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Symposium on 'How and why measure individual variability'

How are genes measured? Examples from studies on iron metabolism in pregnancy

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As the 21st century moves forward, it is becoming more and more apparent that the genetic makeup of any individual strongly influences the way they metabolise nutrients. It is very important, therefore, to understand the techniques and technologies used to assess the contribution genes make to the physiology of an individual. Clearly, it is not possible to provide a comprehensive overview, but in the present review an attempt will be made to show, using examples from the authors' research, how these methods have contributed to this understanding. Studies are being undertaken into Fe transport across the placenta, from the mother to the fetus, and the consequences of maternal anaemia on pregnancy outcome. Levels of gene transcript and protein have been measured using Northern and Western blotting respectively. During the course of this work a new protein has been identified using the available human genome database. Following this 'in silico' or 'cyber biology', techniques such as real-time RT–PCR and RNA interference have been used to examine expression of this gene and its protein. The methods used, briefly how they work and some of their limitations will be explained. The objective of the present review is primarily to give a better perception of how molecular biology can be used in research and to help gain a clearer understanding of some of the techniques used.

Real-time RT–PCR: Short interfering RNA: Multi-Cu ferroxidase

The observation that what might be beneficial for one individual can be harmful for another has made it apparent that the genetic make-up of any individual strongly influences the way they metabolise nutrients. Various dietary factors such as fatty acids, vitamins and minerals are involved in the regulation of gene expression (Carlberg, 1999; Clarke *et al*. 2002; Templeton & Liu, 2003). An example is Fe, an essential micronutrient, important for a large number of biological processes. Fe is an important cofactor for O_2 transport, respiration, the formation of some neurotransmitters, hormones and for DNA synthesis. However, Fe is potentially toxic and organisms have developed tight regulatory systems to control Fe uptake, distribution and storage. An imbalance in nutritional status at the cellular level often leads to an alteration in gene expression, potentially resulting in an alteration in cell proliferation, differentiation or cell death.

There are specific stages when requirements for nutrients change markedly, and pregnancy represents the most important of these stages. The Fe requirement during pregnancy is markedly increased as a result of the demands of the growing baby. The increase in maternal dietary uptake of Fe that occurs is not enough to provide the extra Fe, and the deficit must be met from maternal Fe stores. Once the maternal stores are depleted the mother will develop Fe-deficiency anaemia. In developing countries the percentage of women with Fe-deficiency anaemia can be as high as 80, but even in developed countries it can be up to 20. Fe deficiency during pregnancy has serious consequences for both the mother and the baby. Studies have shown that it can cause fetal growth retardation and that the effects generated in early embryonic development or during fetal development can persist into adulthood. Clearly, it is important to understand why these events occur.

Abbreviations: BLAST, basic local alignment tool; Cp, caeruloplasmin; siRNA, short interfering RNA.
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The authors' research group is studying Fe transport across the placenta from the mother to the fetus, and the consequences of maternal anaemia on pregnancy outcome. In species with haemochorial placentas (e.g. man and rats) there is a fused layer of cells, the syncytiotrophoblast, which forms a selective barrier between the mother and the baby. Nutrients are taken up at the maternal (apical) side, transferred across the cells and out into the fetal (basolateral) side. In order to investigate the mechanism of Fe transport and adaptation to Fe deficiency several experimental models have been used. Fe uptake across the placenta has been studied using rat placenta cells in culture, *in vivo* models and vesicles isolated from the microvillar border of both rat and human placentas. The mechanism of Fe efflux has been studied using human choriocarcinoma cell lines (BeWo cells) that have similar properties to the human placental trophoblast, and can be differentiated into the syncytiotrophoblast.

Iron transport mechanism

Uptake of Fe into the placenta is via transferrin receptor-mediated endocytosis (McArdle & Morgan, 1982, 1984; McArdle *et al*. 1984). Transferrin-bound Fe binds to the transferrin receptor and is internalised in the endosomes (McArdle *et al*. 1985; McArdle & Tysoe, 1988). The endosomes are acidified and Fe, subsequently converted to Fe^{2+}, is released from the endosomes, presumably through the divalent metal ion transporter-1, into the cytoplasm. Fe is then transported across the placenta and into the fetal circulation. Until recently the mechanism of Fe release from tissue has been poorly understood. An investigation using human placenta showed that IREG-1 (also known as metal transporter protein-1 or ferroportin-1) may be located in the basal membrane of the syncytiotrophoblast (Donovan *et al*. 2000), so that the export of Fe into the fetal circulation is through IREG-1 as Fe^{2+} (for review, see McArdle *et al*. 2003).

Iron deficiency

Maternal Fe deficiency, which causes a decrease in Fe levels in maternal liver, results in a smaller decrease in the placenta Fe levels and an even smaller decrease in fetal liver Fe levels (Gambling *et al*. 2001). Clearly, this adaptation is caused by compensatory changes in Fe transport across the placenta and occurs in order to minimise the level of Fe deficiency in the fetus. In Fe deficiency many of the proteins involved in Fe transfer are regulated at both the mRNA and protein levels. In order to determine the mechanism(s) of adaptation in the placenta the expression of transferrin receptor and divalent metal ion transporter-1 in placentas from Fe-deficient rats has been examined using Northern and Western blotting. The expression of transferrin receptor has been shown to increase at both the mRNA and protein levels. Additionally, there is an increase in the expression of the Fe-responsive element–regulated form of divalent metal ion transporter-1. A similar regulation of the transferrin receptor mRNA level has been demonstrated using a human placenta cell line (Gambling *et al*. 2001). Combined with Fe uptake studies these results indicate that the placenta compensates for low Fe levels by up regulating the expression of the receptors for Fe influx. These adaptations will result in increased Fe uptake by the placenta. However, the expression of IREG-1, involved in the transport of Fe from the maternal to fetal side of the placenta, is not up regulated at the mRNA level during Fe deficiency (Gambling *et al*. 2001). Taken together the results indicate that the regulation occurs primarily at uptake rather than efflux.

How were these results obtained?

Changes in nutritional status are known to influence intracellular activities, e.g. transcription of DNA into mRNA, translation of mRNA into protein and post-translational processing steps that may change the activity of the protein (for review, see Clarke & Abraham, 1992). In order to understand the molecular mechanism for the adaptation occurring in the placenta during Fe deficiency, changes in gene and protein expression have been looked at using Northern and Western blotting respectively. The following is a short description of the techniques and their advantages and limitations.

Northern blotting and the ribonuclease protection assay

Nucleic acid labelling and hybridisation to identical target DNA or RNA sequences form the basis for techniques such as Northern blotting and the ribonuclease protection assay. In general, RNA transcripts are separated according to their molecular weight by gel electrophoresis (Fig.1). The chemical nature of the RNA allows it to be immobilised by transfer to membranes. On the membrane

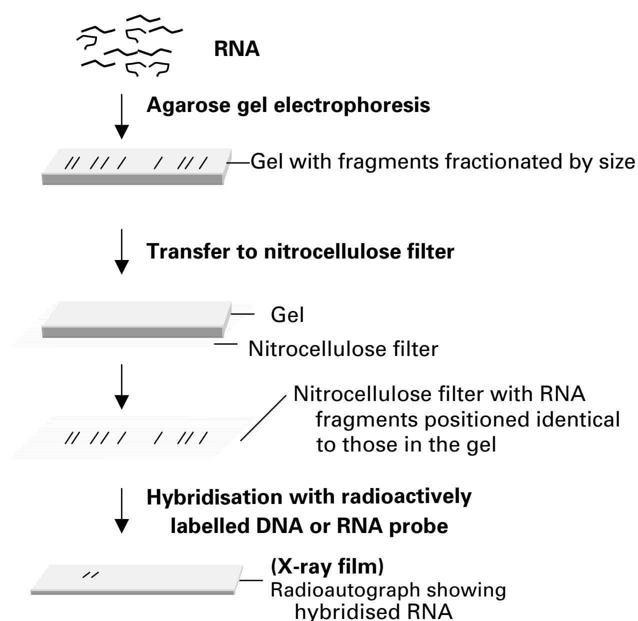

Fig. 1. Northern blotting. RNA is isolated from a tissue sample or cells by standard techniques. The RNA is separated according to size on a denaturing polyacrylamide gel. After electrophoresis, the RNA is blotted onto a membrane. Radioactively-labelled probes bind to RNA that has a complementary sequence. The image of the blot is visualised by exposing the blot to photographic film.

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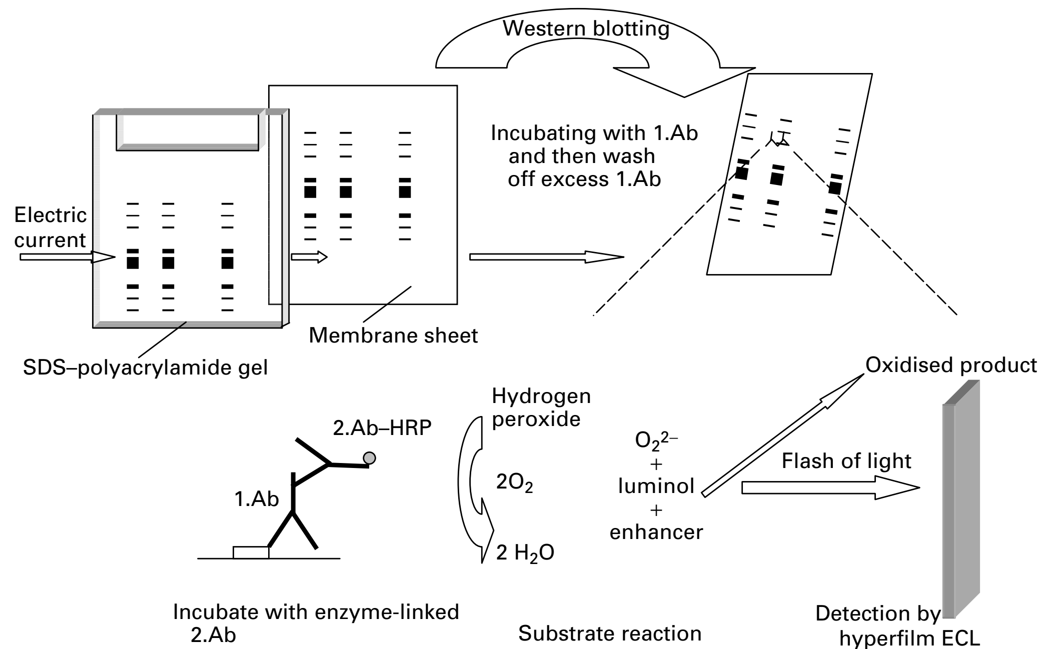

Fig. 2. Western blotting and chemiluminescence. Proteins in a sample are separated according to their size by gel electrophoresis, and blotted onto a membrane for analysis by Western blotting. The membrane is incubated with a primary antibody (1.Ab) specific for the protein of interest. To visualise the interaction between the target protein and the antibody, an enzyme-conjugated secondary antibody (2.Ab; 2.Ab–horse-radish peroxidase (HRP)) is added. The enzyme reacts with a substrate resulting in the emission of light. The image of the blot is visualised by exposing the blot to photographic film. ECL, enhanced chemiluminescence.

the fragments are identified by hybridisation to labelled (radioactive or non-radioactive) RNA, single-stranded DNA or oligodeoxynucleotides that are complementary in sequence to the RNA to be detected (Thomas, 1980). Northern blotting is the only analysis that provides information about the actual size of the mRNA transcript, alternative splice variants of the RNA and the quality of the RNA samples. However, it can be difficult to differentiate between closely-related genes of similar size because they will migrate to similar positions in a blot.

The ribonuclease protection assay is a technique for detecting and measuring specific RNA in a mixture of total RNA. It is based on the ability of ribonucleases to specifically degrade single-stranded RNA while leaving behind an intact labelled RNA probe that is hybridised to its target. This technique is very useful for mapping exon–intron boundaries in a transcript and for examining closely-related transcripts that would migrate to the same spot in a Northern blot analysis (Walmsley & Patient, 1994). A common disadvantage of Northern blotting and the ribonuclease protection assay is their relatively low sensitivity (Rottman, 2002). Additionally, because the mRNA level may not reflect the abundance of protein produced within the cell, changes in target gene expression are typically analysed by looking at the protein product by Western Blotting or other biological assays.

Western blotting

In Western blotting test samples are often unpurified cell homogenates or tissue biopsies containing a mixture of proteins that include the specific target protein. Proteins in a sample can be separated by gel electrophoresis (Gersten, 1996). As the proteins migrate in the gel they are separated according to their size and charge. Typically, smaller proteins migrate faster through the gel than larger proteins. Once the proteins are sufficiently separated in the gel they are transferred onto a nitrocellulose or polyvinylidene difluoride membrane for analysis by Western blotting (Towbin *et al.* 1979). Western blotting is a technique that uses antibodies to detect the presence, relative quantity and molecular weight of a protein in a crude mixture. Following transfer, the membrane is incubated with an antibody to the protein of interest. The amount binding should be proportional to the amount of protein, and control experiments are usually designed to verify this relationship. The amount of primary antibody binding is detected using a secondary antibody conjugated with an enzyme, typically horseradish peroxidase or alkaline phosphatase. This enzyme reacts with a substrate resulting in the emission of light. The image of the blot can be visualised by exposing the blot to photographic film and the protein level quantified by measuring the intensity of the band using densitometry (Fig. 2).

Among other factors, variation in the amount of protein loaded onto the gel and the efficiency of transfer from the gel to the membrane can contribute to errors in the quantification of protein levels by Western blotting. Thus, an accurate estimation of protein concentration using either the Bradford (1976) assay or one of the newly-developed protein assays, such as NanoOrange (Jones *et al.* 2003), is essential. As a consequence of demands by journals and

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referees, it is becoming increasingly common to normalise both the Northern and Western blot data against a gene or protein constitutively expressed in the tissue or cell line of interest rather than using ethidium bromide or total protein staining. Often, the β-actin or the glyceraldehyde-3-phosphate dehydrogenase gene and protein are used as an internal control for loading (Conklin *et al*. 2002). As will be discussed later (p. 486), the use of housekeeping genes and their protein products for normalisation is problematic, as their expression can be affected by the experimental treatment (Quail & Yeoh, 1995; Selvey *et al*. 2001). Western blotting is an excellent tool for looking at protein abundance, but it must always be considered that further measurements of the protein's function may be required. These measurements include the more traditional biochemical approaches such as enzyme activity assays or studies of transport across membranes.

Identifying a new oxidase necessary for export of iron to the fetus

The mechanism of Fe release from the placenta is poorly understood, but for the past few years a combination of computer-based analysis of gene sequences and the application of novel molecular biology techniques has accelerated this research.

It is known that once Fe is transported from the maternal side to the fetal side via IREG1 it is bound to transferrin, which is the plasma Fe-transport protein. For Fe to be bound to transferrin it has to be oxidised from Fe^{2+} to Fe^{3+}. Caeruloplasmin (Cp), a Cu-dependent enzyme, was first shown to be important for Fe efflux from tissue in the 1960s, and was later found to be the enzyme responsible for the oxidation of Fe^{2+} to Fe^{3+} (Roeser *et al*. 1970). Vulpe *et al*. (1999) identified a new multi-Cu ferroxidase involved in Fe release from the intestine into the circulation, termed hephaestin. The identification was partially done by computer database analysis, searching for gene sequences similar to the cDNA sequence of Cp. These two multi-Cu ferroxidases, Cp and hephaestin, play a critical role in the release of Fe from the liver and intestine respectively.

Similar to the Fe transport mechanism in the liver and intestine, Fe transfer across the placenta to the fetus requires an oxidation process. Cp is detectable in human fetal serum from early in gestation (Fryer *et al*. 1993) and has been suggested to be responsible for the oxidation of Fe on the fetal side. However, Harris *et al*. (1999) have found that pups born with no Cp appear to have normal serum Fe levels, but have an impairment of the movement of Fe, leading to accumulation in the liver. The accumulation of Fe would not be expected if Cp is critical for placental release of Fe. Furthermore, an involvement of Cp in Fe release has not been demonstrated (Danzeisen *et al*. 2000).

Instead, it has been shown that yet another oxidase, similar to Cp, is present on the basolateral (i.e. fetal) side of the placenta as a membrane-bound protein (Danzeisen *et al*. 2000). Western blotting has shown that the protein cross-reacts with the antibody for Cp, giving a main band at 100 kDa and a weaker band at 140 kDa. At this stage it had not been established whether this protein is a product derived from the gene for Cp or hephaestin, or whether it is a third member in a family of homologous ferroxidases. The protein level of this Cp homologue has since been shown to be regulated by the Cu and Fe level and to play a role in the efflux process (Danzeisen *et al*. 2002). It has been demonstrated that the ferroxidase is not Cp, the glycosylphosphatidylinositol-anchored form of Cp or hephaestin, but instead is a ferroxidase distinct from the others previously discovered. Recently, DNA sequences of a hephaestin homologue have been placed in publicly-available expressed sequence tag databases. The question arises as to whether these sequences code for the ferroxidase in the placenta cells. To answer this question a new approach has been undertaken, using computer-based analysis of gene sequences and application of novel molecular biology techniques such as real time RT–PCR and short interfering RNA (siRNA). The following is a short description of these methods and some guidelines on how to use them, based on the authors' experiences.

Databases

With the sequencing of the human genome and other organisms, access to most of the protein-coding genes is now available. The majority of the sequence information is accessible on the World Wide Web (Jennings & Young, 1999). One of the major databases is GenBank (http://www.ncbi.nlm.nih.gov) that contains most of the known nucleotide and protein sequences, with supporting bibliographical and biological annotations (Benson *et al*. 1999). The databases and the computer software make it possible to uncover related genes from large-scale gene expression data. Access to full-length sequences provides the material to design primers to facilitate cloning of known or related genes. With the full-length sequences structural models can be produced. Since proteins with similar structure often have similar functions it can help to predict function of proteins with unknown biological activity.

Expressed sequence tags are the major source of new sequences in the databases. Expressed sequence tags are single-stranded short (partial) sequences representing the start (5′ end) or the end (3′ end) of a cDNA. Expressed sequence tag databases are the foundation of many new gene discoveries. One approach for using the expressed sequence tag database is to search for genes related to, or similar to, a known gene by running a basic local alignment tool (BLAST) search.

BLAST is a sequence analysis service provided by the National Center for Biotechnology Information (National Library of Medicine, National Institutes of Health, Bethesda, MD, USA). The BLAST service has expanded enormously and it is beyond the scope of the present paper to provide a comprehensive review. In order to use the service, it is recommended that the home page (http://www.nebi.nlm.nih.gov/BLAST) should be read as well as the references contained therein. Via the BLAST home page there is access to a variety of databases, the two most common of which are the nucleotide and protein databases. With these databases it is possible, for example, to identify sequences, search for similarities between sequences,

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search for a specific motif or find similar proteins to the translated nucleotide sequence. The particular databases that should be used and the BLAST program that should be chosen depend on the nature of the question being asked. Vulpe *et al*. (1999) identified hephaestin as the ferroxidase in the intestine partly by performing a BLAST search of expressed sequence tags with the cDNA sequence of Cp. Alignment of genes and their protein sequences may provide an insight into their biological role, because genes with similar sequences tend to have a similar or related function. Alignment of the predicted protein sequence of hephaestin with the functionally-similar protein Cp has revealed that the cDNA encoded a protein that is 50% identical to the mouse Cp (Vulpe *et al*. 1999). Guidelines relating to the use of databases may be obtained from, for example, Nadeau & Dunn (1998), Claverie (1999) and Turchin & Kohane (2002).

How are changes in gene expression measured?

Improvements in existing techniques have generated highly-sensitive methods such as real-time RT–PCR, a sophisticated tool for the comparison of the levels of gene expression from different samples, as well as for the detection of low-abundance genes. As an alternative to the generation of gene knock-out animals, which is still an expensive and time-consuming way of examining the functional aspects of gene expression, a relatively new technique based on the use of siRNA can be utilised to silence the expression of genes *in vivo* and *in vitro*.

Real-time RT–PCR

PCR was first described in the 1980s by Kary Mullis (Saiki *et al*. 1986, 1988; Mullis, 1990) and has since been refined and modified. It is a flexible method that can be used to compare the levels of gene expression between samples, analyse expression patterns and discriminate between closely-related mRNA. It can also be used to generate inserts for cloning and fragments to be used as probes for Northern blotting. The PCR process uses multiple cycles of template denaturation, primer annealing and primer elongation to amplify DNA sequences. Theoretically, there is a quantitative relationship between the amount of starting target sequence and the amount of PCR produced at any give cycle. In practice, however, many researchers find that replicate reactions can produce an inconsistent amount of PCR product.

One of the newest advancements in PCR technology is quantitative real-time RT–PCR (Higuchi *et al*. 1993). Real-time RT–PCR involves the monitoring of the 'real time' progress of a PCR reaction, whereas traditional PCR measures abundance of the product as a point measurement at the end of the reaction (for reviews, see Bustin, 2000; Wittwer *et al*. 2001; Ginzinger, 2002). By using fluorescent probes or non-specific DNA-binding dyes such as SYBR Green 1, the progress of PCR amplification of a specific sequence can be followed over time as the intensity of the fluorescent signal increases with amplification. This technique is very sensitive, allowing the analysis of samples containing only a few copies of mRNA (Hu *et al*. 2003). In PCR the amplification of DNA is an exponential process, since amplified products from the previous cycle serve as the template for the next cycle of amplification. Eventually, the amplification process reaches a plateau when components in the reaction mixture become rate-limiting. The advantage of real-time RT–PCR is that it monitors the production of the product as it is being amplified, thus allowing the data to be collected during the exponential phase of the PCR reaction, where there is a linear relationship between starting target sequence and amplification product.

RNA extraction

The first step in real-time RT–PCR is the extraction of total RNA from tissue or cells lines. TRI (Helena BioScience, Sunderland, UK) reagent is an efficient and relatively quick method of isolating high-quality RNA, but other RNA isolation kits are also commercially available (e.g. from Stratagene, Amsterdam, The Netherlands; Qiagen, Crawley, West Sussex, UK). If the interest is in the expression profile of a single cell type or the expression of a low-abundance gene problems may arise, as it could be masked or lost because of the bulk of surrounding cells. With new techniques such as laser capture microdissection (Emmert-Buck *et al*. 1996) this problem can be overcome. With laser capture microdissection single cells or cell populations can be isolated from tissue sections with the integrity of the RNA and proteins preserved.

Since accurate estimation of RNA concentration is very important for the absolute quantification of the mRNA copy numbers, any contamination with DNA will result in an inaccurate quantification. Thus, the RNA samples are routinely subjected to DNase treatment to remove any contaminating DNA. To test for contamination with genomic DNA, a PCR of samples that have not been reverse transcribed into cDNA should be run.

RT

As RNA cannot serve as a template for PCR it must be reverse transcribed into cDNA. It is essential for the sensitivity and accuracy of the real-time PCR that the quantity of cDNA produced by RT accurately represents the original amount of RNA in the reaction. Hence, a precise estimation of RNA concentration is vital. The authors prefer to estimate the sample RNA concentration by the use of the Agilent 2100 Bioanalyzer (Agilent Technologies UK Ltd, South Queensferry, West Lothian, UK), which is a capillary gel electrophoresis-based system. This method is not only sensitive and reproducible compared with the traditional measurement of absorbance at 260 nm, but it also has the advantage of simultaneously verifying the integrity of the RNA. It is, however, comparatively expensive and does require specialist equipment.

First-strand cDNA synthesis can be performed using random hexamer primers, oligo dT primers or gene-specific primers. The choice of primers is best made after experimental evaluation of all three primer systems (Lekanne Deprez *et al*. 2002). For first-strand synthesis the Taqman RT reagent kit (Applied Biosystems, Warrington, Cheshire,

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UK) can be used, which contains all reagents needed for the conversion of RNA to cDNA. Other methods and kits may also be suitable. In some cases, where the secondary structure of the RNA might affect the ability of the DNA polymerase to generate a transcript, a change of DNA polymerase and optimisation of the reaction buffer might be needed.

PCR amplification

For PCR amplification in general, the concentration of $MgCl_2$ and the annealing temperature are experimentally determined for each primer combination used. As for the RT reaction, various DNA polymerases are available and the choice of which of these should be used has to be experimentally determined. It is important to remember that comparison of quantitative data from one run to the next requires the use of the same 'system'.

Several companies have developed PCR amplification kits that are easy to use and that minimise the time required to set up the assays. Applied Biosystems, for example, has a SYBR Green 1 Master Mix that contains all the reagents needed for the PCR reaction. Variables such as the Mg concentration and annealing temperature have been standardised, and the Master Mix reagent contains an internal reference dye (ROX) that minimises inter-well variation and normalises for non-PCR-related fluctuation in signal. SYBR Green 1 is a fluorescent double-stranded DNA-binding dye. After the DNA polymerase has elongated the sequence, SYBR Green 1 binds to the double-stranded DNA (Fig. 3). The longer the sequence becomes the more SYBR Green 1 dye binds to the product. Light then excites the SYBR Green 1 and a fluorescent signal of another wavelength is emitted and monitored in real time. The amplification cycle at which the fluorescent signal is detectable above background noise is determined as the threshold cycle (Fig. 4(a)). The higher the starting amount of nucleic acid target the sooner the threshold cycle value is reached. Using a standard curve with, for example, a known amount of RNA or number of copies, the threshold cycle value is used to calculate the initial amount of RNA or copy numbers in the unknown sample (Fig. 4(b)).

The drawback of using SYBR Green 1 dye is that it binds to any double-stranded DNA. Consequently, both specifically and non-specifically amplified DNA will generate signal. To increase the specificity of the reaction, gene-specific fluorescent-labelled probes can be used. Different types of probes exist, e.g. Förster resonance energy transfer probes, Taqman probes or cleavage-based probes. A common feature of these probes is that the fluorescent signal is only generated on amplification of the specific gene of interest and that any increase in fluorescent intensity is proportional to the amount of product produced.

Normalisation

Any variation in the amount of starting material (RNA) between the samples, or in the efficiency of RT and PCR, can contribute to errors in the quantification of mRNA transcripts. Consequently, the need to normalise the data arises. There are several options, but none of them are

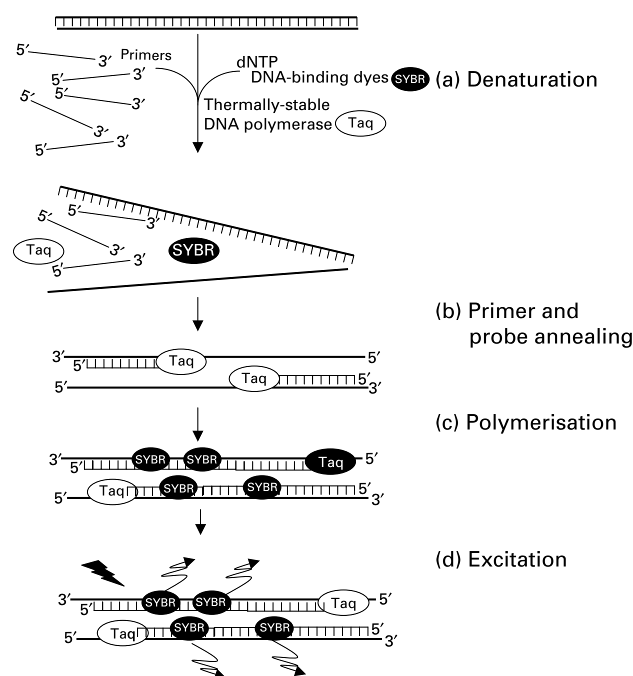

Fig. 3. DNA-binding dye incorporation method in real-time PCR. (a) During denaturation, unbound SYBR Green 1 dye (SYBR) has little fluorescence. (b) At the annealing temperature, a few dye molecules bind to the double-stranded primer–target sequence, resulting in light emission on excitation. (c) During the polymerisation step, more and more dye molecules bind to the newly-synthesised DNA. (d) On excitation of SYBR Green 1 dye with light a fluorescent signal of another wavelength is emitted and monitored in real time. dNTP, deoxynucleoside triphosphates.

ideal solutions. One is to normalise against the level of a housekeeping gene. Housekeeping genes are constitutively expressed in all cells, and expression should not be effected by the experimental treatment. Traditionally, housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase, β-actin and 18S ribosomal RNA have frequently been used for the normalisation of real-time PCR data. However, their use is problematic, as their expression is not always constant and can vary in response to experimental manipulations or show variation in concentration between individuals (Schmittgen & Zakrajsek, 2000; Selvey *et al*. 2001; Tricarico *et al*. 2002). This potential for inconsistency emphasises the importance of validating the housekeeping gene of choice in order to make sure that it is not regulated by the experimental design.

Another option is to normalise to the amount of total cellular RNA. This approach requires an accurate quantification of the RNA samples. As stated earlier (p. 482), the use of the capillary gel electrophoresis-based system gives a quantification of the RNA concentration and an evaluation of the integrity of the RNA in one step.

In some cases caution is required if the data are normalised to total RNA, as the total amount of RNA can be higher in proliferating cells. This factor can affect the results when comparing copy number in normal cells and, for example, cancer cells. As mentioned earlier, ribosomal RNA comprises the majority of the total RNA. Again, it is necessary to confirm that the experimental treatment does

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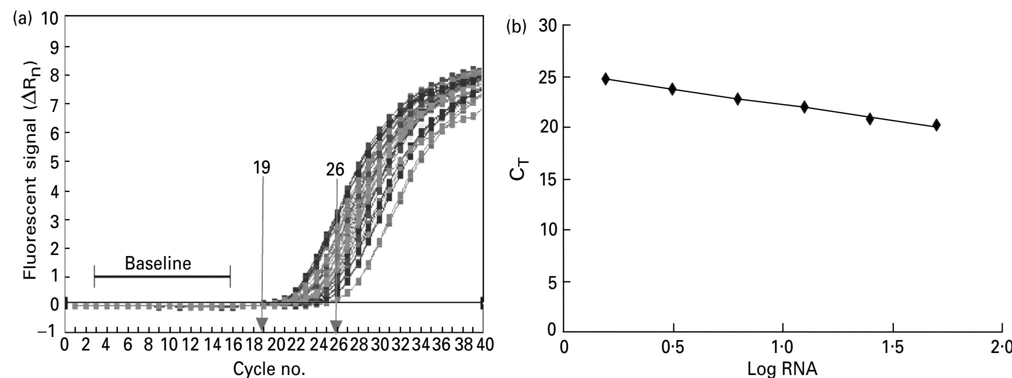

Fig. 4. Amplification of rat transferrin receptor gene in a 2·5-fold dilution of genomic DNA using the Applied Biosystem (Warrington, Cheshire, UK) 7700 system. (a) Amplification plot showing the change in fluorescence of SYBR Green 1 dye *v.* cycle number. The two threshold cycle (Ct) values (19 and 26) demonstrate the difference in threshold value when the starting amount of target DNA is higher and lower respectively (i.e. the higher the starting amount of nucleic acid target the sooner the Ct value is reached). (b) Standard curve showing C_T values *v.* log initial amount of reverse-transcribed RNA. $y = -3{\cdot}0837x + 25{\cdot}266$, R^2 0·9972.

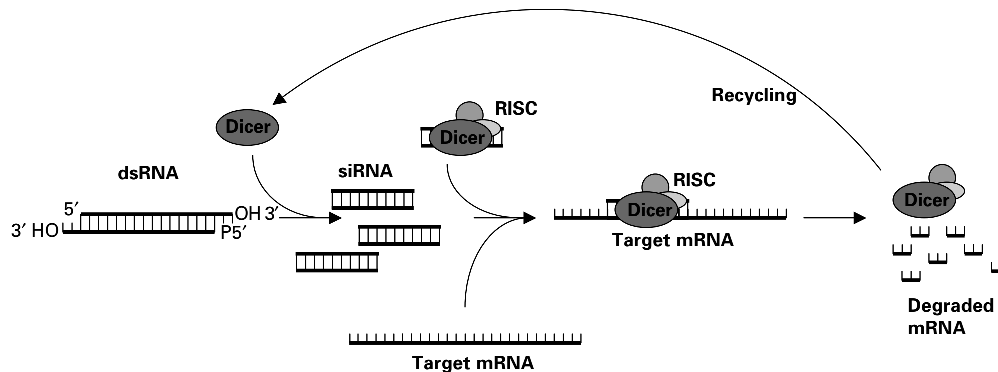

Fig. 5. The molecular mechanism of the small interfering RNA (siRNA) pathway. Double-stranded RNA (dsRNA) is cleaved by the RNase family member, Dicer into siRNA. These siRNA are then incorporated into the RNA-inducing silencing complex (RISC). The siRNA strand guides RISC to mRNA that has a complementary sequence, which results in degradation of the target mRNA.

not affect the expression of structural RNA such as 18S ribosomal RNA.

Short interfering RNA

siRNA are a new tool that can be utilised to evaluate the function of genes (for reviews, see Hammond *et al*. 2001; Hannon, 2002; Dykxhoorn *et al*. 2003). The method, termed RNA interference, describes the use of double-stranded RNA to target specific mRNA for degradation, thereby silencing the expression *in vitro* and *in vivo*. Since the discovery in 1998 that injection of double-stranded RNA in *Caenorhabditis elegans* led to an efficient sequence-specific gene silencing (Fire *et al*. 1998), the siRNA technique has been used to knock-down gene expression in a variety of invertebrate species as well as in mammalian cells (Harborth *et al*. 2001; Sorensen *et al*. 2003). siRNA typically consist of a short (twenty-one to twenty-three nucleotides) double-stranded RNA duplex with two-nucleotide 3′ overhangs. Once transfected into cells, double-stranded RNA are cleaved by the RNase III family member Dicer, into siRNA. These siRNA are incorporated in a multi-protein RNA-induced silencing complex. This complex consists of both RNA and nucleases in a specific arrangement that will act to degrade complementary RNA (Hannon, 2002). The siRNA guide the RNA-induced silencing complex to mRNA with a complementary mRNA sequence, which results in cleavage and degradation of the target mRNA (Fig. 5).

The short interfering RNA sequence

For efficient gene silencing using siRNA, both the length and the sequence are crucial. In mammalian systems the introduction of nucleotide sequences longer than thirty nucleotides can induce an antiviral defence system, leading to a global inhibition of mRNA translation (Elbashir *et al*. 2001) and general loss of host protein synthesis. Thus, the recommended length of sequences for siRNA is between twenty-one and twenty-three nucleotides. The design of

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the siRNA duplex requires knowledge of at least twenty nucleotides in the mRNA-encoding region of the target gene. Selection of the target region is often a trial and error process, but several research groups have generated a set of guidelines that narrow down the choice of siRNA for gene silencing (see Elbashir *et al*. 2002; Dykxhoorn *et al*. 2003).

It is well known that siRNA that target different regions of the same gene can show a marked variation in their efficiency at silencing a gene. The ability of siRNA to knock-down the expression of a gene can be influenced not only by the base composition of the siRNA sequence but also by the secondary structure of the mRNA and the binding of RNA-binding proteins. Thus, multiple regions are generally screened for each target (Elbashir *et al*. 2002).

Transfection of cells with short interfering RNA

The introduction of siRNA into mammalian cells is accomplished using lipid-based reagents (Caplen *et al*. 2001). Each cell system has to be optimised in relation to the concentration of cells plated, the type of transfection reagent and the cells' siRNA : lipid carrier ratio that should be used. In general, it is simplest to use those methods that have been developed for transfection with DNA vectors. However, this approach will not always work. For example, the authors had to try several different agents before a suitable one was found that would allow efficient transfection. It is also important to ensure appropriate control transfections can be carried out. Once again, this area of research is rapidly expanding, and the reader is advised to refer to recent reviews for more information (for example, see Elbashir *et al*. 2001, 2002).

Transient knock-down by short interfering RNA

The transfection of siRNA into cells leads only to a transient knock-down of the gene of interest, as mammalian cells, unlike plants and worms, lack the ability to amplify silencing (Vaucheret *et al*. 2001). Thus, the amount of siRNA needed for an effective transfection and silencing and the duration of inhibition need to be determined experimentally. In actively-dividing cells the duration of silencing is related to the number of cell doublings. In HeLa cells, for example, that have a doubling time of 24 h maximal inhibition is reported approximately 72 h after transfection (Kisielow *et al*. 2002). The duration of inhibition also depends on the abundance and/or the half-life (turnover) of the target protein. Repeated transfection may be required if the target protein has a slow turnover and when doing long-term experiments. Depending on the delivery system used, the variation in the duration of inhibition by the siRNA can also be obtained (Brummelkamp *et al*. 2002; Donze & Picard, 2002; Dykxhoorn *et al*. 2003).

So, what is the present position?

A membrane-bound protein in the placenta that is involved in a process regulated by Cu and Fe levels has been identified. Database searches and computer modelling of the predicted protein structure have been made, based on the alignment of the target sequence with the sequence of Cp. This approach has provided useful information on metal-binding sites, predicted structure and confirmed the finding that this protein is indeed a membrane-bound protein different from Cp and hephaestin. Previous data have demonstrated that both Cu and Fe level regulate the expression of the protein. Real-time RT–PCR has been used to characterise regulation at the mRNA level and a close correlation has been found between protein and gene expression. Finally, the siRNA technique has been proved to be a powerful tool for establishing the final link between the protein and the sequence encoding the protein.

In the present review the focus has been on how a combination of computer-based analysis of gene sequences and application of both traditional and novel molecular biology techniques has been used to identify the nature of a protein important for Fe transport during pregnancy. Although the examples chosen for the most part are those of interest to the authors, it is hoped that the present review will stimulate interest in the productive blending of molecular biology techniques with nutritional investigations. Clearly, the availability of these techniques will accelerate the understanding of nutritional interactions and the importance of nutrients for health and development.

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