CHAPTER 4

REVERSE TRANSCRIPTASE POLYMERASE CHAIN
REACTION TO INVESTIGATE PTP-1B mRNA
EXPRESSION IN HL-60 CELLS

SUMMARY

This chapter describes the use of RT-PCR to study expression of PTP-1B mRNA in proliferating and differentiating HL-60 cells and to generate a product for use as a probe for northern blotting and for cloning.

Total RNA was extracted from HL-60 cells using the Tri-Reagent™ (Sigma) method based on the acid-guanidinium thiocyanate single step procedure of Chomczynski and Sacchi (1987). The RNA extracts were reverse transcribed to cDNA using AMV reverse transcriptase and a segment of PTP-1B cDNA was amplified by PCR. The effect of ATRA and 9-cis RA on PTP-1B mRNA expression was analysed by reverse transcriptase polymerase chain reaction (RT-PCR). Dynamic behaviour was seen but the modulators did not change the dynamics. The high sensitivity of the PCR technique and the exponential increase in PCR product with each cycle of amplification making analysis difficult.
MATERIALS AND METHODS

Polymerase Chain Reaction: BASIC PRINCIPLES

The PCR is an in vitro method based on the amplification of specific DNA or RNA fragments of defined length and sequence. Amplification greater than $10^6$ fold can be achieved from very small amounts of complex template. This is achieved by means of two flanking oligonucleotide sequences (primers), and repeated cycles of amplification with the enzyme Taq DNA polymerase. The invention of the polymerase chain reaction (PCR) technique for nucleic acid amplification has had a major impact on many diverse areas of both basic and clinical research. Since its inception (Saiki et al., 1985), reports on a wide variety of applications for PCR have received much attention in scientific and medical literature. This technology has been shown to have a vast applicability to the diagnosis of human diseases including such diverse areas as infectious diseases, genetic disorders and cancer (Arends and Bird, 1992).

The template DNA is added to a reaction mixture containing two synthetic oligonucleotide primers. Primers are annealed to the complementary strands of target DNA with their 3’ ends pointing towards each other. Extension of the annealed primers is achieved with the enzyme Taq DNA polymerase. Repeated cycling of heat denatured template, annealing of primers to their complementary sequences and extension of the annealed primers with Taq DNA polymerase
results in the amplification of the target DNA (figure 41). The amplified fragment is thus defined by the 5’ ends of the PCR primers (Mullis and Faloona, 1987).

The process results in an exponential accumulation of the specific target DNA, up to several million fold, in a few hours. Between 0.5 and 1µg of DNA, up to 2 Kb in length, can be obtained from 30 to 35 cycles of amplification with as little as 100 ng of starting material. Under standard reaction conditions, the amount of Taq DNA polymerase becomes limiting after 25 to 30 cycles. If further amplification is required, this can be achieved by diluting the amplified DNA up to $10^3$ fold, in a new reaction. Using this technique, $10^9$ or $10^{10}$ fold amplification can be achieved and has been used in conjunction with Southern hybridization to identify a single copy of a target sequence.

The PCR technique is now automated; this is made possible by the thermal stability of the enzyme Taq DNA polymerase and by using an automated thermal cycler.
Figure 41(a): Diagrammatic representation of the polymerase chain reaction

DNA to be amplified is denatured by heating the sample. In the presence of DNA polymerase and excess deoxynucleotide triphosphates, oligonucleotides that hybridize specifically to the target sequence can prime new DNA synthesis. The first cycle is characterized by a product of indeterminate length; however, the second cycle produces the discrete ‘short product’ which accumulates exponentially with each successive round of amplification. This can lead to many million-fold amplification of the discrete fragment over the course of 20 to 30 cycles.
Reverse Transcription-Polymerase Chain Reaction Analysis of PTP-1B

Reverse transcription (RT) of total cellular RNA into cDNA was followed by the amplification of specific cDNA regions to detectable levels during the polymerase chain reaction (PCR). The RT-PCR technique allows the semi-quantitative analysis of gene expression (the transcription of specific mRNA species) within the cell. The PCR product can then also be used for cloning and sequencing the gene product.

Reverse transcription

The synthesis of cDNA from total cellular RNA is achieved by the use of the retroviral enzyme AMV reverse transcriptase. To initiate synthesis, 0.25 µg of oligo dTs were used as primers to bind to the poly-A tails of 0.5µg of total RNA (as prepared in chapter 3). This was achieved by heating them together to 60°C for 5 minutes and then chilling on ice. The mix consisted of 20U of RNase inhibitor, 1mM of each of the 4 dNTPs, 1 x RT buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT and 0.5 mM spermidine), 10U of AMV reverse transcriptase and Baxter water to make a final volume of 20µl. The reaction was allowed to proceed at 37°C for 1 hour and was terminated by heating to 70°C for 5 minutes and cooling immediately on ice.
**PCR primer selection**

PCR primers were selected after careful analysis of the PTP-1B gene sequence using the sequaid computer program (freely available from the internet). Primers were designed such that they were specific for the sequence and did not self anneal to produce primer dimer.

**PTP-1B PCR primers**

Primers were synthesized for us by Genosys Biotechnologies and the Department of Biochemistry, University of Cape Town, DNA Synthetic Laboratory.

**Table 6: PTP-1B primers used and size of products produced**

<table>
<thead>
<tr>
<th>PRIMER SET</th>
<th>mRNA</th>
<th>PRODUCT SIZE (BP)</th>
<th>PRIMER SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PTP-1B</td>
<td>119</td>
<td>F primer 5’-TTTTGGGAGATGGTGTTGGGA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R primer 5’-ATCATCTCTTTTCTTCTT-3’</td>
</tr>
<tr>
<td>2</td>
<td>PTP-1B</td>
<td>753</td>
<td>F primer 5’-TTTTGGGAGATGGTGTTGGGA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R primer 5’-GGGCGAGTCTTTATCCTCCT-3’</td>
</tr>
</tbody>
</table>
Human PTP-1B mRNA sequence (3247 bp)

1  GGGCGGGCCT CGGGCTAAG AGCGCGACGC CTAGAGCGGC AGACGGCGCA GTGGGCCGAG
61  AAGGAGGCGC AGCAGCGGCC CTGGCCCGTC ATGGAGATGG AAAAGGAGTT CGAGCAGTCG
121 GCAAGTGCCG GGAGCTGGGC GGCCATTTAC CAGGATATCC GACATGAAGC CAGTGACTTC
181 CCATGTAGAG TGGCCAAGCT TCCTAAGAAC AAAAACCGAA ATAGGTACAG AGACGGCCAG
241 CCCCCTGACC ATAGTCGGAT TAAACTACAT CAAGAAGATA ATGACTATAT CAACGCTAGT
301 TTGGATAAAA TGGAAGAAGC CCAAAGGAGT TACATTCTTA CCCACGCCCT TTTGCTTAAC

F primer set 1 and 2

361 ACATGCAGTC AGCTTTGGGA GATGGTGTGG GAAGAAAA GCAGGGGTGT CGTCATGCTC

Eae I ↓

421 AACAGAGATGA TGGAGAAAAGG TTCGTTAAAA TGCGCACAAT ACTGAGCACA A
AAAGAAGAA

481 AAAGAGATGA TGGAGAAAAGG TTCGTTAAAA TGCGCACAAT ACTGAGCACA A

R primer set 1 Ava I ↓

541 TCATTATATA CAGTGCGACA GCTAGAAATT GAAAACCTTA CAACGCCAAGAA GCAGTAGAG

601 ATCTTTACATT TCCACTATAC CACATGGGCC GACATTGGAG TCCCTGAATG ACCAGCGCTCA

661 TCTTTGAGCT TCTTTTCAAG AGTCCGAAGAG TCCCTGCAGA CACCGGAGCCC

721 GTTGTTGGGC ACTGCAGTGC AGGCATGGGC AGGTCTGGAA CCTTCTGTCT GGCTGATACC

781 TGCTCTGGCG TGATGGAGAC CAGGAGACAA CTTCTTCCCG TTTGATACAG GAAAGCTGCTG

Pvu II ↓

841 TTGGAAATTG AGGAAGTTTCG GATGGGGTTG ATCCAGACAG CCGACCAG CTGCTGCTTCC

901 TACCTGGCAGT TGATCGAAGG TGCCAAATTC ATCATGGGGG ACTCTTCCGT GCAGGATCAG

Ava I ↓

961 TGGAAAGGAGC TTTGCCACGA GGACCTGGAG CCCCCAC CCGAGCATATCCC CCCACCTCCC

Eae I ↓

1021 CCCGCCACCA ACAGATCTCT GGAAGCGACG AACGGAAATG GAGGGTAGTT ATCCCCAAT

1081 CACCAGTTGG TGAAAGAAGA GACCAGGAGATTG CATTGAGACT GCCCCCTTCC A

R primer set 2

1141 GGAAGCCCCCT TAAATGCGGC ACCCTACTGCG ATCGAAAGCA TGAGTCAGA CACTGAAGTT

1201 AGAAGGCGGG TCGTGAGGGG AAGCTCTCAG ATGCGGCAGG CTGCTCTCCC AGCCCAAGGG

1261 GAGCCCTCAG TGGGCGAGAA GAGCAGGAGG CATGCACTGA GATCTGAGAA GCCCTTCTCG

1321 GTCAACAGTG CGGCACGTAC GCTCCTCACG GCGCGGCGCT ACCTCTGCTA CAGGTTCTCG

1381 TCAACATGCA ACACATAGGC TGACCCCTCT CAACCTCCAC GCCACCCACT GTCCGCGCCTC

1441 GCCCGCAGAG CCCCCCGCGG ACTAGCAGGC ATGGCGGCGTG AGATAGAGGC CGCGGACCG

1501 CGTAGAGAGC CGGGCCGCGG AGCGAGATTG GTTCTGACTA AAAACCATCA TCCCCCGGAT

1561 GTTGTCCTCA CCCCCCTATC TTTTAATTTT TCCCCCTCCC ATTTCTGAGTA CAAATATCCC

1621 AACGCTTTT TTGAGGAGAG TGAAAGAAGG TACCATTGCTG GCGGCCGAGA GGGAGGAGG

1681 CTACACCCGCT CTTGGGGGTC GCCCCACCA GGGCTCCCCG CTGGAGCATC CCAGGGCGGC

...
1741CACGCCAACAGCCCCCGCTTGAACTCGACGGGAGCAACTCTCCACCTAATTATTTTA
1801AACAAATTTTCTCCCCAAGGATCCCATAGTGCTCACTGCTATGACCAATAAATGTA
1861TTAAAAATTTCATGTCACCTCTGCATCAAGGGCTTTATCAAGATCTTGACAAATAATAAAT
1921CTTCCAGGTTGAGGAAAGGCCTTTGGCTGACTGCCGATCGACCCAGAAGCCAGCT
1981GGGAAGGAGGACGCGTTGCAAAGCGTCTGTTATTTAGTGATTTGTAAGCGTGAGAAC
2041TAGAAACAATCTATAATAATGAAACCATGCCGATTTAACCTGCTGTTGAGAATAAAATG
2101ATTACCTTTGTCGGTTTAATTCTCTACCTTCTTGCTGATGATGATGATGCTGTAAGCT
2161CTTCCAGGTTGAGGAAAGGCCTTTGGCTGACTGCCGATCGACCCAGAAGCCAGCT
2221CTTGAATGGAAGACTTTTTGACTCTGCTGTAATATATATATATATATATATATATATAT
2281CCCTAGGCTACCCGATGCTAGCCTCGACCTTGCGACATGCGACATGCGACATGCGACATG
2341TGGCTGACCAATAGGGTGCAGTTTTCCAGGAAAGCCATATGCTGTGCATGCCCGGAC
2401TTTCTTTTCTTCTCGACCTTCTCTCGACCTTCTCTCGACCTTCTCTCGACCTTCTCTCGAC
2461CAGACATTTCCAGGGGTATGGGAAGCCATATGCTGTGCATGCCCGGAC
2521GAGAGCAGGAGCCCGGTGTGCTGCACTCTCATGGGATCACATGGCAGAGAGCAGGAGC
2581ACTCTCTCTTGCAGCGTCTGCCTCGACCTTCTCTCGACCTTCTCTCGACCTTCTCTCGAC
2641AAACAGCCTGCGTGACGCTGCTTGGGACGCGCTTGGGACGCGCTTGGGACGCGCTTGGGACG
2701GCCCGGAGAGGCGCGTGCTGCCTCGACCTTCCACCATGCTCTGCGACCTTGGGAGAGC
2761TTTCTTCTCCTCTTGCAGCGTCTGCCTCGACCTTCTCTCGACCTTCTCTCGACCTTCTCTCGAC
2821GAGAGCAGGAGCCCGGTGTGCTGCACTCTCATGGGATCACATGGGTCGCGGCTGCTGCTGCT
2881GTTAGAGAGAGGTTTGATGGCTCGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
2941TATGAGAAGGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
3001GTGTTTAAATCTTCTGATCTTCTGATCTTCTGATCTTCTGATCTTCTGATCTTCTGATCT
3061AATTCGATAGCTGTTTAAACCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
3121ATGGGATGGCTCGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
3181TGAGTGACATTTTTTCACTGCGGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
3241AAAAAGC

Figure 41(b): Graphical representation of PTP-1B mRNA demonstrating primer binding sites and restriction digestion sites.

Red = forward and reverse primers as labelled
Yellow underline = 119bp PCR product (using set 1)
Grey = 753bp PCR product (using set 2)
The PCR cycling programme

The PCR reaction mixture contained 10 µl of cDNA from the reverse transcription (RT) reaction, 5 µl of 10x MgCl$_2$-free Thermophilic buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0 and Triton X-100), primer pairs at concentration 0.5 µM and Baxter water to make up the reaction volume to 50µl. The cDNA was denatured at 96°C for 5 minutes and cooled on ice before adding 1U of thermostable DNA polymerase. To prevent evaporation 50 µl of nuclease-free mineral oil was layered on top of the PCR mixture.

The reaction mix was heated to an intermediate temperature of 72°C for 2 minutes before being cycled between the denaturing temperature of 93°C for 30 seconds, the primer annealing temperature of 45°C for 30 seconds and the DNA polymerase extension temperature of 70°C for 1 minute. A total of 30 cycles were completed, followed by a final extension at 72°C for 5 minutes.

Visualization of PCR products

The PCR product was electrophoresed for 1-2 hours at 60V on a non-denaturing 1% agarose gel [1 x TAE buffer (pH 7.2) containing 25 mM Tris-HCl, 20 mM sodium acetate and 1 mM EDTA] containing ethidium bromide (1 µg/ml) to allow visualization of the PCR products under UV light. Samples were first
diluted with DNA loading buffer (1 x TAE, 50% glycerol and bromophenol blue). The tank buffer was 1 x TAE.

**Restriction endonuclease reaction of PCR product**

Restriction endonucleases are a group of enzymes that have become essential to gene cloning, analysis and manipulation. Their importance lies in the fact that they are able to ‘cut’ double stranded DNA specifically. The specificity is determined by the sequence of nucleotides which the enzymes recognize and digest. The recognition sites are usually palindromic sequences of approximately 4 to 6 nucleotides and the enzymes cut around the central axis of this sequence to produce ‘blunt’ or ‘sticky’ ended DNA fragments. The size of the fragments produced from a digestion reaction may be used conveniently to determine the restriction map of the template DNA being digested. Blunt or sticky ended DNA fragments may be used for ligation reactions to produce recombinant molecules for gene cloning.

In order to confirm that the PCR products generated in or study were indeed the correct products, specific restriction sites for endonucleases were identified in the sequence and the sizes of the resultant restriction fragments produced compared with molecular weight markers.
Digestion protocol

DNA/ PCR product \( x \mu l \) (±400ng)
10 x buffer \( 1 \mu l \)
Baxter water \( 8-x \mu l \)
Restriction enzyme \( 1 \mu l \) (±10U)

Total volume = 10 \( \mu l \)

Quantitation and data analysis

Agarose gels were photographed and then scanned densitometrically using a Hitachi light camera and Biomed scanner software. PCR product expression was given in terms of peak area (arbitrary units) with a fourth order polynomial fit to the data points.
RESULTS

Optimization of PTP-1B RT-PCR using primer set 1

The use of primer set 1 resulted in the production of a 119bp PTP-1B RT-PCR product (figure 42) that was labelled with $^{32}$P dCTP and used for hybridization to northern blots (chapter 3). The RT-PCR system was optimized for template cDNA and taq polymerase; the best results were obtained when using 200ng of cDNA and 0.5U taq polymerase (figure 42).

Figure 42: Optimization of PTP-1B RT-PCR. Lane 1: molecular weight markers, lane 2: control (0.5 µM primers without template cDNA), lane 3: total cellular DNA extracted from HL-60 cells with 0.5 µM primers and 1U taq polymerase, lanes 4, 5, 6, 7 and 8: contain 20, 40, 80, 140 and 200ng cDNA template, respectively with 0.5U taq polymerase, lane 9: ethanol precipitated 20ng cDNA with 0.5U taq polymerase, lane 10 and 11: 140 and 200ng cDNA template with 1U taq polymerase, respectively.
Optimizing RT-PCR for *taq* polymerase, magnesium chloride and primer set 2 concentration

PTP-1B RT-PCR was optimized for *taq* polymerase, magnesium chloride and primer set 2 concentration. A series of experiments was designed to optimize each of these variables. Results from figures 43 and 44 indicated that 1µM primer, 2mM MgCl₂ and 1U *taq* polymerase were optimal.

Figure 43: 1% Agarose gel electrophoresis of PTP-1B RT-PCR product: optimization of primer concentration. Lane 1: molecular weight markers, lane 2: control (cDNA template without primers), lanes 3, 4, 5, 6: 0.250 µM, 0.5 µM, 1.0 µM and 2.0µM primers, respectively. All reactions used 1 U *taq* polymerase and 2mM MgCl₂.
Figure 44: 1% Agarose gel electrophoresis of PTP-1B RT-PCR product optimization of MgCl₂ and Taq polymerase. Lane 1: molecular weight markers, lane 2: control (no primers), lanes 3-6: 1.0 µM primers, 1 U taq polymerase with 1 mM, 1.5 mM, 2 mM and 3 mM MgCl₂, respectively, lanes 7-10: 1.0 µM primers, 2 mM MgCl₂ and 0.5, 1, 1.5 and 2 U taq polymerase, respectively.
PTP-1B RT-PCR product verification for primer set 2 by restriction digestion

After having optimized the RT-PCR reaction for primer set 2, PCR product (753 bp) formed was verified by restriction digestion with three specific restriction endonucleases, namely: AVA I, PVU II and TRU 9I as shown in figure 45. Digestion of the PCR product, with AVA I gave rise to three fragments (404, 221 and 128 bp), with PVU II two fragments (516 and 237 bp) and with TRU 9I three fragments (611, 73 and 69 bp), respectively.

Figure 45: 1% Agarose gel electrophoresis of PTP-1B PCR product digested with restriction endonucleases. Lane 1: molecular weight markers, lane 2: PCR product, lane 3: PCR product digested with AVA I, lane 4: PCR product digested with PVU II, lane 5: PCR product digested with TRU 9I.
The effect of ATRA and 9-cis RA on the expression of PTP-1B mRNA using RT-PCR

A single band of 119bp PTP-1B RT-PCR product was produced (primer set 1). HL-60 cells were stimulated with either ATRA or 9-cis RA, total RNA extracted, converted to cDNA (200ng) by AMV reverse transcriptase and added to a PCR reaction containing 0.5U Taq polymerase, 1µM primer and 2mM MgCl₂. Below is an example of a 1% agarose gel run with RT-PCR products from cells stimulated with 9-cis RA (figure 46). Results indicate PTP-1B mRNA expression via RT-PCR is dynamic over 120 minutes.

Figure 46: PTP-1B RT-PCR product on a 1% agarose gel. Lane 1: molecular weight markers, lane 2: control (cDNA without PTP-1B primers), lanes 3-10: HL-60 cells stimulated with 9-cis RA sampled every 15 minutes.
The expression of PTP-1B mRNA RT-PCR product was quantitated and analysed graphically (figure 47). Results indicate dynamic behaviour, but there is no change in expression of PTP-1B mRNA on stimulation of cells with either 10mM ATRA or 10mM 9-cis RA.

**Figure 47: Graphical representation of the effect of ATRA and 9-cis RA on PTP-1B mRNA expression.** HL-60 cells were stimulated with either 10mM ATRA or 10mM 9-cis RA, analysed over 120 minutes.
DISCUSSION

The last decade has witnessed tremendous growth in the application of the tools of molecular biology to diagnose human diseases (Coutelle, 1991). DNA probe assays are now commercially available for the detection and identification of a variety of human pathogens, as well as for the diagnosis of human genetic disorders. A manifestation of the rapid growth of DNA technology has been the development of techniques to amplify specific nucleic acid sequences, and to clone these sequences for use in diagnostic applications.

The method for cloning specific DNA sequences to generate probes for identifying variations in or modifications to the cell genome, form the basis of recombinant DNA technology (Sambrook et al., 1989). Recombinant DNA technology depends on two key concepts: (a) cleavage of DNA at sequence specific sites by restriction endonucleases (REs), and (b) hybridization of cDNA sequences. Cloned DNA fragments can be characterized by base pair sequencing and may also be labelled with radioactive nucleotides or non-radioactive chromogens for use as probes.

With all its apparent advantages, PCR is not infinitely powerful or infallible, and does have a number of negative aspects. Eventually, after numerous cycles, the concentration of primers falls due to their incorporation into products of the
reaction, similarly, the concentration of dNTPs and the activity of the DNA polymerase itself, decline. Products of pyrophosphate breakdown may also inhibit further amplification. The specificity of the priming reaction itself depends on several considerations, and the reaction may be inefficient if one of the primers is depleted because of excessive spurious priming (Markham, 1993). Replication error rates have been found to vary from 22 errors in 8000 bases, an error rate of 1 in 400 (Dunning et al., 1988), to 1 single base substitution per 9000 bases (Tindall and Kunkell, 1988).

Contamination may occur with all techniques and is best avoided in PCR by careful laboratory practice. Contamination becomes a major problem if positive controls containing concentrated amounts of mutant sequences are used. The major source of potential contamination then becomes the amplified gene product of experimental cases, and true positives can be a source of contamination causing false positives in later assays (Wright and Wynford-Thomas, 1990). False positives arising from contamination can be avoided by reducing the number of PCR cycles to the minimum required to detect clinically significant doses of microorganisms; positive controls should not be amplified in the same assay and should be prepared separately. False negatives arise when amplification fails for technical reasons. If further analysis is to be performed, such as sequencing, then failed reactions can be repeated or excluded.
Non-specific amplification occurs when primers anneal to other sites in addition to the target site. This can be a source of error, resulting in false positives. A sharp control band on a DNA gel excludes both reaction failure (false negative) and non-specific amplification, provided there are no problems which are specific to the primers or the target sequence.

Accurate quantitation requires internal controls in each PCR reaction, which act as a standard. Often, the primer pair of interest can be used to produce the internal quantitation standard, which in the case of RNA-PCR could be the larger genomic copy (containing introns) corresponding to the amplified cDNA copy. However, differences in the sequence enclosed by the primers can affect amplification efficiency. Hence, any quantitative PCR assay must be re-evaluated for each new sequence of interest, using serial dilutions of a known amount of the target sequence.

The starting template for a PCR reaction can be DNA or RNA. DNA is usually the appropriate template for studying the genome of the cell or tissue or for the detection of DNA viruses. For information on gene expression in a cell or tissue, or the presence of genomic RNA in a retrovirus such as HIV, RNA is the appropriate template. Enzymatic amplification by PCR is well suited for RNA analysis since quantities available for investigation are frequently limited and
often insufficient for more standard methods of analyses, such as northern blotting.

Since DNA polymerase will not work on RNA templates, the RNA retroviral enzyme reverse transcriptase (RTase) is used to catalyse the generation of cDNA from RNA. This cDNA can then be amplified using PCR, in a technique termed reverse transcription-PCR (RT-PCR). Millions of copies of cDNA arise from RNA species, even from a single cell (Rappolee et al., 1989). This has enabled the detection of traces of “illegitimate transcripts” of inappropriate tissue-specific products in cells such as fibroblasts, lymphoblasts and hepatoma cells (Chelly et al., 1989).

RNA can be preferable to genomic DNA for detecting structural changes in long genes. Amplifying the RNA transcript instead of the genomic sequence greatly reduces the length of the DNA to be handled, without losing any of the coding regions where clinically significant deletions may be expected. If there is insufficient sequence information for two primers, the second primer can be poly T, complementary to the mRNA poly A tail (added to the 3’ RNA end of mature RNA species during processing) (Berchtold, 1989). Similarly, an oligonucleotide of known sequence can be joined onto the 5’ end and a complementary primer used (Frohman et al., 1988). The disadvantage of using
only one specific primer is that it increases the chance of non-specific amplification.

The objective of this study was to employ the technique of RT-PCR, also termed message amplification phenotyping (MAPP) (Larrick et al., 1989), to evaluate the mRNA expression for protein tyrosine phosphatase-1B in the human acute promyelocytic leukaemic cell line, HL-60. Results indicate that the high sensitivity of the RT-PCR technique is worth taking advantage of when amplifying single copy sequences, however because of the exponential replication rate both high and low copy sequences became difficult to interpret after 30 cycles of PCR. The other problem experienced was the technical difficulty associated with producing smaller products as compared to larger ones; a high percentage agarose gel had to be used separate the small product from the primer dimer. With all these technical problems associated with RT-PCR it is my opinion that mRNA should be analysed using northern blots, except when high copy numbers are needed as in cloning experiments. A further objective of this study was to clone the PCR product and to generate recombinant protein for possible analytical use.

As with northern blotting temporal variations in expression of PTP-1B mRNA were observed using PT-PCR. However with RT-PCR, ATRA and 9-cis RA had little effect on the oscillation, although the number of experiments was limited.
The cyclic behaviour of PTP-1B mRNA reported here is similar to that observed in MEL cell with H-ras and N-ras mRNA (Hammond et al., 2000). No change in frequency and phasing pattern was noted in PTP-1B mRNA over 120 minutes. Due to the limited time interval studied, it may be that we are missing to see any change in frequency or phasing. Future studied should attempt to study PTP-1B mRNA over a longer period of time since regulation of the dynamics at the mRNA level may be crucial to the control of cell function and transformation.