Sensitive Nonradioactive Dot Blot/Ribonuclease Protection Assay for Quantitative Determination of mRNA

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ABSTRACT

We have developed a simple and sensitive method for the rapid quantitation of mRNA from cell cultures and small tissue samples. The method combines the high sensitivity and specificity of the ribonuclease protection assay with simple handling and rapid execution of dot blotting. The use of digoxigenin-labeled cRNA probes eliminates all problems associated with radioisotopes commonly used in the ribonuclease protection assay. The mRNA preparation is dotted directly onto nylon membranes, and after hybridization the filters are treated with ribonuclease A, which removes the nonhybridized single-stranded RNA. The mRNA hybrid is then revealed by the chemiluminescence technique using labeled anti-digoxigenin antibody, and the signal intensity is quantitated. Comparison with the Northern blotting ribonuclease protection assay revealed that this dot blot technique is almost ten times more sensitive and that its signals are linear over a wide range of RNA concentrations (0.01–10 µg/µL dot). This method seems particularly valuable for simultaneous processing of large numbers of samples containing a wide range of RNA concentrations.

INTRODUCTION

The isolation and quantitation of mRNA are key steps in studies of gene expression. Whereas traditionally Northern blotting has been the method of choice, the ribonuclease (RNase) protection assay (RPA), which was derived from the endonuclease (S1) protection assay (2) and subsequent modifications of it (14,15,21), provides an improved and more sensitive approach (4,7,10,19). Dot blot hybridization was introduced as a simple and efficient method for analysis of nucleic acids in multiple, small cell and tissue samples (18). Modifications of this technique using radioactive probes are widely used for determination of relative mRNA levels (6,8,17,20).

The recent introduction of digoxigenin (Dig)-labeled probes has opened the way for novel alternatives to the use of radioisotopes (9,11). The new probes lack the problems of safety, disposal and short half-lives, and are as sensitive as the radioactive ones (1,12). Indeed, nonradioactive hybridization techniques have received considerable attention because of those advantages (5,13,16).

In the present study, we have developed a simple and rapid dot blot RPA for quantitative determination of mRNA from cell cultures and small tissue samples, combining the high sensitivity and specificity of RPA and the efficiency of Dig-labeled cRNA probes. After blotting and hybridization, the filters are treated with RNase A, which removes the nonhybridized single-stranded RNA. The mRNA hybrid is then revealed by the chemiluminescence (CL) technique using a labeled anti-Dig antibody, and the intensity of signals is quantitated.

MATERIALS AND METHODS

Isolation of RNA and Preparation of Dig-Labeled cRNA Probes

Total cellular RNA was extracted from rat liver by means of guanidinium thiocyanate/phenol/chloroform according to Chomczynski and Sacchi (3).

Dig-labeled cRNA probes were prepared by using the RNA Labeling Kit (Genius™; Boehringer Mannheim GmbH, Mannheim, Germany). Briefly, the subcloned plasmids [pGEM-7Zf(-)] containing a full-length cDNA (1.6 kb) of rat liver catalase were cleaved by HindIII at the sites for antisense sequences. The original plasmid was kindly provided by Dr. T. Hashimoto, Shinshu University, Matsumoto, Japan. The digestion mixtures were subjected to low-melting agarose gel electrophoresis, and the DNA templates were extracted by phenol/chloroform. Two microliters of a nucleotide triphosphate (NTP) mixture containing Dig-labeled uridine 5′-triphosphate (UTP), 1 µg of template DNA and 2 µL of the T7 RNA polymerase were suspended in 2 µL of 10× transcription buffer (provided in the kit), and diethylpyrocarbonate (DEPC)-water was added up to 15 µL. After brief centrifugation (500× g), the mixture was incubated at 37°C for 2 h. The reaction was stopped by 2 µL of 0.2 M EDTA (pH 8.0), and the cRNA probe synthesized was precipitated by adding 2.4 µL of 4 M LiCl and 75 µL of cold 100% ethanol (-70°C, 30 min). The pellet was collected by centrifugation (12 000× g), washed with 50 µL of cold 70% ethanol, dried in vacuum, re-suspended in 50–100 µL DEPC-water and analyzed by denatured agarose gel electrophoresis.

Figure 1. Effect of RNase A digestion on the intensity of signals detected by Northern (top) and dot blot (bottom) analyses. Each lane was loaded with 1 µg of total RNA for the Northern blot, and the same amount of RNA was applied directly to a nylon membrane for the dot blot. Agarose gel electrophoresis and transferring to nylon filter are described in Materials and Methods. Dig-labeled cRNA probe corresponding to rat hepatic catalase was used for hybridization. Probed catalase mRNA was subsequently visualized by means of an alkaline phosphatase-labeled anti-Dig antibody in combination with the CL technique. Column A represents native mRNA before, and Column B, after the treatment with RNase.
Blotting and Hybridization

Identical amounts of total RNA (0.01–10 µg) were either subjected to denaturing agarose gel electrophoresis followed by overnight capillary blotting, or directly dotted in a constant volume of 1 µL onto a nylon membrane (Qiagen, Chatsworth, CA, USA). The membranes were cross-linked by ultraviolet light using a Stratalinker® 2400 (Stratagene, La Jolla, CA, USA) and prehybridized in a blocking solution for 2 h at 68°C according to the protocol of Boehringer Mannheim GmbH (The Dig System User’s Guide for Filter Hybridization).

Subsequent hybridization at 68°C, overnight, was carried out in the same solution by adding 100 ng/mL of the denatured cRNA probe. Thereafter, the membranes were washed twice at room temperature (22°C) in 2× standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) for 5 min each, and again twice at 68°C in 0.1× SSC/0.1% SDS for 15 min each.

Digestion by RNase A

The membranes were equilibrated for 10 min at room temperature in the digestion buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA and 300 mM NaCl). Subsequently, RNase A (Boehringer Mannheim GmbH) was added in a concentration of 1 µg/mL, and the incubation was continued for another 10 min. To remove the RNA fragments, the membranes were washed twice in digestion buffer for 10 min each and in 2× SSC/0.1% SDS for 30 min. To assess the effects of time and temperature, the digestion with RNase was carried out either at room temperature or at 37°C and for different time intervals (5–60 min).

All incubations (at room temperature, 37°C and at 68°C) were performed in a rotating Hybridiser HB-1D (Techne, Wertheim, Germany).

Visualization of Dig-Labeled Hybrids

Dig-labeled RNA hybrids were detected by the CL technique using anti-Dig antibody conjugated to alkaline phosphatase provided by Boehringer Mannheim GmbH. Signals obtained were quantitated by
densitometry using a Chromoscan™ 3 scanner (Joyce-Loebl Ltd, Gateshead, England, UK).

RESULTS AND DISCUSSION

In Figure 1, the results of Northern and dot blots are shown prior to and after the digestion with RNase A. Apparently, the treatment removes most of the unspecific signals due to nonhybridized RNA, leaving only the specific signals due to mRNA-cRNA hybrids. This observation is supported by the fact that RNA cross-linked by ultraviolet rays to a nylon membrane is easily degraded by RNase, if it is not hybridized to the corresponding cRNA probe (data not shown). Interestingly, the quantitation of signals shown in Figure 1 reveals that the amount of RNA digested by RNase is proportionally almost the same in both the North-

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**Figure 2. Effects of time and temperature on the recovery of hybridized catalase mRNA subjected to digestion by RNase A.** One microgram each of total rat hepatic RNA was dotted onto a nylon membrane and hybridized with a Dig-labeled catalase cRNA probe. The dots were subjected to digestion by RNase A at either 37°C or room temperature (RT) prior to the identification of the hybrids by the CL technique. Signals obtained were quantitated by densitometry. The experiment was done in triplicate and values presented are mean value ± SEM.
ern (77%) and the dot blots (71%).

Figure 2 shows the effects of time and temperature on the RNase digestion in dot blots. The digestion for 5 min at room temperature (22°C) of dotted RNA reduced the intensity of the signals to a constant level of about 75%–80% of the original value, which remained unchanged up to 60 min. In contrast, when the digestion was carried out at 37°C, the intensity of signals was progressively diminished to undetectable levels (Figure 2). This suggests that the temperature of the RNase digestion is of crucial importance and should be kept constant for obtaining consistent signals.

To remove the nonhybridized RNA within 10 min (Figure 2), 1 µg/mL of RNase A was sufficient, and similar results were obtained with higher concentrations of RNase A (40 µg/mL) (data not shown).

The sensitivity of the RNase protection assay of dot blotting was compared with that of Northern blotting. Identical amounts of RNA (0.01–10 µg) were dotted either directly onto nylon membranes or subjected to denaturing agarose gel electrophoresis followed by capillary transfer to the membrane. Both preparations were processed identically, as described above for RPA, and the signals were detected by CL. As shown in Figure 3, although at higher RNA concentrations (1–10 µg) both methods give an adequate signal, only the dot blot technique provides reliable signals at concentrations of RNA below 0.1 µg. In Figure 4, the results of quantitation of the dot blot assay signals over a range of 0.01–10 µg RNA are shown, revealing the linearity of the signal over this wide range.

The dot blot RNase protection assay described here provides a simple, direct approach for relative quantitation of small amounts of RNA. Since it involves a direct dotting of the RNA preparation onto nylon membrane, it circumvents most problems and difficulties associated with Northern
blotting, such as denaturing agarose gel electrophoresis and the time-consuming transfer of RNA to membranes. As indicated in an earlier study (21), the specificity of this method is assured by the RNase digestion, which removes the nonhybridized single-stranded RNA. Comparison with Northern blotting RPA revealed that the dot blot technique is about ten times more sensitive and that its signals are linear over a wide range of RNA concentrations (0.01–10 μg). Finally, the use of Dig-labeled cRNA probes eliminates all the problems inherent with radioisotope techniques. The simplicity of this method makes it particularly valuable for simultaneous processing of large numbers of samples containing a wide range of RNA concentrations.

REFERENCES


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