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Joint Clinical Nutrition and Metabolism Group and Macronutrient and Micronutrient Group Symposium on 'Nutrient regulation of gene expression'

Molecular biological approaches to nutrient–gene interactions

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While most of the papers presented in the present Symposium deal with specific aspects of nutrient–gene interactions, the present remit is to provide a brief review of some of the molecular techniques which can aid investigations of these interactions. A more extensive treatment of many of the techniques can be found in Trayhurn & Chesters (1996) and detailed protocols are available in Sambrook *et al.* (1989) and Ausubel *et al.* (1994).

Regulation of gene expression can be subdivided into the following phases:

(A) semi-permanent up- or down-regulation of the gene;
(B) control of gene transcription;
(C) processing of primary transcripts into mature mRNA;
(D) translational control.

The events accompanying embyro development and tissue differentiation involve semi-permanent up- or down-regulation of genes and determine which of a cell's genetic complement of protein-coding sequences can be expressed. While considerable progress has been made in the fields of embryology and tissue differentiation, we still largely lack an understanding of the mechanisms involved. Furthermore, nutrient supply generally has greater impact on rates of development and metabolism than on the pattern of differentiation. Consideration, therefore, will be limited mainly to the molecular techniques used to investigate the final stages of differentiation and the regulation of cellular metabolism.

Principles

Central to studies of nutrient–gene interactions is the isolation and estimation of mRNA. There are several reasons for this. mRNA is the primary product of a gene and mediates its expression as a protein. In many instances, nutrient effects on the expression of proteins reflect changes in the concentration of their mRNA. It is also frequently easier to determine mRNA concentrations than it is to estimate the corresponding proteins. Thus, the initial phase in an investigation of a nutrient–gene interaction often involves estimation of the effects of the nutrient on mRNA concentrations.

Furthermore, mRNA can readily be converted into their equivalent DNA by first synthesizing a complementary strand of DNA (cDNA) and then a second strand complementary to the first. DNA is much easier to manipulate than RNA and individual cDNA can be amplified by cloning in plasmids or by the polymerase chain reaction (PCR). The cDNA can then be sequenced to provide protein sequence information which can be used in structural studies and in the development of immunological methods of protein estimation.

The synthesis of individual mRNA is rarely regulated by direct interaction of a nutrient with a gene. Instead, control is generally mediated through one or more proteins referred to as transcription factors. These bind to regulatory regions of the gene and determine the efficiency of its transcription. Understanding how a nutrient influences expression of a particular gene therefore requires a study of the factors which bind to that gene's regulatory region and a number of techniques to be described such as gel mobility assays and DNAase I (*EC* 3.1.21.1) footprinting are designed to aid this.

Hybridization

Hybridization involves the binding together of paired strands of nucleic acids as a result of specific hydrogen

Abbreviations: cDNA, complementary DNA; PCR, polymerase chain reaction; RT, reverse transcription.

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bonding between the nucleotide bases in the complementary strands. These pairings involve guanine residues in one strand binding to cytosine moieties in its complementary strand, and pairing of adenine with either thymine in DNA or uracil in RNA. Many molecular techniques utilize variations in strength of bonding between the two strands. These result from differences in the base composition of the nucleic acids, since bonding of guanine to cytosine is stronger than that of adenine to thymine or uracil, and from the extent to which the base sequences of the two strands are fully complementary. Increasing solution temperature counteracts the tendency for nucleic acids to hybridize, and can be used to distinguish differing degrees of complementarity. The temperature needed to separate the strands depends also on the prevailing salt concentration and the length of the complementary regions.

Isolation of mRNA

Numerous techniques are available for isolation of total RNA, each of which must address the problem of its relative instability and the virtually universal presence of ribonucleases. These limitations mean that specific care must be taken to eliminate RNAase contamination of apparatus and solutions. The RNA can then be isolated under conditions which denature proteins and allow separation of DNA from RNA (Chomczynski & Sacchi, 1987). For many purposes, preparations of total RNA suffice, but where mRNA is specifically required, this may be isolated either directly from cell homogenates or from total RNA by hybridization of the polyadenylate sequence located at the 3′ end of most mRNA to synthetic polydeoxythymidylate tails attached to a solid matrix (Aviv & Leder, 1972). Following hybridization, the matrix is washed free of unattached RNA under relatively high-salt conditions which stabilize hybridization bonding. The retained polyadenylate-containing mRNA is then eluted from the matrix with a low-salt medium.

Probes

The principles of hybridization are employed to provide the specific probes needed for several molecular biological techniques. Thus, if the sequence of an mRNA is known, it is now readily possible to synthesize chemically a length of DNA complementary to a section of that mRNA. The location of the probe sequence within the mRNA should be selected such that the hybridization of the probe to its target mRNA requires a high temperature to separate the strands, the probe should not self hybridize and should be specific for the particular mRNA. Such selection depends on knowledge of the sequence of the mRNA, which can often be obtained from computerized databases (Devereux *et al.* 1984). However, in making the selection two points need to be remembered. The sequence quoted in the database is that of the mRNA generally with substitution of uracil for thymine; it is not that of the complementary strand, which is actually the strand used during enzymic synthesis of the mRNA.

Second, all enzymic synthesis of nucleic acids involves linking a phosphate group at the 5′ position of one nucleotide to the 3′ hydroxyl of the previous nucleotide. Thus, the 5′ phosphate of the first nucleotide and the 3′ hydroxyl of the last nucleotide in a nucleic acid remain free and the sequence of a nucleic acid is always specified from the end with the free 5′ phosphate to that with the free 3′ hydroxyl. However, note that the two complementary strands of a DNA molecule run in opposite directions. Thus, once the pairings of bases between the mRNA and its potential probe have been determined, the sequence of the probe must be specified in the opposite direction to that of the mRNA.

Probes may also be obtained by PCR, or cDNA molecules can be labelled and used as probes (see pp. 252–253).

Estimation of mRNA

Once a preparation of RNA is available, one of a number of techniques may be employed to quantify specific mRNA species and, thus, the likely concentrations of the corresponding proteins.

Northern blotting

RNA preparations, generally total RNA, are subjected to horizontal electrophoresis in agarose gels under denaturing conditions (Farrell, 1993; Trayhurn, 1996). The separated RNA species are then transferred by capillary- or electro-blotting to a membrane, commonly nylon, under conditions which retain the separation of RNA obtained by electrophoresis (Fig. 1). RNA molecules have an inherent tendency to bind to the membrane and may be permanently fixed to it by subsequent heating or u.v. irradiation. Alternatively, charge-modified nylon membranes may be used which do not require separate fixation of the RNA. Following transfer of the RNA to the membrane, spare nucleic acid-binding capacity on the membrane is blocked by pre-incubation in a solution containing an unrelated DNA. The labelled probe is then added to the pre-hybridization solution and allowed to hybridize to the RNA species of interest. Once hybridization is complete, the membrane is washed successively in solutions of varying salt and detergent concentration to wash off labelled probe not specifically hybridized to the target RNA. The remaining specifically-bound label is then located and quantified to determine the amount of the target mRNA present.

Various methods of labelling the probe have been employed, including incorporation of radioactivity or nucleotides labelled with specific antigens. The former can be detected by autoradiography or with matrix-based gas-flow detector systems. With antigen labels, specific antibodies linked to chemiluminescence reagents are commonly used for quantification.

Once a particular probe has been shown to bind only to the mRNA of interest, electrophoretic separation may be omitted and the RNA bound directly to the membrane in a slot or dot blot apparatus. These systems considerably

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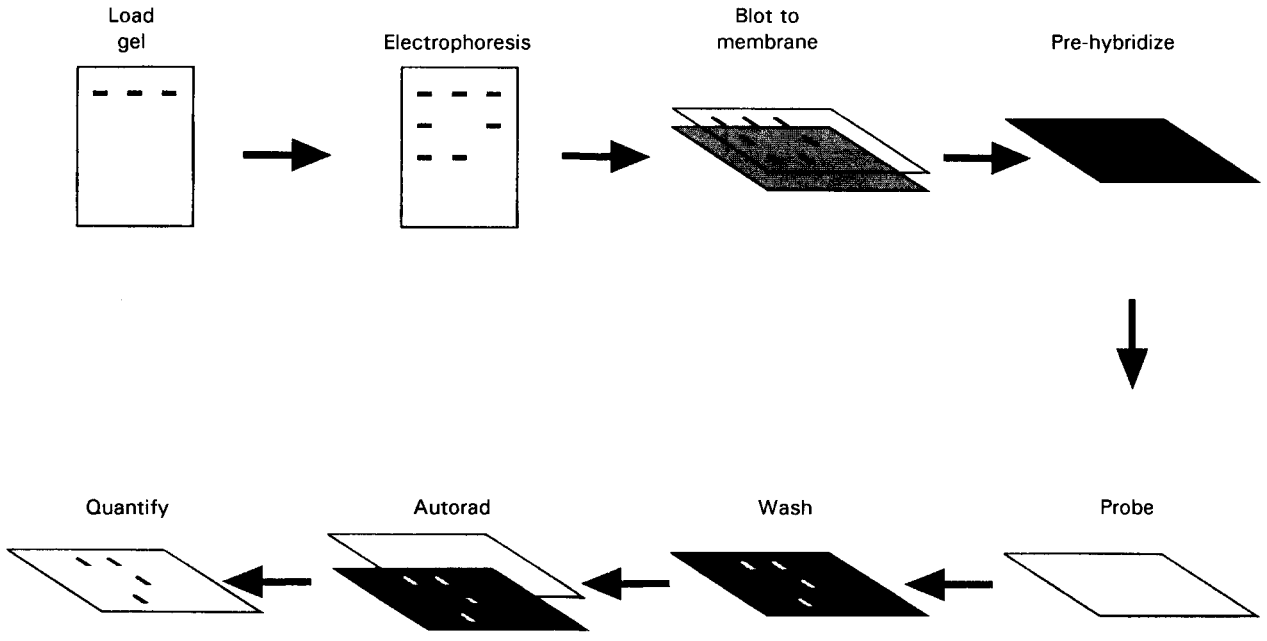

Fig. 1. Northern blotting. The various stages in estimation of an mRNA are illustrated diagramatically from the initial loading of a complex sample of RNA on to a gel through its separation and transfer to a membrane. This is followed by probing for the specific mRNA and estimation of the specifically-bound probe. Autorad, autoradiography.

shorten the time and effort required to measure mRNA and allow many more samples to be processed simultaneously.

Ribonuclease protection assay

Ribonuclease protection assays provide an alternative to Northern blotting and possibly a more sensitive method of estimation of mRNA (Chamberlein & Ryan, 1982; Ausubel *et al.* 1994). They depend on an ability to synthesize a short length of labelled RNA complementary to a section of the mRNA of interest. After hybridization of the complementary RNA to the preparation of mixed experimental RNA, the sample is treated with ribonucleases which destroy any RNA not present as an RNA–RNA duplex. The residual material is then separated on an acrylamide gel, and the original amount of the specific mRNA is estimated by quantifying the label present in the RNA duplex. Any ambiguity in identification of the appropriate band can be resolved by determination of the size of the residual RNA duplex, since this is determined by that of the probe.

In order to provide the RNA probe, a section of the mRNA of interest must first be converted to its cDNA and then inserted into a plasmid vector. The vector is chosen to permit *in vitro* synthesis by a bacteriophage RNA polymerase of RNA complementary to the mRNA. Further details of the techniques involved are given in the following sections.

In situ *hybridization*

Hybridization can also be used to localize specific mRNA within tissue slices (Pardue, 1985). The principles of *in situ* hybridization are essentially the same as those of Northern hybridization, but the probes must be kept small to allow adequate penetration of the slices of fixed tissue. Both radioactive and non-radioactive detection techniques can be used, but fluorescent probes or those labelled with an antigen are often preferable as they facilitate co-localization of separate mRNA.

mRNA kinetics

Often it is advantageous to know whether a nutrient-induced change in the steady-state concentration of an mRNA is mediated by a modification of its rate of synthesis or of its degradation. If inhibitors such as actinomycin D are used to prevent further RNA synthesis, the turnover rate of individual mRNA may be studied by estimating their residual concentrations at various times after addition of the inhibitor.

Alternatively, provided active nuclei can be isolated from the system, their rate of synthesis of individual mRNA may be measured using a nuclear run-on assay. With this, the nuclei are incubated with a labelled precursor of RNA so that the label in the nuclear RNA reflects their rates of synthesis in the nuclei at the time of nuclear isolation. cDNA probes for the mRNA of interest are loaded onto strips of nylon membrane in a slot or dot blot apparatus and then the cDNA are fixed to the membrane by heat or u.v. These strips are then soaked in the solution of RNA isolated from the nuclei to allow each labelled mRNA to hybridize to its corresponding probe. Other labelled RNA are then removed by appropriate washing of the strips, and the activity in mRNA associated with the bound cDNA determined as estimates of the amount of each of the selected mRNA synthesized during the incubation *in vitro*.

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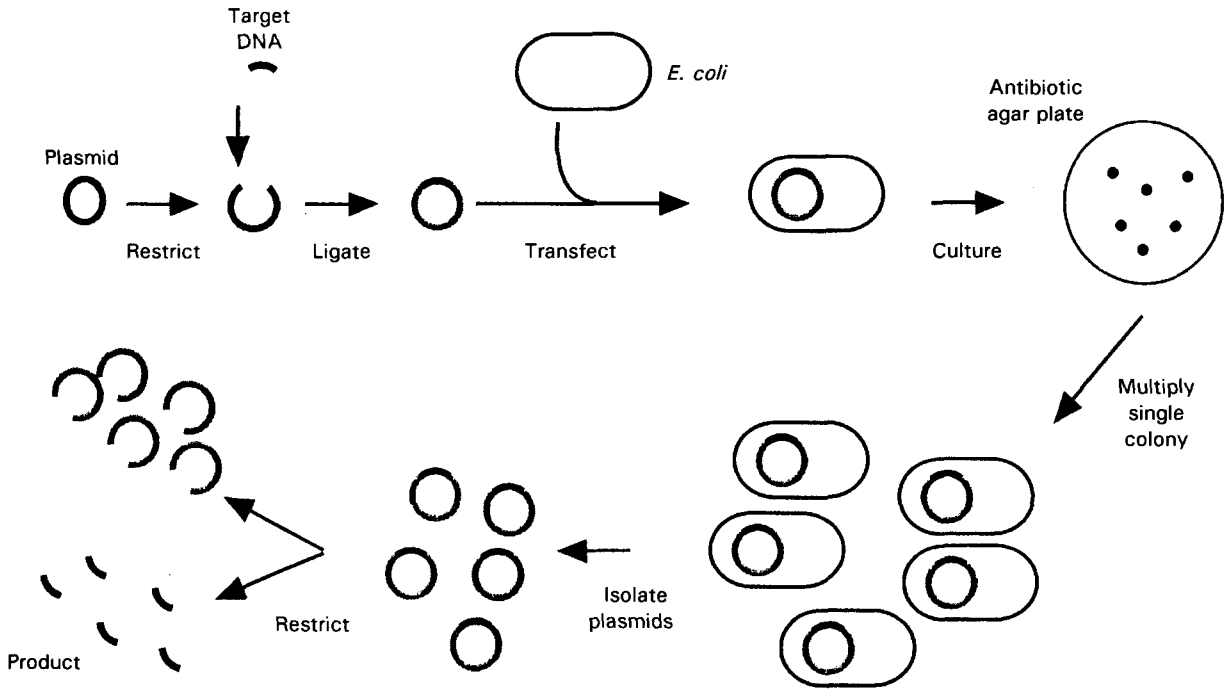

Fig. 2. Cloning of a segment of DNA. The target DNA is ligated into a linearized plasmid and transfected into a suitable bacterium. The latter is cultured, bacteria from individual colonies selected and multiplied and the plasmid DNA isolated for recovery of the inserted DNA. *E. coli, Escherichia coli.*

Complementary DNA

Conversion of mRNA to the corresponding cDNA is a prerequisite of many molecular biological techniques. This involves a two-step process, with initial synthesis by a viral reverse transcriptase of a strand of DNA complementary to the mRNA (Sambrook *et al.* 1989). The mRNA is then partially degraded by a ribonuclease, and this first DNA strand is used to code for the synthesis of its DNA complement, which therefore has a base sequence corresponding to that of the original mRNA.

Cloning

In general, preparations of mRNA will contain molecules coding for many different proteins. To be useful the mRNA must often be isolated, converted to cDNA, separated and then individually amplified (Fig. 2). To achieve this, the cDNA are commonly inserted into bacterial plasmids. These are circular DNA molecules capable of coding for their own replication by host bacteria. In addition, they normally contain antibiotic-resistance genes which convey a selective growth advantage to the bacteria carrying the plasmid. The plasmids also have a multiple cloning site, which allows the circular molecule to be cut specifically with one of several restriction enzymes. Once cut, a cDNA molecule can be inserted between the cut ends of the plasmid, and the combination enzymically ligated to form a modified circular plasmid containing the cDNA.

cDNA synthesis from the mixed mRNA population of a cell combined with ligation into a plasmid vector can provide a library of plasmids, each containing a single cDNA molecule representative of one of the mRNA originally present. These plasmids can then be inserted into a host bacterial species, such as *Escherichia coli*, by one of several methods, and will then be replicated as the bacteria grow.

The process of plasmid insertion, variously called transfection or transformation, is relatively inefficient, possibly between 1 and 10% of the cells acquiring a plasmid. However, if the bacteria are thinly plated on nutrient agar containing an appropriate antibiotic, only those containing a plasmid conferring resistance to that antibiotic will grow. From these, colonies can be isolated, each derived from a single initial cell. All cells of such clones will, therefore, contain copies of the same original plasmid, bearing a specific cDNA which has thus been cloned (Brown, 1990; Hesketh & Partridge, 1996).

After multiplication and harvest of the cloned bacteria, DNA can be extracted and the plasmid DNA separated from the host DNA. This yields greatly increased quantities of the plasmid, from which the original cDNA can be excised by further treatment with restriction enzymes.

If the plasmid vector used to clone the cDNA contains promoter sequences for bacteriophage RNA polymerases, these can be used to produce RNA transcripts of the cDNA *in vitro*. Commonly, separate polymerase promoter sequences are present at either side of the plasmid's multiple cloning site. Appropriate selection of the polymerase used then permits transcription of RNA with a sequence matching that of the original mRNA or its complement. The latter is suitable for use in the ribonuclease protection assays referred to previously.

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Transfection of eukaryotic cells

It is also possible to select as vectors plasmids which contain eukaryotic expression sequences. If these are then transfected into eukaryotic cells, cDNA sequences correctly inserted into their multiple cloning site will be transcribed, and the corresponding proteins expressed (Smith, 1993). Frequently the target proteins are expressed fused with a second protein coded within the expression vector and acting as an aid to isolation of the target protein.

This technology allows the effect of expressing a novel protein on the metabolism of eukaryotic cells to be investigated. Furthermore, it is possible to modify the native form of the cDNA with restriction enzymes and subsequent ligation before or after its insertion into the plasmid vector. Following transfection into the target cells, the functional significance of the modified regions of the cDNA can then be established by comparison of the effect of the modified plasmid with that of the control plasmid.

Polymerase chain reaction

The PCR provides a powerful alternative to cloning for obtaining increased amounts of selected regions of DNA (Bloch, 1991; Chesters, 1996). It depends on being able to select suitable primers based normally on at least a partial knowledge of the sequence of the DNA to be amplified. These oligonucleotide primers are short sequences of twenty to thirty base pairs of single-stranded DNA chemically synthesized to base pair with the extremes of the region of DNA to be amplified. Two primers are used per reaction, one equivalent to the 5′ end of the sequence of interest and the other to the sequence of the 5′ end of the complementary strand of the region to be amplified (Fig. 3). After the strands of the DNA have been separated by heating, they are allowed to cool in the presence of the oligonucleotide primers, which hybridize to the DNA and prime the synthesis of new strands of DNA complementary to each of the original strands. The old and new strands of DNA are then separated again by heating, and each used to prime another cycle of synthesis. Thus, each original strand of DNA gives rise to its own complementary strand of DNA and the amount of DNA theoretically doubles during each cycle.

The synthesis of each new strand by elongation of its primer requires the activity of an added DNA polymerase. The efficiency of PCR depends on the availability of DNA polymerases which are capable of withstanding, without undue loss of activity, the heating required for strand separation. The availability of such enzymes allows the process to proceed automatically through many cycles of amplification without the need to add fresh enzyme after each cycle of heat denaturation of the newly formed DNA duplexes. Relatively large quantities can be obtained of a DNA product with a sequence matching that of the original DNA located between the binding sites for the two primers. Furthermore, since the primers can be chosen to be specific for the target DNA, differential amplification of the target sequence can be achieved without its previous purification. The method is capable of generating detectable amounts of specific DNA given only trace amounts of original template

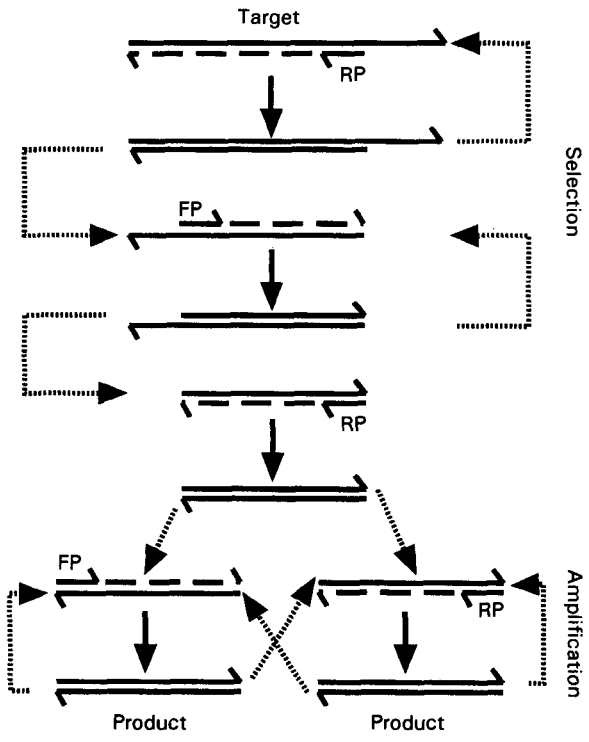

Fig. 3. Polymerase chain reaction. Forward (FP) and reverse (RP) primers are used during the selective phase to copy a section of the target DNA delineated by the primers. During amplification through subsequent cycles, the two strands of the selected DNA are separated and each used to generate replacement complementary strands. (— —) The extent of elongation of the primers; (·····▶) The fate of the products; (——▶) The changes occurring during successive cycles.

DNA, and, therefore, has proved invaluable in instances where the initial sample size is extremely limited.

Reverse-transcription polymerase chain reaction

If the strand of DNA used to initiate PCR is derived from reverse transcription (RT) of mRNA (RT-PCR) and the primers are chosen to be specific for an individual mRNA, this system can be used to amplify specific sections of that mRNA without previous isolation or cloning (Volkenandt *et al.* 1992; Chesters, 1996). The high sensitivity of RT-PCR also permits detection of mRNA at concentrations below those detectable by Northern hybridization or ribonuclease protection assay. However, the exponential nature of the amplification process renders quantitative assessments exceptionally vulnerable to minor variations in the efficiency of the reactions. In general, its use for quantification necessitates comparison of the experimental mRNA with an internal standard. This should preferably be amplified with the same primers, but be distinguished from the target material on the basis of its length or variations in sensitivity to restriction enzyme digestion.

Differential display

Frequently, a specially prepared primer is used for the RT step in RT-PCR in order to selectively amplify the specific

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mRNA under investigation. However, there is a modification to the RT-PCR protocol, termed differential display, in which general primers are used (Alpan *et al.* 1996). The aim of the protocol is to demonstrate differences in mRNA expression between treatment groups. With differential display, three separate RT reactions are performed, using as primers a sequence of deoxythymidylate residues to attach to the polyadenylate tail of the mRNA followed by a single residue which contains one of the bases other than thymine. The PCR stage of the reaction then uses the same reverse primer in combination with one or more arbitary forward primers. During the RT phase, each of the reverse primers hybridizes to a different group of mRNA, as determined by the different 3′ terminal nucleotides of the primers. In the subsequent PCR amplification, the arbitary forward primer binds at different distances from the 3′ end of the DNA strands generated by RT, and this results in products of differing length being obtained from the selection of mRNA reverse transcribed with each of the three initial primers. The products obtained from the different experimental treatment groups with the same primers can then be separated in parallel lanes on an acrylamide gel. Treatment differences in the mRNA expressed are apparent as differences in the banding pattern between lanes and the DNA in such bands can be isolated and sequenced to begin to define treatment differences in the patterns of gene expression.

DNA sequencing

Modern methods of DNA sequencing generally also depend on PCR (Sanger & Coulson, 1975; Rees, 1996). However, for sequencing reactions, only one primer is present and amplification is limited to a single strand of the DNA. Amplification proceeds linearly rather than exponentially, with a fresh copy of the region of interest synthesized during each cycle. Included with the deoxynucleotides required for DNA synthesis is a small proportion of their di-deoxy derivatives. Incorporation of a di-deoxy analogue in place of its normal nucleotide results in termination of the extension of that DNA molecule, and this occurs randomly at each of the nucleotide locations within the DNA being synthesized. Suitable electrophoretic analysis allows determination of which of the four di-deoxynucleotides resulted in termination of the molecule at each base position, and hence the sequence of the DNA can be deduced. Any one such determination generally yields the sequence of not much more than 400 bases, but suitable selection of primers allows overlapping segments of the DNA to be amplified, thus permitting the sequence of long stretches of DNA to be built up.

Regulation of transcription

The extent to which a particular coding sequence is transcribed generally depends on sequences in a region located 5′ to the transcription start site. Within this promoter region lie a number of binding sites for both general and specific transcription factors which function to modify the basic rate of transcription of the gene (Watson *et al.* 1987). The nature of these sites and their functional significance can be studied by inserting the corresponding cDNA or genomic coding sequence into a plasmid vector and observing the effect of placing either their native or modified 5′ regulatory sequences immediately upstream of the coding region. Often such experiments would be difficult to interpret if the native coding sequence were employed, since the products from the introduced plasmid would be identical to those from the endogenous gene. In such cases, the regulatory sequence under investigation is often attached to the coding region for a protein which is not normally expressed by the target cell. Thus, both bacterial chloramphenicol acetyltransferase (*EC* 2.3.1.28) and luciferase (*EC* 1.14.14.3) genes have frequently been used as 'reporter' genes, since their expression within mammalian cells is entirely due to the inserted plasmid, and variations in their activity reflect that of the promoter under test.

Gel mobility shift assays

An alternative approach to studying transcription-factor binding to upstream regulatory regions of genes involves incubating the region of DNA in question with nuclear protein extracts (Garner & Revzin, 1981). The DNA may be chemically synthesized or derived by restriction enzyme digestion of appropriate cloned DNA, but in either case, it must be labelled either radioactively or non-radioactively before use. The protein–DNA mixture is then subjected to electrophoresis through a gel and the distribution of the labelled DNA visualized. The conditions of electrophoresis are such that unbound DNA probe will move relatively unhindered through the gel but, if it complexes with transcription factors present in the nuclear extract, movement of the enlarged complex will be retarded. The pattern of retardation bands on the gel gives an indication of the number and size of the complexes.

Purified transcription factors may also be used in mobility assays to test their ability to complex with the promoter. By using modified versions of the native DNA sequence it is possible to determine which regions are critical for complex formation.

Methylation analysis

Methylation analysis requires initial treatment of adenine and guanine residues in the DNA from the upstream regulatory region with dimethyl sulfate to an extent sufficient to induce an average methylation of one base per molecule (Hendrickson & Schleif, 1985). The DNA is then end-labelled and used for gel mobility shift assay. Following separation of the bands, their associated DNA is isolated and the average level of methylation at each adenine and guanine residue in the uncomplexed DNA compared with that of the DNA in the band of interest. Since methylation of a base within the binding site will generally inhibit attachment of a transcription factor to that site, the factors will only bind to those molecules which happened not to be methylated within their binding site. Bases which appear methylated in the native DNA, but not

where it is complexed with protein, can be assumed, therefore, to lie within the binding site of the transcription factor, and thus help to define the location of that site.

DNAase I footprinting

DNAase I footprinting provides another approach to locating transcription-factor binding sites and is based on transcription factors protecting DNA within their binding site from being degraded by DNAase I (Galas & Schmitz, 1978). The target DNA is end-labelled and then incubated with the relevant transcription factor or nuclear extract. Once binding is established, the mixture is treated briefly with DNAase I and then run on a sequencing gel. DNAase I is an endonuclease which will hydrolyse bonds at random within the DNA. Electrophoresis of DNAase I-treated unprotected DNA will, therefore, generate a ladder of fragments of decreasing size. However, in those regions to which the transcription factors bind, the DNA will be protected from hydrolysis and, when run on a gel, gaps will appear in the sequence ladder in the regions to which the factor bound.

Southern blotting

Most of the techniques referred to previously help to define factors modulating mRNA concentrations. However, differences in genomic DNA which underlie variations in protein expression between individuals can be examined by Southern blotting (Southern, 1975; Kelly, 1996). With this technique genomic DNA isolated from the subjects is first split with restriction enzymes into characteristic fragments. The DNA is then separated by electrophoresis on an agarose gel, blotted and probed essentially as for Northern blotting but with important technical differences. The resultant blots can then be hybridized with appropriate probes to detect differences in length or sequence of the DNA restriction fragments.

DNAase I hypersensitivity

Finally, DNAase I hypersensitivity can provide clues to the factors influencing tissue specific expression (Eissenberg *et al.* 1985). Most cellular DNA is heavily complexed with proteins which restrict its transcription. These proteins also inhibit access of DNAase I to the DNA. However, there exist islands within the chromatin where the DNA remains relatively open to hydrolysis. These are thought to be sites of genomic regulation and can be identified by their susceptibility to DNAase I.

To obtain meaningful results, the DNA needs to be retained in as near its naturally complexed state as possible. This may be achieved by DNAase I treatment of nuclei isolated as gently as possible from the tissues of interest, or by direct treatment of permeabilized cells. Following brief DNAase I attack the DNA is isolated, cut into large sections by restriction enzymes and examined by Southern blotting, using a labelled probe designed to bind to the section of genomic DNA being investigated. If there are no DNAase I-sensitive sites within the sector to which the probe binds, a single, full-length band of DNA will be observed. However, sites of DNAase I sensitivity in the chromatin will be revealed as shorter segments on the gel, since the remainder of the cleaved DNA fragment will have been removed during electrophoresis. The patterns of sensitivity so revealed have been shown in many cases to be tissue specific, and probably represent sites of attachment of tissue specific factors.

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References

Alpan RS, Sparvero S & Pardee AB (1996) Identification of mRNAs differentially expressed in quiescence or in late G1 phase of the cell cycle in human breast cancer cells by using the differential display. *Molecular Medicine* **2**, 469–478.

Ausubel FM, Brent R, Kingston RF, Moore DD, Seidman JG, Smith JA & Struhl K (1994) *Current Protocols in Molecular Biology*. New York: John Wiley.

Aviv H & Leder P (1972) Purification of biologically active messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proceedings of the National Academy of Sciences USA* **69**, 1408–1412.

Bloch W (1991) A biochemical perspective of the polymerase chain reaction. *Biochemistry* **30**, 2735–2747.

Brown TA (1990) *Gene Cloning*. London: Chapman and Hall.

Chamberlein M & Ryan T (1982) Bacteriophage DNA-dependent RNA polymerases. In *The Enzymes*, vol. 15, pp. 87–108 [P Boyer, editor]. New York: Academic Press.

Chesters JK (1996) Polymerase chain reaction. *Proceedings of the Nutrition Society* **55**, 599–604.

Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159.

Devereux J, Haeberli P & Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acid Research* **12**, 387–395.

Eissenberg JC, Cartwright IL, Thomas GH & Elgin SCR (1985) *Annual Review of Genetics* **19**, 485–536.

Farrell RE Jr (1993) *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*. London: Academic Press.

Galas D & Schmitz A (1978) DNase footprinting; a simple method for the detection of protein DNA binding specificity. *Nucleic Acid Research* **5**, 3157–3170.

Garner MM & Revzin A (1981) A gel electrophoretic method for quantifying the binding of proteins to specific DNA regions. *Nucleic Acid Research* **9**, 3047–3060.

Hendrickson W & Schleif R (1985) A dimer of AraC protein contacts three adjacent major groove regions of the Ara I DNA site. *Proceedings of the National Academy of Sciences USA* **82**, 3129–3133.

Hesketh JE & Partridge K (1996) Gene cloning: studies of nutritional regulation of gene expression. *Proceedings of the Nutrition Society* **55**, 575–581.

Kelly KF (1996) Southern blotting. *Proceedings of the Nutrition Society* **55**, 591–597.

Pardue ML (1985) In situ hybridization. In *Nucleic Acid Hybridization: A Practical Approach*, pp. 179–202 [BD Hames and SJ Higgins, editors]. Oxford: IRL Press.

Rees WD (1996) DNA sequencing. *Proceedings of the Nutrition Society* **55**, 605–612.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanger F & Coulson AR (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology* **94**, 441–448.

Smith DB (1993) Purification of glutathione S transferase fusion proteins. *Methods in Molecular and Cellular Biology* **4**, 220–229.

Southern E (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.

Trayhurn P (1996) Northern blotting. *Proceedings of the Nutrition Society* **55**, 583–589.

Trayhurn P & Chesters JK (1996) Molecular biological techniques in nutritional science. *Proceedings of the Nutrition Society* **55**, 573.

Volkenandt M, Dicker AP, Banerjee D, Fanin R, Schweitzer B, Holikoshi T, Danenberg K, Danenberg P & Bertino JR (1992) Quantitation of gene copy number and messenger RNA using the polymerase chain reaction. *Proceedings of the Society for Experimental Biology and Medicine* **200**, 1–6.

Watson JD, Hopkins NH, Roberts JW, Steitz JA & Weiner AM (1987) *Molecular Biology of the Gene*. Menlo Park, CA: Benjamin Cummings.

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