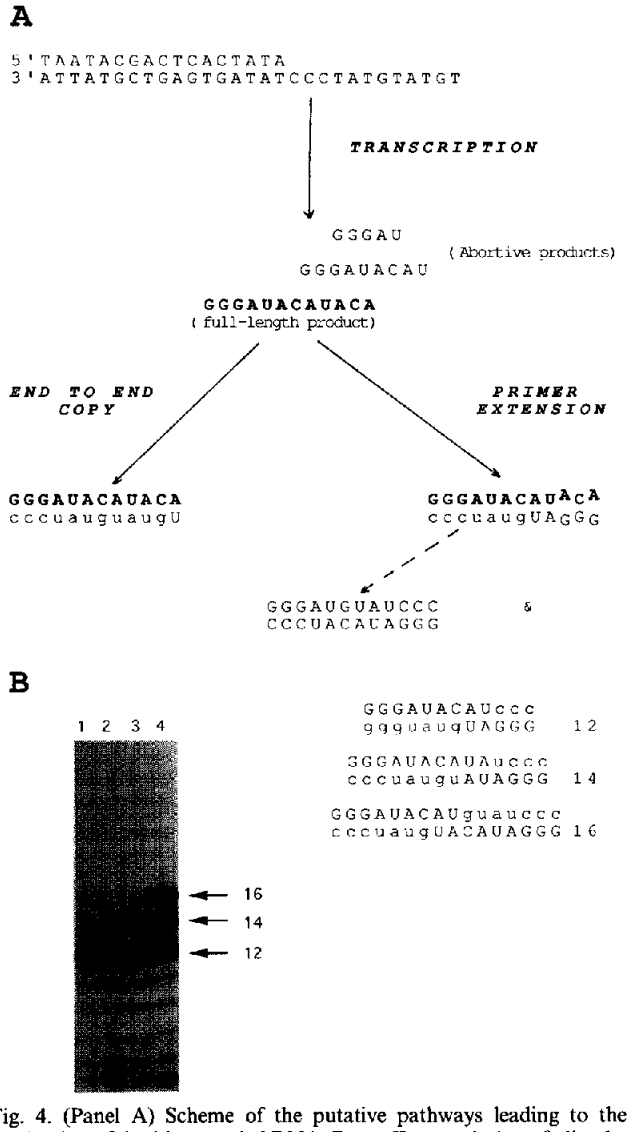

Fig. 4. (Panel A) Scheme of the putative pathways leading to the production of double-stranded RNA. Run-off transcription of oligodeoxyribonucleotides produces the expected full-length product (bold capital letters) together with shorter abortive products (standard capital letters). Besides a direct end-to-end copy of the full-length transcript (left), imperfect annealing of abortive products to the full-length product can lead to double-stranded RNA by primer extension of the abortive product (extensions are indicated with lower case letters) followed by hybridization of two such extended products to give a perfect RNA duplex. Alternatively, hybridizations between abortive products can give rise to double-stranded RNA of various sizes. (Panel B) Transcription products obtained with various concentrations of T7 RNA polymerase, illustrating the presence of longer transcription products predicted by the primer extensions occuring between abortive products proposed in the scheme depicted in panel A. Final concentrations of T7 polymerase were 16 (lane 1), 19 (lane 2), 22 (lane 3), 29 μg/ml (lane 4). Transcription was for 30 min with 1 mM each NTP. Sizes are indicated to the right.

penultimate base (alkaline hydrolysis of our transcript has indicated that the longest species in the mixture of RNAs is 12 nucleotides long, but cannot exclude the possible presence of an RNA one nucleotide shorter, as sometimes, oligonucleotides with slightly different sizes but of different base composition have identical mobilities in denaturing sequencing gels [20]). Since changes of the penultimate nucleotide did not prevent the production of double-stranded RNA, initiation at the very 3′ end with BrUTP seems more plausible. Attempts to distinguish between the various possible mechanisms depicted in Fig. 4, by enzymatic sequencing of the mixture of sense and antisense transcripts have failed mostly because of their double-stranded nature, which prevents many positions to be efficiently cleaved by the nucleases. In these attempts, however, we detected the presence of two U at positions near the 5′ end and compatible with the sequence expected for the antisense produced by an end-to-end copy mechanism (data not shown) indicating that this latter is undoubtly at work for the production of antisense in our experimental conditions, but its overall contribution relative to the primer extension mechanism remains to be established.

In conclusion, extra care has to be taken when designing RNAs to be synthesized by transcription with T7 RNA polymerase when BrUTP is planned to be substituted for UTP in that reaction, and, in any case, a complementary analysis of the final product seems to be highly desirable at all times.

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