A Highly Efficient Method for Long-Chain cDNA Synthesis Using Trehalose and Betaine

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Obtaining full-length cDNA is important for many molecular biology methods like cDNA library construction or RACE-PCR (rapid amplification of cDNA ends). We have found that the inclusion of betaine alone and in combination with trehalose in reverse transcription results in longer cDNA synthesis products. As shown on the 14-kb long clathrin mRNA with real-time PCR, a combination of 2 M betaine and 0.6 M trehalose leads to almost 9 times more cDNA with a length of 12.5 kb. This is due to the ability of betaine to resolve secondary structures of the RNA, thereby decreasing its melting temperature. The application of betaine in combination with trehalose should prove useful in all laboratory methods relying on full-length cDNA.

The efficiency of first-strand cDNA synthesis from mRNA in the reverse transcription procedure is a major prerequisite for subsequent protocols like reverse transcriptase polymerase chain reaction (RT-PCR), 5′ RACE-PCR (rapid amplification of 5′ cDNA ends), or the construction of cDNA libraries.

The formation of secondary structures such as loops or hairpins by self-complementary regions or other conformational features associated with GC-rich template sequences within the mRNA may lead to the sudden termination of cDNA synthesis due to the sensitivity of some polymerases to stop at certain sites. This undesired effect counts for reverse transcriptases (1–3) as well as for low- and high-temperature polymerases (4, 5) and results in the occurrence of strong stops in sequencing reactions or inefficient amplicon yields in the polymerase chain reaction (PCR).

Betaine (trimethylglycine) is a naturally occurring methyl group donor which has also been shown to play a major role in the saline tolerance of several bacteria when induced to osmotic stress (6, 7). The action of betaine is twofold: While drastically stabilizing proteins against thermal unfolding (8), it can also reduce or eliminate the base pair composition dependance of DNA thermal melting transitions by destabilizing the DNA helix (9, 10). The latter effect has been used to overcome sequence- or structure-induced problems encountered in sequencing (11) or PCR (12, 13).

Trehalose is an endogenously synthesized stress protectant in Escherichia coli (14). Like betaine, its main function is to protect proteins from thermal denaturation by reducing fluctuations within the main backbone of the protein (15, 16). The heat-stabilizing or even heat-activating properties of this compound have proved useful for the enhancement of several enzymatic reactions applied in molecular laboratory work, i.e., cDNA synthesis, DNaseI or RNaseI digestion, restriction enzyme digestion (17), or mRNA display (18).

Here we report the application of a combination of these two compounds for gaining efficiency in first-strand synthesis that is superior to the use of trehalose alone. This results from the ability of betaine to decrease the melting temperature not only of DNA, but also of RNA, leading to less formation of secondary structures within the RNA template at temperatures employed in the process of reverse transcription, as shown by RNA melting curves conducted on a complex mRNA pool and the highly GC-rich and structured mRNA of the bovine oxytocin receptor. We were able to quantify this enhancement in efficiency by phosphor-imaging of labeled cDNA and real-time quantitative
PCR for two housekeeping genes and for different regions at varying distances from the reverse transcription priming site of the 13.8-kb-long mouse clathrin mRNA.

MATERIALS AND METHODS

Reverse Transcription

A total of 5 µg of total RNA was reverse transcribed after being primed with 500 ng Oligo-dT 25 in a total volume of 30 µl using 5× first-strand buffer (250 mM Tris–HCl, 375 mM KCl, 7.5 mM MgCl 2, pH 8.3, at 20°C), 10 mM dithiothreitol, 500 µM dNTPs, and 200 U MMLV reverse transcriptase (Gibco BRL, MD) according to the manufacturer's protocol at 42°C. For more efficient first-strand synthesis, 2 M betaine (Sigma, Deisenhofen; made from 5 M stock), 0.6 M trehalose (Sigma; made from 2 M stock), or a combination of these two compounds was added. For this purpose, a 10× first-strand buffer, attained by evaporation of the 5× first-strand buffer in a speedvac and reconstitution in half the volume of water, was used. The following temperature profiles were applied: 2 M betaine: 42°C for 90 min; 0.6 M trehalose: 42°C for 30 min, then 60°C for 1 h; 2 M betaine + 0.6 M trehalose: 42°C for 30 min, then 60°C for 1 h. A 5-µl aliquot was reverse transcribed in the presence 0.2 µl [α-32-P]dCTP (100 µCi/µl, Amersham). All experiments were done in triplicate.

Denaturing Gel Electrophoresis

The labeled reverse transcription samples were electrophoresed in a 1% agarose gel overnight in 30 mM NaOH, 1 mM EDTA at 1 V/cm. The gel was dried on top of a nylon membrane (Nytran, Schleicher & Schuell) to prevent losses during the drying process. The membrane/gel sandwich was exposed to a phosphorimage screen for 16 h. The screen was imaged at 50-µm resolution with the Storm phosphoimager (Molecular Dynamics, Sunnyvale, CA) and total counts as well as the distribution of the counts were analyzed with ImageQuan software.

Real-Time Quantitative PCR

A total of 2 µl from the triplicate reverse transcription reactions was diluted 20-fold, and 2 µl was used for a real-time quantitative PCR using the LightCycler instrument (Roche, Basel) and the FastStart kit (Roche). The primer pairs corresponding to different regions of the clathrin mRNA and to two different housekeeping genes employed for the PCR reactions are shown in Table 1.

The resulting PCR products were confirmed by electrophoresis in a 1.3% agarose gel in TBE buffer. The crossing-point of the real-time fluorescence curve was calculated by the accompanying data analysis software package.

RNA Melting Curve

The RNA melting curves were conducted in a closed peltier-heated cuvette system (Gilford 2600 photometer) with 12 µg mouse muscle mRNA that had been purified three times with Oligotex latex beads (Qiagen, Germany) or 12 µg of a 297-nt cRNA fragment from the bovine oxytocin receptor (19) that had been synthesized by in vitro transcription and column-purified. The melting curves were conducted in 0.5 M NaCl/1× phosphate-buffered saline (PBS) or 0.5 M NaCl/1× PBS/2 M betaine. A melting curve with 0.5 M NaCl/1× PBS/2 M betaine without RNA was used as the control and subtracted from the RNA-containing samples.

The cuvette was heated at 1°C/min with data points collected every 0.5°C. The Tm of the melting curves was calculated by nonlinear fitting of the data points (Boltzmann sigmoidal approximation). In order to be certain that the resulting hyperchromicity was not due to RNA degradation, the samples were cooled to room temper-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Orientation</th>
<th>Sequence</th>
<th>Distance from poly-(A) (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clathrin1</td>
<td>Sense</td>
<td>5'-gctactgagcacaacacacagc</td>
<td>2435</td>
<td>65</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>5'-tataaacaccgtggagtgac</td>
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<tr>
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<td></td>
<td>Antisense</td>
<td>5'-tgacgtaaggcagaatggac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clathrin3</td>
<td>Sense</td>
<td>5'-gtttctcttgccgaggatccatt</td>
<td>12053</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-tgtagtgagcgagtgcccttgac</td>
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<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Sense</td>
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<td>1037</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>5'-acacactgtctaggagtggac</td>
<td></td>
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</tr>
<tr>
<td>GAPDH</td>
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<td>928</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5'-tgccgggacccgatgggatgg</td>
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</table>
nature for 6 h and the experiment was repeated. The optical density of the samples after cooling was within 3% variance of the initial absorbance values. All melting curves were performed in triplicate.

Calculation of RNA Secondary Structure

The secondary structure of the in vitro transcribed RNA fragment of the bovine oxytocin receptor was calculated by the Zucker algorithm (20) and displayed with RNAdraw V1.1 software (21). A temperature of 42°C was the basis of the calculation, as this is the optimal temperature for reverse transcription with MMLV reverse transcriptase (RT).

RESULTS AND DISCUSSION

Many of the modern techniques used in a molecular biology laboratory rely on intact and efficient synthesis of long-length cDNA from RNA. For producing cDNA libraries, it is desirable to have as many full-length cDNA clones as possible, as this will save the tedious work of cloning the missing sequences. For 5’ RACE-PCR, having a full-length cDNA is an absolute prerequisite for obtaining the complete 5’-end containing the transcription start site. Many improvements have been made in recent years for improving the efficiency of the reverse transcription procedure. These are based mainly on two different strategies. The first strategy was to engineer reverse transcriptases to delete the intrinsic RNAse H activity, which results in longer length cDNA products as a consequence of the elimination of RNA degradation (i.e., Superscript II reverse transcriptase from Gibco BRL). The other strategy is the isolation of reverse transcriptases with an elevated optimal enzymatic temperature. The main disadvantage yet of these enzymes is the remaining intrinsic RNAse H activity.

A recent article describes the application of the thermostabilizing compound trehalose in the application of cDNA synthesis (17). It was shown that trehalose not only stabilizes the MMLV-RT up to 60°C but also increases enzymatic activity at normal temperature (42°C). More recently, we have begun to apply betaine to the reverse transcription reactions. Betaine decreases the melting temperature of DNA by reducing the thermal stability of mainly the G–C nucleotide pairing (10). We concluded that this effect should also be applicable in resolving secondary RNA structures resulting from intramolecular base pairing and that a combination of both compounds could be even more effective.

Total RNA from mouse muscle was reverse transcribed in the presence of 2 M betaine, 0.6 M trehalose (17), and a combination of both. In earlier experiments we had found that 2 M betaine is the optimal concentration for reverse transcription and that higher concentrations had a negative effect on cDNA synthesis (data not shown). The reason is unclear, but the increasing viscosity acting as a “molecular brake” could be a possible explanation.

FIG. 1. (A) [32P]dCTP-labeled cDNA from different reverse transcription conditions electrophoresed on a denaturing agarose gel and exposed to phosphoimage screens. Lane A, control (no betaine, no trehalose); Lane B, 2 M betaine; Lane C, 0.6 M trehalose; Lane D, 2 M betaine and 0.6 M trehalose. The molecular weight annotation corresponds to the marker bands (1-kb ladder), and the white arrows indicate regions of cDNA synthesis terminations that seem to have been resolved under the different experimental regimes. (B) Densitometric presentation of the autoradiograph from (A). The distance of migration was plotted against the RFU (relative fluorescence units) from the phosphorimager scan. Graph 1, control (no betaine, no trehalose); Graph 2, 2 M betaine; Graph 3, 0.6 M trehalose; Graph 4, 2 M betaine and 0.6 M trehalose. Vertical straight and dotted lines represent 3 and 7.5 kb molecular weight, respectively.
Figure 1A shows the autoradiograph from these different conditions. Two major effects can be observed under these experimental regimes. The most prominent is the increase in average size of the synthesized cDNA in the presence of betaine alone (lane 2) and trehalose alone (lane 3). The second effect is the elimination of several strong bands that appear in the control lane and in the lane with trehalose alone, as depicted with white arrows. These bands appear to be the product of stops during the synthesis of cDNA, possibly from secondary structures present in some highly expressed mRNAs. In the lanes of cDNA reverse transcribed in the presence of betaine (lanes 2 and 4), these bands decrease in substantial amounts. As a general observation, the overall impression of these lanes is more homogeneous. We applied densitometry on these autoradiographs and quantified the distribution of signal in various size windows of the synthesized cDNA. In Fig. 1B the densitometric plots show the distribution of the incorporated radioactivity expressed as RFU (relative fluorescence units) from the phosphorimaging process, separated by two lines delimiting the different molecular sizes of the cDNA (straight line, 3 kb; dotted line, 7.5 kb). The “area under the curve” was quantified, and the times fold RFU in comparison to the control was calculated (Table 2). Several conclusions can be drawn from the data. The inclusion of betaine or trehalose in the reverse transcription mix leads to an increase in the synthesis of higher molecular size cDNA. In the low range under 3 kb trehalose is superior to betaine and the addition of betaine does not seem to have a substantial effect. In the medium range between 3 and 7.5 kb this effect is more pronounced. Remarkably, this is not the case in the high range over 7.5 kb. Only a combination of the two compounds results in a fivefold incorporation of radioactive label. A cooperative effect of these two compounds seems likely, resulting in a substantial increase of long-chain products.

To demonstrate the benefits of the combinational approach we examined the yield of cDNA products from mouse clathrin mRNA by applying primers specific for regions at varying distances from the priming site. The crossing-point from real-time quantitative PCR was taken as the appropriate marker to investigate the length of the attained products within this one defined mRNA species. The crossing-point is the number of cycles needed for entering the exponential amplification phase of PCR and is used by the accompanying software package as the best parameter for reliable quantification. As demonstrated in Fig. 2, there is no difference in the amount of synthesized cDNA for regions that are 3 or 4.5 kb distant from the priming site. At 12.5-kb distance, however, there is a substantial increase in cDNA synthesis product with betaine and trehalose alone and also an observable

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**TABLE 2**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total RFU</th>
<th>cDNA &lt; 3 kb</th>
<th>3 kb &lt; cDNA &lt; 7.5 kb</th>
<th>cDNA &gt; 7.5 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 M betaine</td>
<td>1.22 ± 0.08</td>
<td>1.05 ± 0.12</td>
<td>1.94 ± 0.35</td>
<td>2.64 ± 0.31</td>
</tr>
<tr>
<td>0.6 M trehalose</td>
<td>2.28 ± 0.21</td>
<td>1.32 ± 0.22</td>
<td>9.44 ± 0.62</td>
<td>2.70 ± 0.26</td>
</tr>
<tr>
<td>2 M betaine + 0.6 M trehalose</td>
<td>2.17 ± 0.32</td>
<td>1.30 ± 0.19</td>
<td>9.15 ± 0.71</td>
<td>4.96 ± 0.55</td>
</tr>
</tbody>
</table>

Note. Values are shown as fold area in comparison to the control curve (no betaine, no trehalose).

**FIG. 2.** Schematic representation of the PCR for evaluating the efficiency of the first-strand synthesis with primers specific for different regions of the mouse clathrin mRNA. Diagrams illustrate the number of PCR cycles to be run until the “crossing point.” C, Control; B, 2 M betaine; T, 0.6 M trehalose; B + T, 2 M betaine + 0.6 M trehalose.
additive effect of these two compounds when used in combination. The average cycles to the crossing point are $31.5 \pm 0.7$ for the control and $27.7 \pm 0.3$ for betaine and trehalose in combination. With a PCR efficiency of 1.74 (as calculated by the slope in the linear amplification phase) this counts for roughly 8.5 times more of the long-range transcription product. In similar experiments with primers for regions not so distant from the priming site for GAPDH or $\beta$-actin, we could observe no differences for GAPDH but a decrease of $3.0 \pm 0.4$ cycles to the crossing point for $\beta$-actin, thus estimating almost 4.5 times more product. The differences in the gain of efficiency between these two products might be explainable by different structural features of the mRNA.

In mRNA, the occurrence of secondary structures is a highly organized feature that is influenced by the surrounding conditions (i.e., ionic strength) and its sequence composition. The transition between the ordered and an unordered state results in a disruption of stacked base pairs. This leads to hyperchromicity as a consequence of the separation of overlapping $\pi$ elec-
trons from the heterocyclic bases (22) and can be conveniently monitored by UV absorbance changes at increasing temperatures.

To investigate the possible resolving effect of betaine on the secondary structure of mRNA, we conducted melting curves in the presence of 2 M betaine. We used mRNA from mouse muscle and an in vitro transcribed 297-nt cRNA fragment of the bovine oxytocin receptor transcript as examples for a complex mRNA pool and for a single mRNA species. The latter was chosen because of its high GC content (74%) and a highly stable and complex predicted secondary structure with a free energy of −349.32 kJ and several stem regions with an overall self-complementarity of 88 nucleotides, of which 55 are GC pairs (Fig. 3A). The two melting curves conducted either in the absence or in the presence of 2 M betaine display the ability of betaine to resolve secondary structures of RNA. In the case of the complex mRNA pool (Fig. 3B) an increase of hyperchromicity at almost all temperatures can be seen in comparison to the control. The calculated $T_m$ is 72.6 ± 0.8°C for the control and 67.1 ± 0.7°C for 2 M betaine. The overall hyperchromicity increases from 16.6 ± 0.8% to 18.6 ± 0.6% at 96°C. In the case of the oxytocin receptor mRNA fragment (Fig. 3C) the same observation applies. Increasing hyperchromicity can be seen; the calculated $T_m$ is 86.6 ± 2.3 and 78.9 ± 2.8°C for control and 2 M betaine, respectively. The overall hyperchromicity increases from 20.9 ± 1.2 to 25.6 ± 2.1%. Melting curves were also conducted in the presence of 0.6 M trehalose, but no effect on RNA melting could be observed (data not shown). Trehalose exhibits this effect of increasing the cDNA length thus solely by activating the reverse transcriptase (17) and not by influencing RNA secondary structure.

Both curves (complex and single species) display a multiphasic pattern. Each transition found within the curves can be interpreted as a portion of this molecule melting under the given condition. Since RNA structures consist of different regions containing duplexes, bulges, loops, or single-stranded regions, melting these regions leads to transitions that can be characterized as independent peaks when plotting the first derivative of the extinction versus temperature. In the case of the oxytocin receptor mRNA fragment, the predicted stems may contribute to the multiphasic curve pattern. In the case of the complex mRNA with a markedly reduced transition pattern, there either seem to be some common features inherent to all mRNAs (i.e., poly(A)-tail) or the transitions are a result of some secondary structures typical of a few very highly expressed mRNAs in the muscular tissue (i.e., GAPDH, β-actin).

In this paper we demonstrate an efficient way of reverse transcribing mRNA to cDNA by using a combination of betaine and trehalose that, by exhibiting cooperative effects, leads to a dramatically enhanced yield of long-range products. This method should prove useful for all techniques depending on the synthesis of full-length cDNA.

REFERENCES


