Proceedings of the Nutrition Society (1996), **55**, 583–589

Northern blotting

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INTRODUCTION: WHY MEASURE AN mRNA?

Northern blotting is one of the key techniques in molecular biology, its principal aim being the measurement of a specific messenger RNA (mRNA). Before discussing Northern blotting in detail, it is appropriate to consider the question of why one should wish to measure an mRNA. There are in practice two main reasons. The first is to determine which tissues express a particular gene, and this can give some indication of the physiological function of the encoded protein. For example, the recently-described *ob* (obese) gene is expressed in white adipose tissue, which is the basis for the view that the protein product (leptin) acts as a signal for the size of the fat depots. The second principal reason for measuring an mRNA is to determine the factors which regulate the expression of a given gene, be they nutritional, hormonal, or environmental.

There are three techniques for measuring an mRNA. The first, and most extensively used, is Northern blotting. A second method is the RNase protection assay, which is generally considered to offer improved sensitivity. The third method utilizes the reverse transcriptase polymerase chain reaction; this provides considerable increases in sensitivity over Northern blotting and the RNase protection assay, and may be useful for measuring very low levels (a few copies) of an mRNA in a tissue. Given that Northern blotting is the principal method of measuring an mRNA, this technique is outlined in the present paper. For a detailed discussion of the various stages of the procedure, reference can be made to recent reviews (Sambrook *et al.* 1989; Düring, 1993; Farrell, 1993).

PRINCIPLES OF NORTHERN BLOTTING

The underlying principle of Northern blotting is that RNA are separated by size and detected on a membrane using a hybridization probe with a base sequence complementary to all, or a part, of the sequence of the target mRNA. Fig. 1 provides a schematic representation of Northern blotting; the separate steps in the process are summarized in Fig. 2. It should be noted that although the term 'Northern blotting' applies strictly only to the transfer of RNA from a gel to a membrane, the whole procedure is often loosely referred to by this general descriptor.

The initial step (step 1) is to extract total RNA from a tissue, using chaotropic agents such as guanidinium isothiocyanate. Such agents disrupt cells and denature proteins (including RNases), as well as solubilizing RNA. In some cases, a separate step for the isolation of mRNA from total RNA may be included (step 2), this giving increased sensitivity. The improvement in sensitivity stems from the fact that only a few per cent of the total RNA extracted from a tissue is mRNA. The isolation of mRNA from total RNA uses a poly-A+ selection procedure, involving an oligo-T column, or beads primed with oligo-T, to bind to the poly-A+ tail of mRNA.

The extracted RNA species, whether total RNA or poly-A+ selected, are then

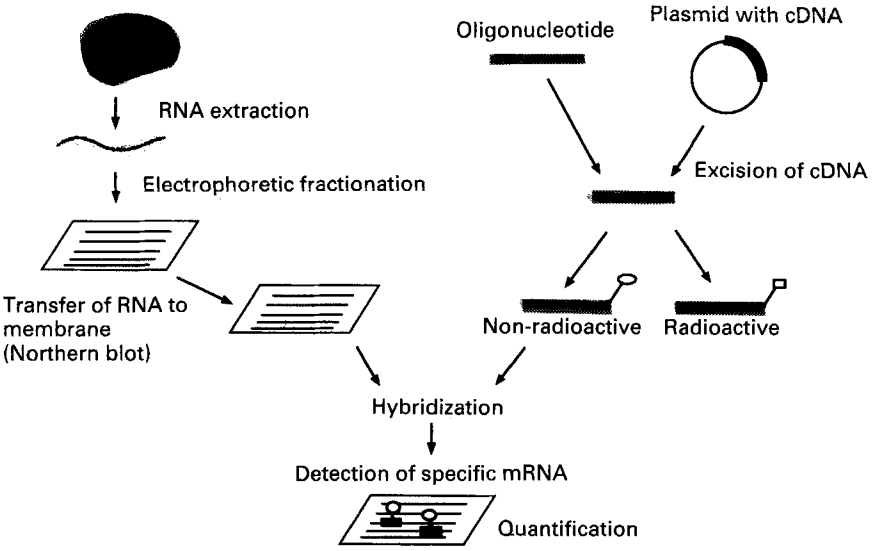

Fig. 1. A schematic representation of Northern blotting.

STEP 1
Extraction of RNA (chaotropic agents)

(STEP 2)
Isolation of mRNA, by poly-A+ selection

STEP 3
Separation of RNA species (agarose gel electrophoresis)

STEP 4
Blotting onto a nylon membrane (capillary, vacuum)

STEP 5
Immobilization of RNA on the membrane (baking, u.v. light)

STEP 6
Preparation of the hybridization probe (cDNA, oligonucleotide)

STEP 7
Hybridization with probe; post-hybridization stringency washes

STEP 8
Detection (normally by film) and quantification (densitometry)

Fig. 2. Outline of the different stages of Northern blotting.

separated on the basis of molecular size by agarose-gel electrophoresis (step 3). This is followed by blotting onto a nylon membrane (step 4). Nylon membranes, particularly positively-charged membranes, are normally used in preference to nitrocellulose because of their high binding capacity for nucleic acids and their greater robustness on handling. Two main alternatives are available for blotting, either capillary or vacuum. The traditional approach is to employ capillary blotting, this requiring no special equipment. However, vacuum blotting is being increasingly used, since it offers advantages in terms of speed (1–2 h *v.* 4–18 h) and reproducibility. A blot provides a precise reflection on a membrane of the separated RNA species from a gel. The gel itself is too fragile to be

probed directly, and hybridization probes will not readily penetrate gels.

Following blotting, the RNA must be immobilized on the membrane (step 5), either by baking in an oven or by exposure to u.v. light. This results in the covalent linkage of RNA to the membrane, which prevents the nucleic acid from being washed away during the subsequent processing. The hybridization probe must then be prepared (step 6), and the probe hybridized with the membrane (step 7). This is followed by post-hybridization stringency washes, which ensure that the probe is bound specifically to the target mRNA and that there is negligible non-specific binding to other mRNA or the nylon membrane itself. Hybridization signals are then detected, usually by film, and quantified where required by densitometry (step 8).

Choices have to be made in the selection of a hybridization probe and the nature of the detection signal, and these are considered in detail in the next section.

HYBRIDIZATION PROBES

Nucleic acid hybridization requires that a probe is complementary to all, or part, of the sequence of the mRNA of interest. It depends upon the strict base pairing between C (cytosine) and G (guanine), and between A (adenine) and T (thymine). In general, the minimum size for a probe to ensure specificity is approximately twenty-five bases, providing that there is a complete match between the probe sequence and the sequence of the target mRNA; this can be modulated, however, by the stringency conditions. With a probe of approximately thirty bases in length, the probability that the same sequence occurs in the mammalian genome by chance is of the order of 1 in 1 billion (see Sambrook *et al.* 1989).

There are two main forms of hybridization probe, the customary approach being to use a complementary DNA (cDNA). Alternatively, anti-sense oligonucleotides (generally thirty to forty bases in length) can be designed from sequence data and synthesized. Oligonucleotides offer some advantages in terms of simplicity (obviating the need for plasmid isolation, for example), and reduced hybridization times. Despite being widely used for *in situ* hybridization studies, anti-sense oligonucleotides have not been extensively employed in Northern blotting applications. Oligonucleotides, like cDNA, are DNA molecules, but 'riboprobes' based on RNA can also be employed. Riboprobes may increase sensitivity compared with DNA probes, but they are less stable in the sense of being subject to breakdown by RNases (see p. 586).

Detection is achieved either through the use of radioactivity or by non-radioactive strategies. Most laboratories continue to employ radioactivity, probes being labelled with ^{32}P (or ^{33}P). There are several advantages to using radioactively-labelled probes; the procedures are well-established and robust, and there is a high level of sensitivity. There is, nonetheless, growing interest in non-radioactive protocols, reflecting the disadvantages of radioisotopes (see Kricka, 1992; Düring, 1993). These disadvantages include safety, instability of probes (reflecting the short half-life of the isotopes used), and increasing difficulties with the disposal of waste. Colour dyes may be employed in non-radioactive detection, although these generally have low sensitivity. At present, the main non-radioactive approach is based on chemiluminescence, and this is gaining in popularity. Detection may also be based on fluorescence, but this requires a substantial investment in dedicated instrumentation.

In the case of chemiluminescent detection, the breakdown of specific chemilumi-

nescence substrates is catalysed by alkaline phosphatase (*EC* 3.1.3.1) or horseradish peroxidase (*EC* 1.11.1.7), with the emission of light. These enzymes may be conjugated directly to a probe, or more commonly a probe is labelled with a ligand (e.g. digoxigenin, fluorescein, biotin) which is then localized by an antibody (or avidin or streptavidin if biotin is the ligand) to which alkaline phosphatase or horseradish peroxidase is attached.

X-ray film is the usual means by which hybridization signals are collected, and this can be used with both radioactivity and chemiluminescence. Quantification is achieved by densitometry, and is based on 'arbitrary units' and relative changes between experimental groups and controls. Alternatively, phosphor storage screens together with a molecular imager may also be employed. This offers reduced exposure times, together with the ability to quantify over several orders of magnitude at the same time as obtaining the image.

A simplified, non-radioactive procedure

With a view to increasing the accessibility of Northern blotting for nutritional and physiological studies, particularly for laboratories with limited facilities and expertise in molecular biology, we have recently established a rapid, non-radioactive procedure (Trayhurn *et al.* 1994). This couples anti-sense oligonucleotides (30–35-mer) as hybridization probes with chemiluminescence detection. The approach involves designing anti-sense oligonucleotides from published sequences accessed via the gene databanks (European Molecular Biology Laboratory, or Genbank), and labelling the oligonucleotides with the digoxigenin ligand (Boehringer Mannheim). An antibody to digoxigenin, conjugated with alkaline phosphatase, is used to detect hybridization of the digoxigenin-labelled oligonucleotide to the target mRNA. High-sensitivity chemiluminescence substrates, such as CSPD or CDP-*Star* (Tropix, USA), provide detection within a few minutes of exposure of membranes to film (Trayhurn *et al.* 1994, 1995*a*).

The general strategy of combining chemiluminescence-based detection with anti-sense oligonucleotides as probes has been successfully utilized for the rapid detection by Northern blotting of a number of mRNA in mammalian tissues. These include the mitochondrial uncoupling protein, facilitative glucose transporters (GLUT 1–4), lipoprotein lipase (*EC* 3.1.1.34), and the *ob* gene (Trayhurn *et al.* 1994, 1995*b*).

PROBLEMS: RNases

All biochemical techniques require meticulous care in their execution. A particular problem in the case of Northern blotting relates to the potential degradation of RNA through the action of RNases. These enzymes are widely distributed in tissues, and environmental contamination is possible at most stages of Northern-blot analysis, with fingers being a major source of contamination. The problem can be overcome by baking, or sterilizing, glassware and solutions, and by employing specific RNase inhibitors such as diethyl pyrocarbonate (DEPC). The use of protective latex gloves is essential.

The other key problem is high background on membranes, reducing the signal:noise ratio. Careful adjustment of the post-hybridization wash conditions is important in minimizing background, particularly with chemiluminescence-based detection.

NORTHERN BLOTTING IN NUTRITIONAL STUDIES

Northern blotting has been extensively used to examine the expression of specific genes

in nutritional science. Three recent examples from the work of my group are presented as illustrations. Fig. 3(A) shows a Northern blot where a range of tissues from mice have been probed for the mRNA for the *ob* gene, using a 33-mer anti-sense oligonucleotide. Signals are obtained exclusively in white adipose tissue, and not in any of the other tissues examined. Thus, the *ob* gene is expressed in a highly tissue-specific manner, and codes for a protein that is particular to adipose tissue. This clearly provides clues as to the general nature of the function of the encoded protein, as described previously.

An example of the nutritional regulation of gene expression is given in the Northern blot in Fig. 3(B), where the level of *ob* mRNA in white fat is greatly reduced following fasting and rapidly restored on subsequent refeeding (Trayhurn *et al.* 1995*b*). Fig. 4 shows a Northern blot illustrating both tissue specificity of gene expression and tissue specificity of regulation. Expression of the gene encoding metallothionein-1 is induced in brown adipose tissue and the liver by exposure to cold, being greater after 24 h than after 6 h (Beattie *et al.* 1996). Zn, a major inducer of metallothionein-1, has a much greater effect on the liver than 24 h of cold exposure. In marked contrast to the liver, the injection of Zn does not lead to a major induction of metallothionein-1 gene expression in brown adipose tissue. Unlike brown fat, the metallothionein-1 gene appears not to be expressed in white adipose tissue, following either cold exposure or the administration of Zn.

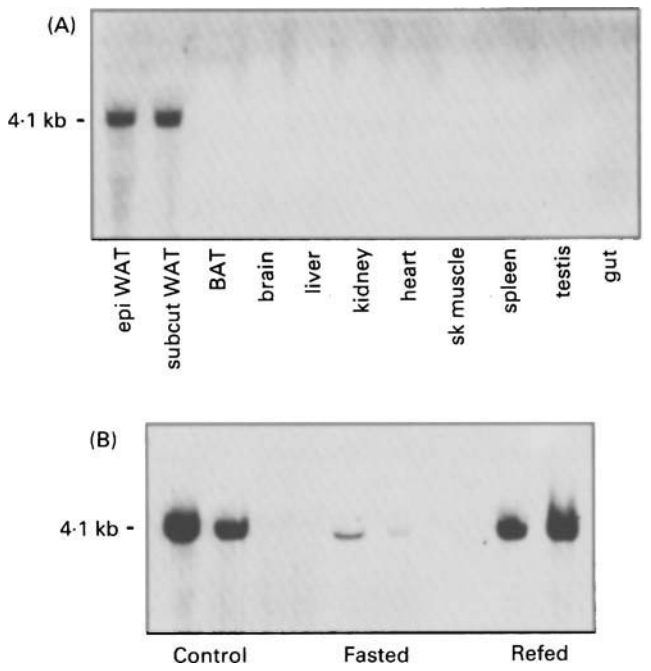

Fig. 3. Northern blot showing (A) the tissue specificity of expression of the *ob* gene, and (B) nutritional regulation of the expression of the gene. (A), Tissues were removed from a mouse, total RNA extracted, and 10 μg added to each lane of an agarose gel; (B), mice were fasted for 24 h, and some were refed for 6 h; control animals received continuous access to food. Portions (10 μg) of total RNA from the epididymal fat pads were loaded onto each lane of the gel. A 33-mer anti-sense oligonucleotide, end-labelled with digoxigenin, was used as a probe, with detection by chemiluminescence (Trayhurn *et al.* 1995*b*). BAT, brown adipose tissue; WAT, white adipose tissue; subcut, subcutaneous; sk, skeletal; epi, epididymal.

https://doi.org/10.1079/PNS19960051 Published online by Cambridge University Press

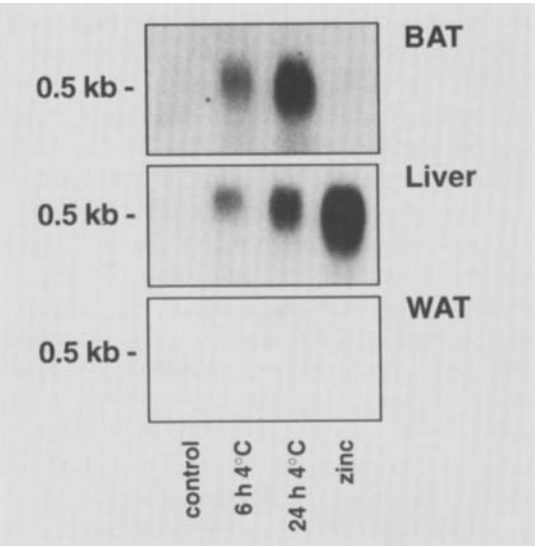

Fig. 4. Northern blot showing tissue specificity in the regulation of expression of the metallothionein-1 gene. Rats were either exposed to cold (4°) for 6 or 24 h, or injected with zinc (10 mg/kg body weight) and killed 6 h later. Total RNA was extracted from liver, brown adipose tissue, and the epididymal white fat pads; 10 μg RNA were loaded onto each lane of the gel. A 28-mer anti-sense oligonucleotide, end-labelled with digoxigenin, was used as a probe, with detection by chemiluminescence (Beattie *et al.* 1996). BAT, brown adipose tissue; WAT, white adipose tissue.

SUMMARY

Northern blotting is the main method for examining the expression of genes through measurement of an mRNA. Tissue specificity and the factors which regulate expression can both be determined by Northern blotting. A cDNA radioactively labelled with ^{32}P is the most-commonly-used hybridization probe. Convenient, non-radioactive detection protocols are, however, increasingly available. A combination of anti-sense oligonucleotides as probes, together with chemiluminescence-based detection provides a rapid and simplified approach to Northern blotting, increasing the accessibility of this important procedure for nutritional studies.

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Printed in Great Britain