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Synthesis and Automated Detection of Fluorescently Labeled Primer Extension Products

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ABSTRACT

Common methods for determination of transcription initiation sites are based on the extension of radioactively labeled primers or internal labeling of extension products with reverse transcriptase. Here, we describe a modification of this procedure, adapted for automated detection of extension products by using fluorescent primers and an optimized reaction protocol. This facilitates high-throughput screening to detect mRNA start-points of large DNA regions and significantly reduces the time required to determine sites of transcription initiation.

INTRODUCTION

Synthesis of run-off cDNA copies starting at synthetic oligonucleotides annealed near the expected 5' ends of specific mRNA species is a common strategy used to determine transcriptional start-points. In most of the current protocols, detection of the extension products after electrophoretic separation on sequencing gels relies on terminal labeling of the primers or internal labeling of the extension products using [γ - ^{32}P]ATP or [α - ^{35}S]ATP, respectively (1,5,6). Because radioactively labeled products are not suitable for on-line detection by available systems for automated nucleotide sequence analysis, conventional autoradiography and data processing methods must be used. Moreover, depending on the copy numbers and the expression levels of the genes under investigation, standard primer extension protocols often require substantial amounts (50 to 100 μg) of total RNA. Here, we describe and demonstrate an efficient method for nonradioactive automated primer extension analysis (NAPE) that is effective even with minor amounts of RNA

(5–10 μg). In addition, NAPE does not depend on the use of radioactivity and avoids time-consuming primer labeling and gel-processing steps. The essence of NAPE is the inclusion of fluorescent oligonucleotides in a primer extension reaction, allowing for subsequent on-line detection of the reaction products using a nucleotide sequence analyzer. On-line detection methods now make it possible to use the high processivity of reverse transcriptase for detecting large transcripts with maximum detectable lengths depending primarily on mRNA secondary structures.

MATERIALS AND METHODS

Total RNA was prepared according to a modified method of Chomczynski (1–3) from *Lactobacillus gasseri* NCK102, a prophage-cured derivative of strain ADH (7), 40 min after infection of the bacteria with phage ϕadh , or from overnight cultures of *L. gasseri* NCK102-adh, a derivative of NCK102 that had been relysogenized with ϕadh (4). The primers, a 22-mer and a 24-mer, carrying the IRD800 fluorescent label at their 5' ends (both from MWG-Biotech, Ebersberg, Germany), were complementary to downstream regions of ϕadh -specific transcripts starting at the early promoters P_{tec} and P_{rad} , respectively (4). Extension reactions were carried out by using a commercially available reverse transcriptase RNA sequencing kit and avian myeloblastosis virus reverse transcriptase (AMV-RT) (both from Amersham Pharmacia Biotech, Braunschweig, Germany). The primer (0.1–1.5 μM final concentration) was annealed with total RNA (5–10 μg) in 10 μL H_2O (RNase-free) by heating at 90°C for 2 min and subsequently cooled to 30°C within 30 min in a Model TC1 Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA). For extension of the primer, annealing reaction mixtures were combined with 20 U of reverse transcriptase in 37.5 μL reaction volumes, containing 26.7 mM Tris-HCl (pH 8.3), 4.25 mM MgCl_2 , 26.7 mM NaCl, 0.54 mM dithiothreitol and 195.6 μM of each of the four dNTPs. After an incubation at 42°C for 90 min, reactions were

stopped by adding 3.3 μL 0.2 M EDTA (pH 8.0) containing 3 ng of RNase A (DNase-free) per μL , and a second incubation step at 37°C for 30 min followed. In this step, remaining RNA was removed, possibly affecting the electrophoretic mobility of the synthesized cDNA. Nucleic acids were cautiously concentrated by mixing the samples with 4 μL of 100 mM spermine tetrahydrochloride, incubating at ambient temperature for 10 min, centrifuging at 10000 $\times$ g for 40 min and dissolving the precipitates in 2 μL of alkaline sample buffer (95% [vol/vol] formamide, 10 mM EDTA, pH 9.0, 0.1% xylene cyanole, 0.1% bromophenol blue). Aliquots (0.8 μL) of the samples, after being heated at 70°C for 2 min, were applied to 4.3% polyacrylamide-urea LongRanger™ Sequencing Gels (FMC BioProducts, Rockland, ME, USA) that were 66-cm long and made with a 64-well shark-tooth comb. Gels were electrophoresed under standard conditions in a LI-COR DNA 4000L Nucleotide Sequence Analyzer (MWG-Biotech), and the data generated were processed by using the software supplied with the system.

RESULTS AND DISCUSSION

To evaluate the potential of our new method for reliable determination of transcription initiation sites, we applied NAPE to reproduce a recent extension experiment in which we had mapped the early promoters P_{tec} and P_{rad} of the temperate *L. gasseri* bacteriophage ϕadh using radioactively labeled primers (4). When we performed NAPE with the same primers carrying a fluorescent label, the lengths of the resulting extension products (150 and 86 nucleotides [nt], respectively) corresponded exactly to the initiation sites of the P_{tec} and P_{rad} transcripts, which we had previously found using the conventional procedure (4). This clearly demonstrated that NAPE is an accurate and convenient alternative to existing primer extension protocols.

In the model experiment shown in Figure 1A, 10 μg of total RNA, isolated from cells infected with phage ϕadh , were sufficient to allow for the produc-

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tion of a suitable amount of specific extension product. With 5 μg of RNA, the intensity of the signal was much weaker, and with 1 μg of RNA the band was almost invisible. One might suspect that the presence of multiple copies of phage DNA in infected bacteria accounted for the high sensitivity of NAPE in this experiment. We therefore used 10 μg of RNA isolated from a strain carrying the ϕadh prophage, to perform a similar primer extension (Figure 1B). In this case, we used a suitable primer to detect the transcription initiation site of the immunity re-

pressor gene *rad* (4), which is probably the only ϕadh function expressed in lysogenic cells. This experiment clearly demonstrates that even low amounts of specific RNA, e.g., RNA transcribed from a single copy of the phage genome, are sufficient to yield a detectable signal in NAPE.

As a favorable consequence of using fewer than 10 μg of RNA, the RNase digestion step included in conventional primer extension protocols (8) can be omitted.

The amount of labeled primer used for NAPE proved to be a critical para-

meter. With primer concentrations above 0.9 μM , diffuse spots of nonspecific background fluorescence occurred in the gels at various positions (Figure 1C). The intensity of the specific signal produced by the extension product, on the other hand, decreased with decreasing primer concentration. Nonspecific background spots probably resulted from aggregation of the fluorescent primer at elevated concentrations. In the case of conventional procedures relying on incorporation of radioactive label during extension of unlabeled primers, such aggregates would be invisible.

Figure 1. Primer extensions done to determine optimum RNA and primer concentrations for mapping experiments. (A) Mapping of the ϕadh *P_{tec}* transcript with variable amounts of RNA. RNA (lane 1, 1 μg ; lane 2, 5 μg ; lane 3, 10 μg), isolated from ϕadh -infected *L. gasseri* NCK102, was used in extension reactions containing a constant amount (0.9 μM) of a primer (41% G+C), complementary to nt 150 through 172 of the *P_{tec}* transcript (4). Reference sequencing reactions (lanes A, C, G and T), performed with the SequiTherm EXCEL™ Long-Read™ DNA Sequencing Kit (Epicentre Technologies, Madison, WI, USA), were initiated by the same primer on ϕadh DNA (450 ng) as a template. The sequence of the coding strand, encompassing the 3' end of the major extension product (★) is shown to the right. (B) Mapping of the ϕadh *P_{rad}* transcript by NAPE. RNA (10 μg) isolated from the ϕadh lysogen *L. gasseri* NK102-adh, was used in an extension reaction containing a primer (33% G+C, 0.9 μM final concentration), complementary to nt 86 through 110 of the *P_{rad}* transcript (4) (lane 1). Reference sequencing reactions (lanes A, C, G and T) initiated by the same primer were performed as in Figure 1A. The sequence of the coding strand, encompassing the 3' end of the major extension product (★) is shown to the right. (C) Influence of primer concentration on NAPE. A constant amount (10 μg) of the same RNA and decreasing concentrations (lane 1, 1.5 μM ; lane 2, 0.3 μM ; lane 3, 0.1 μM ; lane 4, 0.01 μM) of the same primer as in Figure 1A were used for NAPE. In addition to the signal specific for the *P_{tec}* transcript (II) (see Figure 1A), nonspecific background fluorescence (I) was observed with primer concentrations above 0.9 μM . Reference sequencing reactions (lanes A, C, G and T) are as in Figure 1A.

In the model experiment shown in Figure 1A, an optimal background-to-signal ratio was obtained with primer and RNA at concentrations of 0.9 μ M and 0.27 μ g/ μ L, respectively.

From a previous attempt to perform nonradioactive primer extension by use of fluorescent oligonucleotides, it was suspected that rigorous extraction of the extended samples by phenol-chloroform resulted in substantial loss of the fluorescent label (B. Fartmann, MWG-Biotech, personal communication). Therefore, NAPE includes only one single precipitation step using spermine as the denaturing agent. This treatment was found to be sufficient for appropriate purification of the extension products and does not affect the stability of the label. Pellets of cDNA obtained by precipitation with spermine are readily dissolved in the alkaline sample buffer used for sequencing gels. The activity of AMV-RT, under the conditions of NAPE, was found to be remarkably high so that cDNA products up to 570 nt in length were produced in readily detectable amounts. This, together with the potential of the LI-COR sequence analyzer for separating and detecting polynucleotides of more than 1000 bases, allows for the use of primers that hybridize at widely varying distances from expected transcription start sites in a single gel run. With our system, it was possible to analyze up to 13 different primer extension reactions on a single gel. This allows for rapid and efficient promoter analysis, that could be especially useful for verifying and completing preliminary transcription maps generated using hybridization studies with large DNA fragments or complete genomes.

In conclusion, this new method for nonradioactive primer extension analysis offers the possibility of quickly and reliably screening large DNA regions for functional promoters. To save processing time, the method makes use of commercially available primers, omits dispensable centrifugation and purification steps, and does not require any further processing of gels or autoradiographs.

Although the method was optimized for the LI-COR 4000L apparatus, it should also work with any DNA sequence analyzer that can handle fluorescent primer extension products.

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