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University of Windsor

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PART I:
HIGHLY SENSITIVE HYBRIDIZATION ASSAYS FOR PROSTATE-SPECIFIC ANTIGEN mRNA BASED ON TIME-RESOLVED FLUORESCENCE AND BIOLUMINESCENCE

PART II:
FLUOROMETRIC AND TIME-RESOLVED IMMUNOFUOROMETRIC ASSAYS FOR PROTEIN-TYROSINE PHOSPHATASE AND KINASE ACTIVITY

by
Barbara Galvan

A Dissertation
Submitted to the Faculty of Graduate Studies and Research through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada
1996
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ABSTRACT

PART I:
HIGHLY SENSITIVE HYBRIDIZATION ASSAYS FOR PROSTATE-SPECIFIC ANTIGEN mRNA BASED ON TIME-RESOLVED FLUORESCENCE AND BIOLUMINESCENCE

PART II:
FLUOROMETRIC AND TIME-RESOLVED IMMUNOFUOROMETRIC ASSAYS FOR PROTEIN-TYROSINE PHOSPHATASE AND KINASE ACTIVITY

by
BARBARA GALVAN

Bioanalytical assays, namely nucleic acid hybridization assays and immunoassays, provide powerful tools for scientific investigation. In order for such techniques to be useful in a routine clinical setting, the procedures should be highly sensitive, simple and efficient to perform, and adaptable to automation. The objective of my doctoral research was to develop clinically applicable bioanalytical assays for the determination of cancer-associated analytes.

More specifically, the first part of this DISSERTATION describes hybridization assays which were developed for the detection and quantification of prostate-specific antigen (PSA) mRNA, characteristic of prostate cancer cells. By using the polymerase chain reaction (PCR) combined with time-resolved fluorometric or bioluminescent detection systems, these assays can detect mRNA representative of one PSA-expressing cell amidst one million PSA-negative cells. These methods can facilitate the early detection of prostate cancer cells in the bloodstream, thereby aiding in the correct staging and treatment of prostate cancer patients.

The quantitative PCR method, which was developed by analyzing PSA mRNA in
parallel with the mRNA of the housekeeping gene, β-actin, allows for the monitoring of relative changes in PSA mRNA levels. This method may aid in the study of prostate cancer by permitting the correlation of PSA mRNA levels in patients, over time, with types of treatment, aggressiveness of tumours and patient outcomes.

The second part of the DISSERTATION describes fluorometric and time-resolved immunofluorometric assays developed for the determination of the oncogenically relevant protein-tyrosine kinase (PTK) and phosphatase (PTP) activities.

The first assay developed for the determination of PTP activity utilized Tb$^{3+}$ and conventional fluorometry. Since Tb$^{3+}$ forms fluorescent complexes with phosphotyrosine (P-Tyr) but not tyrosine (Tyr), PTP activity (ie. the dephosphorylation of P-Tyr) was determined by measuring the decrease in fluorescence.

For the time-resolved immunofluorometric PTP and PTK assays, synthetic substrates containing Tyr residues were immobilized onto microtitre wells. The P-Tyr groups (formed upon incubation with PTK, or remaining following the PTP reaction) were detected using an anti-phosphotyrosine antibody and an alkaline phosphatase-conjugated secondary antibody. The methods proposed here are safer, more practical, and offer superior sensitivity to established isotopic methods.
ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Dr. Theodore K. Christopoulos, for his sound intellectual leadership which he provided throughout the course of my research studies.

To Drs. K. Adeli and R. J. Thibert, I would like to express my sincerest gratitude for their ever-present willingness to guide me through the intricacies of the Clinical Chemistry program.

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To Miss Susan Bortolin, the best colleague a friend could ask for, thanks for all the laughs and for weathering the storms with me right from the start.

My sincerest gratitude goes out to Mrs. Patricia Aroca for being such an excellent role model on both a professional and personal level.

For the financial support provided throughout the course of my graduate studies, I wish to acknowledge the Natural Sciences and Engineering Research Council of Canada and the Faculty of Graduate Studies and Research at the University of Windsor.

Finally, to all the students, staff and faculty in the Department of Chemistry and Biochemistry at the University of Windsor, I wish you all the best.
DEDICATION

To my parents, Ignazio and Nives,  
my brother, Adrian, and sister, Nancy,  
for their love, encouragement and support

and

To Hartt,  
my dearest friend,  
for his strength, warmth, intelligence and, above all, his love
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## PART I:

HIGHLY SENSITIVE HYBRIDIZATION ASSAYS FOR PROSTATE-SPECIFIC ANTIGEN mRNA BASED ON TIME-RESOLVED FLUORESCENCE AND BIOLUMINESCENCE

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<td>ALP</td>
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<tr>
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<td>deoxythymidine 5' triphosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine 5' triphosphate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>FSA</td>
<td>fluorosalicylate</td>
</tr>
<tr>
<td>FSAP</td>
<td>fluorosalicylphosphate</td>
</tr>
</tbody>
</table>
h  hour
kb  kilobase
kDa  kilodalton
min  minute
M-MLV  Moloney murine leukemia virus
mRNA  messenger ribonucleic acid
PBS  phosphate-buffered saline
PCR  polymerase chain reaction
PGT  poly (Glu, Tyr)
pp60c-src  cellular homologue of the Rous sarcoma virus transforming protein, pp60v-src
PSA  prostate-specific antigen
PTK  protein-tyrosine kinase
PTP  protein-tyrosine phosphatase
RCML  reduced carboxyamidomethylated and maleylated lysozyme
RNA  ribonucleic acid
RT-PCR  reverse transcriptase-polymerase chain reaction
s  second
SA  streptavidin
SDS-PAGE  sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ss  single-stranded
Taq  Thermus aquaticus
TdT  terminal deoxynucleotidyl transferase
Tris  tris(hydroxymethyl)methylamine
tRNA  transfer ribonucleic acid
UNG  uracil-N-glycosylase
UV  ultraviolet
Part I

HIGHLY SENSITIVE HYBRIDIZATION ASSAYS FOR
PROSTATE-SPECIFIC ANTIGEN mRNA BASED ON TIME-RESOLVED
FLUORESCENCE AND BIOLUMINESCENCE
CHAPTER 1

GENERAL INTRODUCTION

1.1 PSA and Prostate Cancer

1.1.1 The Prostate Gland

The prostate is generally described as a chestnut-size gland belonging to the male reproductive system. It is situated at the neck of the bladder, surrounding the urethra as it exits the bladder (shown in Figure 1.1). The urethra is the tubular passage through which urine and seminal fluid is carried to the exterior of the body. The prostate consists of two lateral lobes and a median lobe, and is enclosed in a thin but firm, fibrous capsule (1).

During ejaculation, fluids from the prostate and seminal vesicles combine with sperm, carried by the ejaculatory ducts, to form the seminal fluid (semen). The prostate gland, whose function is dependent on a constant supply of testicular androgens from the circulation, contributes 15-30% of the seminal fluid. The prostatic epithelial cells are specialized in the production of prostatic fluid, a slightly acidic aqueous solution of inorganic compounds (e.g., sodium, calcium, zinc and potassium), organic compounds (e.g., citrate, cholesterol and inositol), and proteins, including proteolytic enzymes which account for the liquefaction of the coagulated semen. These substances are thought to nourish the ejaculated sperm, and provide a suitable environment for their mobility, thus creating conditions conducive for fertilization. In total, human prostatic secretion contains about 57 known major proteins, one of which is prostate-specific antigen (2, 3).

1.1.2 Prostate-Specific Antigen

Prostate-specific antigen (PSA), then named gamma-seminoprotein, was first discovered in seminal fluid in 1971 while searching for a specific semen marker which could be used for forensic purposes (4). In 1979, PSA was isolated from prostatic tissue (5) and shortly thereafter, it was detected in human serum (6). Since the finding that it is elevated in the serum of prostate cancer patients, PSA has been the pre-eminent clinical
FIGURE 1.1  
Anatomical Location of the Prostate

Legend

The prostate gland is generally described as having the shape and size of a chestnut or walnut, and is situated just below the bladder, where it fits like a collar around the urethra (the tube that carries urine from the bladder, through the penis and out of the body). The prostate consists of three lobes, two lateral lobes and a middle lobe, and is enclosed in a thin but firm fibrous capsule. [Adapted from O'Toole (1).]
FIGURE 1.1

- Seminal Vesicle
- Rectum
- Prostate
- Anus
- Bladder
- Urethra
- Penis
- Testicle
turnour marker in the management of these patients (7, 8).

Structurally, PSA is a 33 kDa single chain glycoprotein comprised of 237-amino acid residues and approximately 7% carbohydrate by weight (9). It is primarily produced and secreted by the columnar epithelial cells of the prostate (10). Briefly, PSA mRNA is translated into a 261 amino acid preproprotein in which a hydrophobic signal sequence of 17 amino acids and a 7-amino-acid propeptide precede the mature protein (11, 12). It enters the secretory pathway when the signal sequence is removed in the endoplasmic reticulum. The resulting inactive proprotein (zymogen) is exocytosed into the lumina of the prostate ducts. The release of the 7 amino-terminal residues results in the mature, enzymatically active form of PSA which is secreted into the seminal fluid at concentrations of 0.5-2 mg/mL (13).

The complete gene encoding PSA has been sequenced and localized to chromosome 19. It is about 6 kilobases (kb) in length, consists of 5 exons and 4 introns, and has extensive homology with the genes encoding the kallikrein family of serine proteases. The human glandular kallikrein-1 (hK2), also expressed in human prostatic tissue, is most closely related to PSA, having approximately 80% gene sequence conservation (11, 14). Like the kallikreins, PSA is a serine protease. It shows chymotrypsin-like activity favouring cleavage on the carboxy-terminal side of leucine or tyrosine (14).

The primary biological role of PSA is to increase sperm motility via the liquefaction of the seminal coagulum formed at ejaculation. This is achieved by PSA through the cleavage of the major coagulum-forming proteins, seminogelin I, II and fibronectin (15, 16). Although the majority of the PSA in seminal fluid is enzymatically active, approximately 20-30% is inactive, primarily due to clipping between residues 145-146 (lysine-lysine) (17). The nicked PSA remains connected by internal disulfide bonds, but does not complex to any protease inhibitor.

As an active serine protease, free PSA would be harmful in circulation. In serum
the predominant form of PSA (60-90%) is covalently complexed with α₁-antichymotrypsin (ACT), resulting in the inactivation of the enzyme. A small amount (10-40%) of the PSA in serum is free (F-PSA) (18). Although the F-PSA in serum has not been fully characterized, this uncomplexed and enzymatically inactive PSA is thought to represent either the internally clipped PSA, the 244-amino acid proform (zymogen), or even the hK2 (19). A minimal portion (< 0.1%) of the total PSA in serum has also been shown to complex with α₂-macroglobulin (20). However, unlike the F-PSA (33 kDa) and PSA-ACT (100 kDa), the PSA-α₂-macroglobulin (800 kDa) is not detected immunologically by current commercial PSA immunoassays (20).

PSA is present in normal, benign hyperplastic, malignant prostatic tissue, and metastatic prostate carcinoma. In the serum of normal healthy men, PSA concentrations are below 4 μg/L (20). As women have no prostate, it was assumed for many years that women do not produce PSA in any tissue and that PSA is not present in the female blood circulation. In the last two years, PSA immunoreactivity has been reported in breast tumours (21), in some other tumours, such as lung (22, 23), in healthy female breast (24), in amniotic fluid and in maternal serum (25).

Although these findings demonstrate that PSA is neither male- nor prostate-specific, and may indicate a new biological function for PSA in nonprostatic tissues (20), PSA is still a valuable marker for prostate cancer screening, diagnosis and especially for postsurgical monitoring of prostate cancer patients (10).

1.1.3 Prostate Cancer

Prostate cancer is the most frequently diagnosed non-skin malignancy and the second leading cause of cancer-related deaths (behind lung cancer) in North American men. More than 60% of the cases occur in men older than age 70 (26). The continuing shift of the demographic pattern of western countries towards an older-aged population is leading to an increase in the number of patients in which prostate cancer is diagnosed. Additionally,
improved diagnostic methods and greater public awareness have resulted in earlier diagnoses. In a number of countries, screening for prostate cancer in men over fifty as a part of annual medical examinations is becoming the norm. As a result, prostate cancer is expected to continue as a very important issue in the future (27).

Although the exact mechanisms that give rise to prostate cancer are unknown, the presence and action of testicular androgens, and advanced age are recognized as risk factors. In addition, genetic, dietary and environmental factors are also thought to be involved in the etiology of this disease (14, 27).

The symptoms of prostate cancer may be similar to those caused by benign prostatic hyperplasia, BPH (a noncancerous enlargement of the prostate which occurs in almost 80% of men by the age of 80 years) (27). Because of its position around the urethra, enlargement may interfere with the normal passage of urine from the bladder. There may be difficult or painful urination, dribbling of the urine, occasional blood in the urine, and painful ejaculation. Approximately 25% of men with BPH require prostate surgery (usually transurethral resection) to relieve the symptoms of BPH (27). In late stages of cancer, the patient may experience back pain, or pain in other bones due to advanced disease. Unfortunately in many cases, prostate cancer remains clinically silent until reaching an advanced state (27).

Prostate cancer varies widely in its clinical aggressiveness. Although prostate cancers are usually slow-growing, in some patients the disease metastasizes (spreads to another site in the body) rapidly, killing the patient within one year of initial clinical presentation (28). Unfortunately, no accurate and specific methods exist to distinguish the more aggressive form of prostate cancer from the latent form of the disease (27).

1.1.3.1 Diagnosis of Prostate Cancer

In the traditional method of diagnosis, the rectal wall adjacent to the prostate is probed, with a gloved finger, for abnormalities in the prostate's size, contour or
consistency. Since the finger reaches only half of the prostate, this digital rectal examination (DRE) can miss half of the tumours that are big enough to feel, and all of those that are too small (29, 30).

Occasionally, prostate cancer is detected as a by-product of treatment for BPH. Whenever the transurethral resection of the prostate is performed to relieve the patient of urinary troubles, the excised tissue is analyzed under a microscope for evidence of malignancy (29, 30).

The PSA blood test constitutes a third means of detecting prostate cancer. PSA concentrations that exceed 4 µg/L suggest cancer might be present; levels greater than 10 µg/L are especially suggestive. The PSA test is useful in detecting prostate cancers that are microscopic and cannot be detected by the DRE. Unfortunately, an elevated PSA reading is by no means proof that cancer is present. Factors other than cancer, such as development of BPH, inflammation of the prostate (prostatitis), and mechanical pressure on the gland, can cause the concentration to rise; but generally not above 10 µg/L. An estimated 3-21% of BPH patients have PSA values greater than 10 µg/L (10). The positive predictive value of PSA testing is low (30-35%). Thus many men who are tested will go on to have an unnecessary transrectal ultrasound (TRUS) and prostate biopsy (31). Conversely, in many men who have cancer, the PSA concentration is normal at the time of diagnosis (29). It is estimated that approximately 40% of men with organ-confined disease (cancer has not spread beyond the prostate), the ideal candidates for therapy with the best chance of being cured, have serum PSA concentrations less than 4 µg/L (10).

In order to improve the diagnostic sensitivity and specificity of serum PSA, it has been suggested to use PSA density (32). This quantity, which is estimated by dividing the serum PSA concentration by the prostatic volume, adjusts the serum PSA concentration for the size of the prostate. Other methods which have been suggested include the use of PSA velocity (the average annual change in serum PSA concentrations) (33), and the use of age-
specific reference ranges for serum PSA concentrations (34).

Detection of a possible cancer is only the first step in diagnosis. When a DRE or an elevated serum PSA concentration reveals that a cancer might be present, the result is usually followed up with a TRUS. This procedure can often pinpoint the location of a tumour and can aid in the next step of diagnosis: biopsy of the prostate and study of the suspicious tissue under a microscope. If the microscopic analysis confirms the presence of a malignancy, physicians try to determine its stage of advancement (29, 30).

1.1.3.2 Staging of Prostate Cancer

A common stage classification system divides tumours into four lettered stages (see Table 1.1). Stages A, B and C include cancers that have not metastasized, whereas stage D consists of tumours that have already metastasized. Prostate cancer has usually been found to spread first to the lymph nodes that are immediately downstream from the prostate gland, then it appears in the bones and other organs (29, 30).

The first three stages are distinguished from one another by the size of the tumours. Stage A malignancies are microscopic and can be divided into two subclasses. Stage A1 tumours are confined to one small area of the prostate and are composed of relatively well differentiated tissue. Despite some abnormalities in the cancerous cells (e.g., enlarged nuclei) they, like healthy gland cells, are of uniform size and closely packed. Stage A2 cancers are more diffuse and consist of more moderately to poorly differentiated tissue. Stage B malignancies are palpable (i.e., they are large enough to be felt as a nodule during a DRE) but rarely cause discomfort. Stage C tumours have spread through most or all of the gland, making the prostate very hard. Typically, these tumours have pushed past the borders of the prostate into the surrounding structures (29, 30).

To "stage" tumours, information obtained from the DRE, TRUS and the biopsy is combined with information provided by other noninvasive tests. For instance, a computed tomographic (CT) scan of the abdomen and the pelvis, or a biopsy of the lymph nodes
## TABLE 1.1

The Stages of Prostate Cancer*

<table>
<thead>
<tr>
<th>STAGE OF DISEASE</th>
<th>STANDARD THERAPY</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STAGE A</strong></td>
<td></td>
</tr>
<tr>
<td>MICROSCOPIC</td>
<td></td>
</tr>
<tr>
<td>CANCER WITHIN</td>
<td></td>
</tr>
<tr>
<td>PROSTATE GLAND</td>
<td></td>
</tr>
<tr>
<td><strong>A1</strong> Cancer is confined to one site and is well differentiated</td>
<td>Observation, radiation or radical prostatectomy</td>
</tr>
<tr>
<td><strong>A2</strong> Cancer occurs in many sites or is moderately to poorly differentiated</td>
<td>Radiation or radical prostatectomy</td>
</tr>
<tr>
<td><strong>STAGE B</strong></td>
<td></td>
</tr>
<tr>
<td>PALPABLE LUMP</td>
<td></td>
</tr>
<tr>
<td>WITHIN THE</td>
<td></td>
</tr>
<tr>
<td>PROSTATE GLAND</td>
<td></td>
</tr>
<tr>
<td><strong>B1</strong> Cancer forms a small, discrete nodule in one lobe of gland</td>
<td>Radiation or radical prostatectomy</td>
</tr>
<tr>
<td><strong>B2</strong> Cancer forms a large nodule or multiple nodules, or involves multiple lobes</td>
<td></td>
</tr>
<tr>
<td><strong>STAGE C</strong></td>
<td></td>
</tr>
<tr>
<td>LARGE MASS</td>
<td></td>
</tr>
<tr>
<td>INVOLVING ALL OR</td>
<td></td>
</tr>
<tr>
<td>MOST OF THE</td>
<td></td>
</tr>
<tr>
<td>PROSTATE GLAND</td>
<td></td>
</tr>
<tr>
<td><strong>C1</strong> Cancer occurs as a continuous mass that may have extended somewhat beyond the gland</td>
<td>Radiation, sometimes combined with hormonal therapy</td>
</tr>
<tr>
<td><strong>C2</strong> Larger cancer occurs as a continuous mass that has invaded structures surrounding the gland</td>
<td></td>
</tr>
<tr>
<td><strong>STAGE D</strong></td>
<td></td>
</tr>
<tr>
<td>METASTATIC</td>
<td></td>
</tr>
<tr>
<td>TUMOUR</td>
<td></td>
</tr>
<tr>
<td><strong>D1</strong> Cancer appears in the lymph nodes of the pelvis</td>
<td>Hormonal therapy and palliative therapy for pain and other discomforts</td>
</tr>
<tr>
<td><strong>D2</strong> Cancer involves tissues beyond the lymph nodes, usually including the bones</td>
<td></td>
</tr>
</tbody>
</table>

* Adapted from Garnick (29)
from the pelvis may be performed in order to search for metastatic cancer (29, 30). In
general, PSA concentrations are proportional to the clinical stage of the disease and can aid
in classification but cannot solely be relied upon for staging (9). Discovery of metastasis is
important because metastatic malignancies call for a therapeutic approach different from
those applied to less advanced tumours. Approximately 65% of prostate cancers detected
are clinically localized (organ-confined), but only one half of these (one third of the total)
are confirmed to be organ-confined at surgery (35, 36).

1.1.3.3 Treatment of Prostate Cancer

Most patients with stage A or B disease (except perhaps older individuals with stage
A1 disease) are treated with one of two potentially curable therapies. These treatments,
which are thought to be about equally effective, involve surgical removal of the prostate
gland (a radical prostatectomy) or irradiating the gland to destroy the cancerous cells within
it. Both radiation and surgery can lead to considerable impotence and to incontinence
(inability to control the passage of urine from the bladder), bowel injury and, rarely, to
death (29, 37). The surgical risks often decline however, if the patient is young, has
minimal tumour burden, and if the operation is performed by a team having extensive
experience with the nerve-sparing technique pioneered by Patrick C. Walsh and his
colleagues at the John Hopkins University School of Medicine in the early 1980’s. In this
procedure surgeons avoid cutting into two bundles of nerves and blood vessels that are
needed for a penile erection and that touch the surface of the prostate gland. Radiation is
often preferred for men who are too frail to withstand surgery. Because surgery cannot
fully eradicate tumours that have pushed their way past the borders of the prostate gland,
the treatment of choice for stage C disease is also radiation (29).

Since neither surgery nor radiation is likely to cure metastatic, stage D disease,
individuals with such advanced cancer are better served by systemic therapy aimed at
slowing the progression of metastatic deposits and at easing pain and other symptoms
(e.g., weight loss and muscle weakness) (29). The attempt to inhibit progression of advanced cancer by initiating hormonal therapies is based on the finding that male hormones (androgens) can markedly accelerate the growth of prostate cancer and that withdrawal of such hormones can retard its growth. Androgen levels in the body can be reduced by removing the testes (a bilateral orchiectomy), where 95% of testosterone, the main male hormone, is made. They can also be lowered by various drugs which interrupt biological pathways that lead to the synthesis of testosterone and to its action in prostate cells. An example of this is the drug finasteride, which blocks the enzyme 5-α-reductase from converting testosterone to its active form, dihydrotestosterone (29). Such a blockade prevents testosterone from fueling the growth of tumours derived from cancerous prostate cells and can also shrink tumours, at least for some time. Unfortunately, almost all metastatic tumours eventually become resistant to hormonal therapy and then progress rapidly. Usually the patients die within two to five years after metastases are discovered (29).

The measurement of serum PSA concentrations is perhaps most useful for monitoring cancer therapy. Highly sensitive PSA assays have shown that after prostate removal, and after allowing for the PSA to be eliminated from circulation, PSA concentrations fall below 0.02-0.06 μg/L. PSA concentrations above 0.1-0.3 μg/L, 3-6 weeks after radical prostatectomy, are indicative of residual disease. Successful radiation and anti-androgen therapies also cause significant reductions in PSA concentrations (20).

1.2 The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an in vitro technique used to generate a high concentration of a specific nucleic acid sequence relative to that of other sequences in the reaction mixture (38). It was conceived and demonstrated to be a practical method for manipulating nucleic acid fragments by Dr. Kary Mullis in 1983 while working for the Cetus Corporation in California (39, 40). The protocol for PCR was not published until
1985 (41). The invention grew from a theoretical scheme to perform limited dideoxynucleotide sequencing of unique human genes using synthetic oligonucleotides and DNA polymerase for the purpose of diagnosing common human disease mutations. This powerful method has revolutionized the practice of DNA technology as it allows for the detection and characterization of nucleic acids of interest without the interference from the presence of other DNA fragments. Today, just over a decade after its introduction, PCR is used by investigators in such diverse fields as diagnostics, oncology, evolution, and environmental and forensic sciences.

1.2.1 The Principles of PCR

PCR involves an enzymatic amplification of DNA by a repetitive cyclical process which results in an exponential increase in the total number of DNA copies. Each cycle of PCR consists of three reactions: target denaturation; primer annealing; and the extension of annealed primers by DNA polymerase. All three reactions are carried out in the same vessel and with the same temperature-stable reagents. Changes in incubation time and temperature mediate the initiation of the separate reactions. The repetitive cycling is therefore self-contained and fully automated.

The PCR mixture consists of the target DNA (i.e., the specific DNA sequence which one is interested in amplifying), two single-stranded (ss) oligonucleotides (primers) designed to be complementary to known sequences of the target DNA, deoxyribonucleoside triphosphates, DNA polymerase and a magnesium-containing buffer. In the first step of a PCR cycle, the reaction mixture is heated to 90-95 0C. At such elevated temperatures, double-stranded (ds) DNA is rendered ss via the disruption of the hydrogen bonds holding the strands together. Following this target denaturation step, the temperature is reduced to between 40-72 0C thereby allowing the two short DNA primers to anneal to complementary sequences on opposite strands of the target DNA. These primers are chosen to bind to regions which flank the target DNA, and are oriented so that
their 3'-OH ends point towards one another along the intervening sequence. The third step of the cycle involves an increase in temperature to 72 °C which is optimal for the DNA polymerase activity. The synthesis of new DNA complementary to the target DNA occurs through the extension of each annealed primer (in the 5' to 3' direction) by the polymerase. Since the DNA products synthesized in one cycle can serve as templates in the next, the number of target DNA copies approximately doubles at every cycle. The short products, which accumulate exponentially during PCR, are of uniform size. Their length is equal to the sum of the lengths of the two primers plus the distance in the target DNA between the primers. The theoretical abundance of the short products is represented by $2^n$, where $n$ is the number of PCR cycles performed. This translates to approximately a million-fold amplification in 20 cycles of PCR. The strands that are synthesized as copies of the original template (long products) are bounded at the 5' end by a primer while the 3' end is determined by the position at which the DNA polymerase terminates its synthesis. As these strands with indeterminate 3' termini can only be generated by the copying of the original template, their accumulation is linear ($2n$). The overall consequence is that the long products are relatively rare compared to the short products. The principle of PCR amplification is illustrated in Figure 1.2.

The size and homogeneity of the PCR product can be assessed by agarose gel electrophoresis and ethidium bromide staining. Further sensitivity can be achieved by subjecting the gel to Southern blotting and performing hybridization with a specific DNA probe.

1.2.2 The Reagents of PCR

Although the results of PCR will be good in most cases, its overall success is largely dependent on the intelligent, or more often empirical, selection of the few critical reagents.
FIGURE 1.2
The Polymerase Chain Reaction Amplification Scheme

Legend

The basic PCR cycle consists of three steps performed in the same closed container but at different temperatures. The elevated temperature in the first step melts the double-stranded target DNA into single strands. As the temperature is lowered for the second step, the two oppositely directed oligonucleotide primers anneal to complementary sequences on the target DNA, which acts as a template. During the third step, the Taq DNA polymerase enzymatically extends the primers covalently in the presence of deoxyribonucleoside triphosphates, the building blocks of new DNA synthesis. This extension step is carried out at 72 °C, the optimum temperature for Taq DNA polymerase activity. The process of denaturation, annealing and extension is repeated many times, resulting in the exponential accumulation of strands that contain the sequence between the priming sites. In the first cycle the products are defined at the 5' phosphate terminus (depicted as open circles) by the primer but are not defined at the 3' hydroxyl terminus (represented by solid circles). These products are termed long products. After the third cycle the majority of products will be double-stranded, blunt-ended DNA strands ("short products") that are defined at both termini. [Adapted from Gibbs (39) and Eisenstein (42).]
FIGURE 1.2

Double-Stranded Target DNA

Cycle 1

Denature and Anneal primers

Primer Extension

Cycle 2

Denature and Anneal primers

Primer Extension

Cycle 3

Denature and Anneal primers

Primer Extension

Cycle n

2^n Copies

Short Product

Long Product
1.2.2.1 The Target

A broad range of nucleic acid sources are suitable targets (i.e., templates) for PCR amplification. PCR can amplify ds or ss DNA and, with the reverse transcription of mRNA into a cDNA copy, RNA can also serve as a target. The target sequence in genomic DNA or cDNA, optimally with a length of 100-1000 base pairs (bp) but possibly as long as 10 kilobases (kb), should be known in sufficient detail to design primers that will anneal to its ends (43).

1.2.2.2 The Primers

Unfortunately, the approach to the selection of efficient and specific primers remains somewhat empirical. There is no set of rules that will ensure the synthesis of an effective pair of primers. Yet, it is the primers more than anything else that determines the success or failure of an amplification reaction.

The recommended primer is between 20-30 bases in length, and has a GC content of 40-60%. Intrastrand complementary regions, such as runs of 3 or more G's or C's, are inclined to form secondary structures (e.g., hairpin loops) and thus should be avoided especially at the 3' end of the primer. Interstrand complementary regions at the 3' ends of the primers should also be avoided in order to prevent the formation of "primer dimers". This amplification artifact is a ds fragment whose length is equal to the sum of the two primers, and appears to occur when one primer is extended by the polymerase over the other primer. Whenever possible, the two primers selected should have very similar melting temperatures. This aids in ensuring that a given thermal profile is optimally efficient and specific for both primers. In general, concentrations ranging from 0.05 to 0.5 µmol/L of each oligonucleotide primer are used in each PCR mixture. The concentration of primer should be high enough as to not limit the reaction (43).

Sequences not complementary to the target may be added to the 5' end of the primers. These exogenous sequences become incorporated into the ds PCR product and
provide a means of introducing restriction sites, regulatory elements (e.g., promoters), or
detection labels at the ends of the amplified target sequence (37, 39).

1.2.2.3 The DNA Polymerase

DNA polymerases catalyze the addition of a nucleotide from a nucleoside
triphosphate to the free 3'-OH group of a base paired polynucleotide so that DNA chains
are extended only in the 5' to 3' direction. The initial studies that relied on PCR to amplify
specific targets of DNA utilized the Klenow fragment of Escherichia coli DNA polymerase
I. Although widely used, this polymerase is not a thermostable enzyme, and its
inactivation at the high temperatures necessary for strand separation made it necessary to
add fresh enzyme after the denaturation step of each PCR cycle. This requirement was
eliminated by the purification of a thermostable DNA polymerase from the thermophilic
bacterium Thermus aquaticus. Thus by using Taq DNA polymerase and a simple
automated thermal cycling device, the PCR could be accomplished in a single tube
containing all the necessary reagents. The availability of thermostable polymerases has
simplified the procedure for PCR and, as will be discussed later, it has increased the
specificity and efficiency of the amplification reaction. Moreover, longer segments of DNA
could be amplified, probably due to a reduction in the secondary structure of the target
strands at the elevated temperatures (72 °C) used for primer extension with Taq DNA
polymerase. Taq possesses a 5' to 3' exonuclease activity but does not contain a 3' to 5'
exonuclease (proofreading) activity. The concentration of enzyme typically used in PCR is
about 2.5 units per 100 μL reaction (44).

1.2.2.4 The Deoxyribonucleoside Triphosphates

Each of the deoxyribonucleoside triphosphates (dNTPs), more specifically dATP,
dCTP, dGTP and dTTP, is usually present in the PCR reaction mixture at a concentration
of 50 to 200 μmol/L. Higher concentrations are usually avoided because of the tendency to
promote misincorporations by the polymerase (43). Magnesium present in the PCR
mixture is bound by the dNTPs and is required for optimal polymerase activity. As with the primers, modified dNTPs can also be incorporated into the PCR products and used as detection labels.

1.2.3 Specificity of PCR

PCR commonly shows great specificity. Amplification of a single target sequence from a complex mixture of DNA, initially present in very low amounts, often results in a single product of exactly the expected size and sequence despite the numerous opportunities for primers to anneal to nontarget sequences (38). This specificity is largely due to two important features of the PCR protocol. The first of which is the requirement for annealing of the two primers, correctly oriented, within a span of less than about $10^4$ nucleotides. While the probability of mispriming (i.e., the binding of the primers at regions other than the two specific sites) is high; the likelihood of the nonspecific events occurring in the correct orientation, and close together is very low. In addition, after a few cycles, the amount of target has increased so much that less favourable competing events progressively become less frequent. The second feature involves the use of the thermostable Taq DNA polymerase, which allows the annealing and extension temperatures to be raised. Limited increases in these temperatures will reduce the likelihood of the nonspecific priming but will not reduce the efficiency of the correct priming events in which the primers and target sequences are perfectly matched. In addition, the concentrations of enzyme and primers as well as the annealing time, extension time, and the number of cycles can also affect the specificity of the PCR (39).

One practical approach that has been used to minimize nontarget amplification involves the manual addition of an essential reagent (e.g., the polymerase or primers) to the reaction mixture only after it has reached elevated temperatures (>70 °C) (45). This "hot-start" approach has been reported to not only improve specificity but also to minimized the formation of the primer-dimer (45). Nested PCR may also be employed to improve the
specificity of reactions that do not otherwise yield homogeneous products. This protocol uses a two-step reaction scheme beginning with amplification by the outer primer set, followed by a second round of PCR using a set of primers which bind inside the first PCR product. The overall lower complexity of the target for the second PCR ensures a more homogeneous final product (39).

1.2.4 The Efficiency of PCR

The theoretical amplification of PCR ($2^n$) would be achieved only if the reaction could be performed with 100% of its theoretical maximum efficiency. In practice however, PCR is operating with less than its maximum potential. The difference between the theoretical and actual amplification efficiency is largely due to an initial lag phase and the eventual saturation of the reaction (39).

The initial lag phase is attributable to a number of factors including the unavailability of DNA target due to strand breaks, lack of its dissociation from other macromolecules, structural constraints, or a tendency of the long parental strands to reanneal. A low efficiency during the first few PCR cycles will greatly influence the overall yield of the reaction (39).

At saturation the desired amplification fragment gradually stops accumulating exponentially and enters a linear or stationary phase. In most cases, this amplification "plateau" is an unavoidable and inherent limitation of the PCR reaction. The point at which a PCR reaches its plateau depends primarily on the amount of target DNA originally present in the sample, and the number of amplification cycles. The causes of the plateau effect include saturation of the DNA polymerase, competition between primer/template and template/template annealing, competition for the primers by nonspecific amplification products, and incomplete denaturation of the template at high product concentrations. Destruction or inactivation of reactants, (e.g., dNTPs and polymerase) due to repeated exposure to high temperatures, and inhibition of the reaction by end products, such as
pyrophosphate and ds DNA, may also contribute to the plateau effect. The primers and dNTPs are added in excess thus should not be exhausted and should not limit the final reactions (43).

1.2.5 PCR Contamination

Given the ability of PCR to generate large numbers of DNA copies from a target sequence, contamination of the PCR reaction mixture with products of an earlier PCR reaction ("product carryover"), exogenous DNA, or cellular material can create problems both in research and diagnostic applications (46).

Some of the common, however, very valuable precautions followed to minimize the risk of contamination include the prealiquoting of reagents, the physical separation of the reaction preparation from the area of reaction product analysis, and the use of dedicated pipettes (46). Irradiation of pipettes, pipette tips, glassware and some reagents (e.g., water) with UV light to destroy contaminating DNA may also be performed prior to setting up the PCR reaction. The inclusion of PCR negative controls (in which no target DNA is added to the reaction mixture) allows for the detection of contamination.

One of the approaches used to minimize PCR contamination involves the selective destruction of PCR product carryover. To distinguish PCR products from sample target DNA, deoxyuridine triphosphate (dUTP) is substituted for dTTP in the PCR mixture, and is thus incorporated into the amplification products. If contamination is suspected the enzyme uracil N-glycosylase (UNG) is added to the reaction mixture prior to thermal cycling. UNG catalyzes the excision of uracil from any potential single- or double-stranded PCR carryover DNA present in the reaction mixture prior to the first PCR cycle. The resulting abasic polynucleotides are susceptible to hydrolysis in slightly alkaline solutions (i.e., the PCR buffer) and elevated temperatures, and thus cannot function as DNA templates. Since UNG itself is inactivated at the elevated temperatures used in PCR, the amplification products generated during the thermal cycling are not destroyed and can
accumulate as usual. This elegant strategy for eliminating PCR product carryover still allows amplified DNA to serve as a target for probe hybridization (46).

1.2.6 Variations of PCR

Several variations on the basic PCR protocol have been developed. Two of these variations, namely reverse transcriptase-PCR and quantitative PCR, were applied to the work presented here and are briefly described below.

1.2.6.1 Reverse Transcriptase-PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR), also known as RNA PCR, involves amplification from mRNA rather than DNA targets. This approach requires the conversion of the mRNA target to cDNA with reverse transcriptase and an oligonucleotide primer, such as the poly dT primer. RT-PCR is a very useful variation of PCR because it allows for the analysis of gene expression (39).

1.2.6.2 Quantitative PCR

Quantitative PCR or quantitative RT-PCR methods relate the magnitude of an analytical signal (obtained from the amplification product) to the initial number of target DNA or mRNA molecules present in the sample prior to amplification. Unlike qualitative PCR methods, which only detect the presence or absence of a particular target, quantitative PCR can provide sensitive and quantitative determinations of the amount of target relative to a standard, or can detect changes in target expression over time (39).

1.3 Time-Resolved Fluorescence

Bioanalytical assays such as nucleic acid hybridization assays and immunoassays frequently require ultrasensitive detection methods to successfully detect minute quantities of the analyte of interest present in a biological sample. The necessary sensitivity has been achieved in the past through use of radioactive labels such as $^{32}$P and $^{125}$I as tracers. However the hazards and limited shelf-life associated with the use of radioactive labels has made their replacement with ultrasensitive nonisotopic detection systems a priority (47).
During the last decade, several highly sensitive, nonisotopic detection systems using fluorescent or luminescent labels, or enzymes which liberate fluorescent or luminescent products have been reported (48). The state-of-the-art nonisotopic detection systems have better sensitivity than the ones using radioactive labels (49). Among the most sensitive nonisotopic methodologies is time-resolved fluorescence.

Time-resolved fluorescence originated from the realization that the sensitivity of conventional fluorescence measurement is restricted, to approximately $10^{-9}$ M, by high background signals. These background signals are attributable in part to luminescence from cuvettes, instrument filters and lenses, and sample matrix. Serum autofluorescence due mainly to albumin-bound bilirubin overlaps with the emission spectrum of conventional fluorophores, such as fluorescein. The fluorescence lifetime, $\tau$ (defined as the time required for the fluorescence emission to decay to $1/e$ of its initial intensity following excitation) of such background fluorescence is short (nanoseconds to a few microseconds). Since the conventional fluorophores also have short fluorescence lifetimes (5-100 ns), and the measurement of their fluorescence emission must be performed very shortly after excitation, the background fluorescence is also incorporated into this measurement. Scattering of the excitation light is also a major contributor of the high backgrounds. Another problem associated with conventional fluorophores is the concentration quenching, also known as the inner filter effect. Significant overlapping of their excitation and emission spectra (small Stokes shifts) results in the absorption of fluorescence by fluorophore molecules adjacent to the emitting fluorophore (50).

The use of time-resolved fluorometry significantly diminishes these high background signals, thereby improving the signal-to-background ratio and assay sensitivity. In order to apply time-resolved fluorometry, however, labels possessing long fluorescence lifetimes are necessary. The lanthanide chelates fulfill this requirement, having fluorescence lifetimes of 10-1000 $\mu$s. These complexes also exhibit large Stokes
shifts (approximately 290 nm), narrow emission spectra (10 nm bandwidth) and no overlap between excitation and emission spectra. In practice, when lanthanide chelates are used in combination with time-resolved fluorometry, the observed background fluorescence is only associated with the nonspecific binding of labelled reagents to the solid phase (e.g., microtitre Wells).

1.3.1 The Principle of Time-Resolved Fluorometry

The principle of time-resolved fluorometric measurement is simple and straightforward. In each cycle of measurement, the sample is first excited with a short pulse of light from a laser or a flash lamp. The excited molecules in the sample emit either short- or long-lived fluorescence, both of which decay as an exponential function of time. The short-lived fluorescence dissipates to zero in less than 100 μs. If no measurements are taken during the first 100-200 μs after excitation, all short-lived fluorescence background signals and scattered excitation radiation are completely eliminated. The long-lived fluorescence, arising from the lanthanide chelate may then be measured with very high sensitivity. The principle of time-resolved fluorosence measurement, as it applies to the CyberFluor 615 Immunoanalyzer, is illustrated in Figure 1.3.

The CyberFluor 615 Immunoanalyzer is the time-resolved fluorometer used throughout this work. As its excitation source, this instrument uses a nitrogen laser with an excitation wavelength of 337.1 nm and a pulse duration of 3 to 4 ns. Each 50 ms cycle is divided into 200 μs of delay time and 400 μs of counting time. The measurement time per sample (well) is 1 s, which corresponds to 20 flashes per well (51).

1.3.2 Energy Transfer and Lanthanide Fluorescence Emission

Europium (Eu³⁺), terbium (Tb³⁺), samarium (Sm³⁺) and dysprosium (Dy³⁺) are the only four of the 15 lanthanide elements which fluoresce when excited by ultraviolet (UV) radiation (49). These ions emit characteristic radiation in the visible region (metal-ion fluorescence). Inorganic salts of these lanthanides emit only weak fluorescence, however,
FIGURE 1.3
The Principle of Time-Resolved Fluorometry

Legend

In each 50 ms cycle of measurement, the CyberFluor 615 Immunoanalyzer first excites the sample with a short pulse (3-4 s) of light from a nitrogen laser ($\lambda=337.1$ nm). The excited molecules in the sample emit either short- or long-lived fluorescence, both types of which decay as an exponential function of time. There is a measurement delay time of 200 $\mu$s after excitation, during which all short-lived fluorescence background signals and scattered excitation radiation are completely eliminated. The long-lived fluorescence, arising from the lanthanide chelate is then be measured (during a 400 $\mu$s counting time) at a wavelength of 615 nm. Following a 49.4 ms recovery time, there is a subsequent pulse of excitation light and the measurement cycle begins again. The measurement time per sample (well) is 1 s, which corresponds to 20 flashes per well. [Adapted from Diamandis (51).]
upon the formation of a chelate between the metal ion and an appropriate organic ligand this fluorescence is dramatically enhanced (50). The excitation light is absorbed at a wavelength characteristic of the ligand and emitted as a line spectrum characteristic of the metal ion as a result of an intramolecular energy transfer from the ligand to the central metal ion (see Figure 1.4). Upon excitation, the organic ligand absorbs energy and is raised from its singlet ground state, \( S_0 \), to any one of the vibrational multiplets of the first singlet excited state, \( S_1 \). The molecule rapidly loses its excess vibrational energy through nonradiative deactivation processes and falls to the lowest level of \( S_1 \). At this stage the ligand may relax by a radiative \( S_1 \) to \( S_0 \) transition (ligand fluorescence) or may pass to one of the triplet states, \( T_1 \), through intersystem crossing. From the triplet state, the molecule can return to the ground state by means of a spin-forbidden \( T_1 \) to \( S_0 \) radiative transition (molecular phosphorescence) or a nonradiative process. Alternatively, if the ligand is chelated to an ion, such as the lanthanide, intramolecular energy transfer can occur from the triplet excited state of the ligand to an appropriate 4f energy level (resonance level) of the central metal ion. This ion can, in turn, move up to its own excited singlet state and subsequently emit characteristic radiation (ion fluorescence) (50). Not all ligands that chelate lanthanides can trigger metal ion fluorescence. Additionally, the intensity of metal ion fluorescence is quite variable, depending on the structure of the triggering ligand (49).

In order to achieve efficient ion fluorescence, the following requirements must be fulfilled: firstly, competition from deactivating ligand transitions \( S_1 \) to \( S_0 \) or \( T_1 \) to \( S_0 \) (either radiative or nonradiative) should be minimal. Secondly, the energy of the ion resonance level should be just below that of the triplet-state level of the ligand, so that the probability of the triplet to resonance level transition (\( T_1 \) to \( d \)) is high. Finally, the probability of the nonradiative deactivation of the resonance level should be small compared with that of the radiative transition (50, 52). The above requirements can be fulfilled to a variable extent by certain organic ligands and the four previously mentioned lanthanide ions
FIGURE 1.4
The Energy Transfer Mechanism

Legend

Upon excitation, the organic ligand absorbs energy and is raised from its singlet ground state, S$_0$, to any one of the vibrational multiplets of the first singlet excited state, S$_1$. The molecule rapidly loses its excess vibrational energy through nonradiative deactivation processes and falls to the lowest level of S$_1$. At this stage the ligand may relax by a radiative S$_1$ to S$_0$ transition (ligand fluorescence) or may pass to one of the triplet states, T$_1$, through intersystem crossing. From the triplet state, the molecule can return to the ground state by means of a spin-forbidden T$_1$ to S$_0$ radiative transition (molecular phosphorescence) or a nonradiative process. Alternatively, if the ligand is chelated to an ion, such as the lanthanide, intramolecular energy transfer can occur from the triplet excited state of the ligand to an appropriate 4f energy level (resonance level) of the central metal ion, which in turn can move up to its own excited singlet state and subsequently emit characteristic radiation (ion fluorescence). [Adapted from (50).]
(Eu$^{3+}$, Tb$^{3+}$, Sm$^{3+}$ and Dy$^{3+}$). The emission spectra of these ions have more than one emission line, but the major bands appear at 613 (Eu$^{3+}$), 546 (Tb$^{3+}$), 597 (Sm$^{3+}$) and 483 (Dy$^{3+}$).

1.3.3 The Fluorosaliclylate-Tb$^{3+}$-EDTA Ternary Complex

Due to its superior detectability, europium has been the lanthanide label of choice for assays using time-resolved fluorometric detection. Tb$^{3+}$ and Sm$^{3+}$ have primarily been used as secondary labels in multi-analyte assays. The advantage of Tb$^{3+}$, however, is that it can form fluorescent complexes with a wider variety of organic ligands, many of which are freely soluble in water (53). It is known that, at a pH of 12-13, organic ligands, such as salicylate, coordinate around terbium in the presence of EDTA forming highly fluorescent ternary complexes (47). These complexes fluoresce at wavelengths characteristic of Tb$^{3+}$, following ligand excitation and an internal transfer of energy, as described in the previous section. The presence of EDTA in these ternary complexes serves to chelate Tb$^{3+}$ with high affinity keeping it soluble in water and preventing its precipitation as hydroxide salts at such a strongly alkaline pH.

Slight modifications of the coordinated ligand (i.e., salicylate), especially on the hydroxyl group, will usually dramatically diminish or totally abolish its ability to form fluorescent complexes. Thus, salicylate or similar compounds can be modified to become enzyme substrates which can be converted, by an enzyme, from a compound that does not form a highly fluorescent chelate with Tb$^{3+}$, to a product which does form such a chelate. The use of enzymatic amplification, in bioanalytical assays, significantly enhances their sensitivity due to the intrinsic signal amplification provided by the activity of the enzyme in producing a large number of detectable product molecules per molecule of enzyme (47, 54). The necessary alteration of the chemical properties of the substrate or conversion to product, which results in lanthanide fluorescence, may derive from a combination of factors: alteration of the position of its absorption spectrum, such that the substrate does
not absorb the excitation light efficiently whereas the product does; alteration of the position of its excited state energy levels such that the substrate does not efficiently transfer the excited energy to the chelated lanthanide, whereas the product does; and alteration of its chelating properties such that the substrate does not efficiently chelate the lanthanide whereas the product does. The alteration of at least one of these properties by the enzyme of choice allows detection of the product molecule only, even in the presence of a very large excess of substrate molecules (47).

The phosphate ester of fluorosalicylic acid (FSAP) was found to be the optimal substrate for time-resolved fluorometric assays using alkaline phosphatase (ALP), as the enzyme label, and Tb$^{3+}$ as the fluorescing lanthanide. Dephosphorylation of this substrate by ALP produces fluorosalicylate (FSA) which, unlike FSAP, is able to form a highly fluorescent ternary complex with Tb$^{3+}$-EDTA at alkaline pH. The FSA- Tb$^{3+}$-EDTA complex is coordinated in eight positions to the terbium ion and has a fluorescence lifetime of 1.6 ms (47). It has an excitation maximum of 336 nm (characteristic of the FSA ligand) and emission maxima at 491, 548, 587 and 624 nm (characteristic of the Tb$^{3+}$) (53). An intact hydroxyl group on the FSA molecule is essential for this highly fluorescent complex to form. The fluoro-substituent increases the solubility of FSA, and also maximizes the shift between the absorption maximum of the substrate (280 nm) and the excitation maximum of the FSA- Tb$^{3+}$-EDTA chelate (336 nm). The dephosphorylation of FSAP by ALP and the complexation of FSA with Tb$^{3+}$-EDTA to form the fluorescent ternary complex, FSA- Tb$^{3+}$-EDTA, is summarized in Figure 1.5.

1.4 The Bioluminescence of Aequorin

Bioluminescence is a special type of chemiluminescence occurring in living organisms. Bioluminescent molecules generate light through a biochemical reaction and, in contrast to fluorescent and phosphorescent molecules, do not require optical excitation. The physical event of the light emission in bioluminescence and chemiluminescence is
FIGURE 1.5

Formation of the Fluorescent Ternary FSA-Tb$^{3+}$-EDTA Complex

Legend

The dephosphorylation of fluorosalicylphosphate (FSAP) by alkaline phosphatase (ALP) produces fluorosalicylate (FSA). Unlike FSAP, FSA is able to form a highly fluorescent ternary complex with Tb$^{3+}$-EDTA at alkaline pH. The FSA-Tb$^{3+}$-EDTA complex is coordinated in eight positions to the terbium ion and has a fluorescence lifetime of 1.6 ms. It has an excitation maximum of 336 nm (characteristic of the FSA ligand) and emission maxima at 491, 548, 587 and 624 nm (characteristic of the Tb$^{3+}$) [Courtesy of T.K. Christopoulos.]
FIGURE 1.5

\[
\begin{align*}
\text{FSAP} & \quad \text{ALP} \quad \text{pH 9-10} \quad \text{FSA} \\
\text{H}^+ & \quad \text{HPO}_4^{2-} \\
\end{align*}
\]

Fluorescent Complex

\[
\begin{align*}
\text{Tb}^{3+} \quad \text{EDTA} \quad \text{pH 13}
\end{align*}
\]
similar to fluorescence in that it occurs from an excited singlet state and the light is emitted when the electron returns to ground state (55). The advantages of bioluminescence and chemiluminescence assays include sensitivity (attomole and subattomole detection limits), speed (signal is generated in a few seconds and in some cases is stable for several hours), use of nonhazardous reagents, and involve simple procedures (56). It is now well accepted that labels based on the use of bioluminescence and chemiluminescence technology can be used to develop ultrasensitive assays as alternatives to radioisotope and enzyme-mediated detection systems.

One of the most promising bioluminescent labels is the 22,000 Da photoprotein aequorin. It was first isolated from the jellyfish, <i>Aequorea victoria</i>, in 1962 (57). Aequorin is a complex composed of apoaequorin (a single polypeptide chain of 189 amino acids), the cofactor coelenterazine (243 Da), and molecular oxygen. Within apoaequorin there is a hydrophobic region postulated to be the coelenterazine-binding site, and three Ca<sup>2+</sup>-binding sites (58). Upon binding of Ca<sup>2+</sup> to these sites, a conformational change in the protein takes place, resulting in the oxidation of coelenterazine to coelenteramide and a concomitant flash of blue light (\(\lambda_{\text{max}}=469\) nm) which lasts for approximately 3 s (59). The excited state coelenteramide is the emitter in the reaction (60). Aequorin may be regenerated from apoaequorin by incubation with coelenterazine, dissolved oxygen, EDTA, and \(\beta\)-mercaptoethanol (61). The principle of the aequorin bioluminescence reaction is presented in Figure 1.6.

Due to its sensitivity to Ca<sup>2+</sup> and harmlessness in biological systems, for many years, aequorin has been used as an intracellular Ca<sup>2+</sup> indicator (62). The potential utility of using aequorin as a nonradioisotopic reporter molecule in various assay formats has only been exploited recently particularly due to the availability of recombinant protein. Recombinant aequorin, produced by purifying apoaequorin from recombinant <i>E. coli</i> bacteria (59, 60) followed by reconstitution of the complex <i>in vitro</i> with pure
FIGURE 1.6

The Principle of Aequorin Bioluminescence

Legend

Aequorin consists of a 189-amino-acid polypeptide complex that includes a reactive group, coelenterazine and oxygen. Upon addition of calcium ions, the photoprotein undergoes a conformational change. Filling the calcium-binding sites (I, II and II) results in the oxidation of bound coelenterazine using the protein-bound oxygen. The energy from this oxidation reaction is released as a flash (approximately 2-3 s) of blue light with a maximum emission wavelength of 469 nm. The R1, R2 and R3 of the coelenterazine represent p-hydroxyphenyl, benzyl and p-hydroxybenzyl groups, respectively. [Adapted from Shimomura (63).]
FIGURE 1.6

\[ \text{H}_2\text{N} \quad \text{Ca}^{2+} \]

\[ \text{HOO} \quad \text{Ca}^{2+} \quad \text{Ca}^{2+} \]

\[ \text{HOOC} \quad \text{Ca}^{2+} \quad \text{Ca}^{2+} \]

\[ \text{Aequorin} \quad \text{Aequorin} \quad \text{Aequorin} \]

\[ \text{CO}_2 \quad \text{LIGHT (\text{\textit{\(\lambda_{max}=469\text{ nm}\)}}}) \]
coelenterazine, circumvents the laborious and expensive extraction procedures required to purify native aequorin from jellyfish. The availability of recombinant aequorin has also facilitated its conjugation to other proteins such as antibodies or streptavidin, or small molecules such as biotin. The subsequent use of these conjugates in bioluminescence-based assays has allowed for the design of various assay configurations.

Streptavidin and biotin conjugates of aequorin, which only became commercially available in 1995, have already proven to be extremely versatile and sensitive bioluminescent labels for use in a variety of analytical methods (e.g., Western blot, Southern blot and immunoassays). Because of the relatively high quantum yield of the reaction (approximately 15%), aequorin can be detected at the attomole level using commercially available luminometers capable of simultaneously injecting a solution of calcium salt and measuring the light produced (59). The aequorin luminescence reaction was shown to be linear over a broad range spanning from attomoles to picomoles of recombinant aequorin (59). As mentioned previously, excitation light sources are not required for this reaction to produce a signal, thus background associated with fluorescence spectroscopy and sample autofluorescence is eliminated. The extremely low background, due to nonspecific binding of reagents, and the broad linear range of aequorin conjugates make them more sensitive than chemiluminescent, fluorescent and colourimetric enzyme-mediated techniques (59). Furthermore, since the aequorin reaction can be detected within two seconds of the addition of Ca$^{2+}$, longer substrate incubation steps are avoided and the turnaround time of assays is significantly improved.

1.5 Research Proposal

The presence or absence of extracapsular prostate cancer is often the critical factor in therapeutic decisions regarding prostate cancer. Once the cancer has spread beyond the prostate, the benefits of performing a radical prostatectomy are limited. This spread presupposes that prostate cancer cells penetrate the capsule of the prostate and are shed into
the bloodstream prior to attaching to distant sites. The detection of these prostatic cells in the peripheral blood of patients with prostate cancer is, therefore, considered as an early indication of metastasis, and could aid in determining the appropriateness of removing the prostate.

Unfortunately, the sensitivities of current staging modalities are inadequate and cannot detect these stray cancerous cells. In fact, up to half of the patients who are believed to have organ-confined disease and thus undergo a radical prostatectomy are found at the time of surgery to actually have extraprostatic disease. The obvious need for a more sensitive staging technique capable of more accurately identifying patients with extraprostatic disease has spawned the development of RT-PCR methods using primers specific to PSA mRNA which is expressed by the prostate cells.

In these methods, PCR products are analyzed by agarose gel electrophoresis and ethidium bromide staining or by Southern blot. The Southern blot technique involves the transfer of electrophoretically separated PCR products onto a nylon membrane and detection of the PCR product of interest via hybridization with a specific probe. Although the Southern blot procedure offers a better sensitivity than achieved with the ethidium bromide staining, it is time-consuming, tedious and not suitable for automation.

The purpose of my research, presented in the first part of the DISSERTATION, was to develop highly sensitive, nonisotopic assays for the detection and the quantification of PSA mRNA. The analyses of the PCR products are conducted entirely in microtitre wells using a detection system based on either time-resolved fluorescence or bioluminescence. The combination of these state-of-the-art techniques, create assays that are highly sensitive, time-efficient, simple to perform, and that can be easily adapted to the routine clinical laboratory.

The assay described in Chapter 2 involves the detection of PSA PCR products using time-resolved fluorometry. In this assay, a biotin-tailed probe specific for the PSA
PCR product is immobilized onto streptavidin-coated microtitre wells. PSA PCR products labelled with the hapten, digoxigenin (DIG), are captured onto the wells by a specific hybridization reaction with the immobilized PSA probe. Detection is performed via an alkaline phosphatase-conjugated anti-digoxigenin antibody (anti-DIG-ALP). The ALP hydrolyzes the substrate, fluorosalicylphosphate (FSAP), releasing fluorosalicylate which forms highly fluorescent complexes with Tb$^{3+}$-EDTA. These long-lived fluorescent complexes are then measured using a time-resolved fluorometer. The hybridization of the PSA PCR product with the specific probe allows for the confirmation of the amplified product identity.

Chapter 3 describes the development of the quantitative RT-PCR method for PSA mRNA which involves the simultaneous analysis of the mRNA of β-actin, a housekeeping gene. In this method, PSA and actin cDNA, co-synthesized by reverse transcription, are subjected to PCR amplification using PSA-specific and actin-specific primers. The DIG-labelled PCR products are then analyzed by two separate hybridization assays; one of which is performed with an actin-specific probe and the other with a PSA-specific probe. Both of these probes are complementary to sequences spanning an exon/intron junction of the specific mRNA, ensuring that genomic DNA is not detected by the assay. The PCR products are analyzed by time-resolved fluorometry using the anti-DIG-ALP and FSAP substrate.

In Chapter 4, three configurations of an extremely sensitive and quick bioluminescent detection system, have been introduced for DNA hybridization assays. Although applied to the detection of PSA mRNA, these methods can easily be adapted to any mRNA of interest. All three assays involve the capturing of the PCR product between a DIG-tailed PSA probe, immobilized onto anti-DIG-coated microtitre wells, and a biotin-tailed PSA probe. The biotin moieties on the second PSA probe are then detected directly with a streptavidin-aequorin conjugate, with streptavidin and biotinylated aequorin, or with
streptavidin-biotinylated-aequorin preformed complexes. A luminometer is used to inject a Ca\(^{2+}\)-containing solution into the wells and instantaneously measure the resultant luminescence of aequorin. This detection system provides a high degree of sensitivity while reducing the turnaround time of the assays by eliminating the longer incubation periods required when using enzymes and substrates.

The time-resolved fluorometric and bioluminescent assays developed for the detection of PSA mRNA should prove very useful for detecting metastatic cancer cells in the peripheral blood and lymph nodes of patients with prostate cancer. In addition, we believe that the quantitative method would be very valuable in the study of prostate cancer spread. By allowing for the monitoring of PSA mRNA levels in patients over time, this method could be used to correlate PSA mRNA levels to types of treatment, aggressiveness of tumours, and the clinical outcome of patients.
CHAPTER 2
DETECTION OF PROSTATE-SPECIFIC ANTIGEN mRNA BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION AND TIME-RESOLVED FLUOROMETRYa

2.1 Introduction

As the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in the North American male population (26), prostate cancer has become a major concern in both the scientific community as well as the general population. Since prostate-specific antigen (PSA) is produced by the prostate epithelial cells, the cells involved in prostatic oncogenesis, the measurement of serum PSA has been a valuable marker for diagnosis, screening and clinical management of patients with prostate cancer (9, 64).

Since the therapeutic approaches for prostate cancer depend on the stage of the malignancy, after the initial diagnosis it is very important to assess whether the cancer is organ confined or has already spread beyond the prostate. Unfortunately, the sensitivity of current staging techniques is not adequate and up to 40% of patients who have undergone prostatectomy are found at the time of surgery to have metastasis (35, 36).

Polymerase chain reaction (PCR) (65, 66) has emerged as a powerful technique able to detect a few tumour cells in the presence of a vast excess of normal cells. This is accomplished by amplifying nucleic acid sequences that are specific and characteristic of tumour cells and subsequently analyzing the amplified fragments. During the last 3-4 years, reverse transcription-polymerase chain reaction (RT-PCR) assays for the PSA mRNA have been developed and applied to the detection of metastatic prostate cancer cells in lymph nodes and in peripheral blood (67-71). It has been shown that RT-PCR

offers higher sensitivity than standard histological and immunohistochemical methods for
detection of lymph node metastasis. Furthermore, Katz et al. (70), using RT-PCR in
whole blood, found that 78% of metastatic prostate cancer patients had circulating cancer
cells and 38% of patients with clinically localized disease were positive by RT-PCR
performed on peripheral blood specimens prior to surgery (70). A significant observation
in all these studies is that circulating PSA-expressing cells were not detected in any of the
patients who did not have prostate cancer. Thus, the detection of PSA mRNA is emerging
as a new test which may be useful for the correct staging and the clinical management of
patients diagnosed with prostate cancer. These issues have recently been reviewed (72).

Up to now all the assays for PSA mRNA have been based on the analysis of PCR
products by agarose gel electrophoresis and ethidium bromide staining or by Southern blot.
In this work, we report the first microtitre well-based hybridization assay for the detection
of PSA mRNA amplified by RT-PCR. The probe is immobilized in microtitre wells and
the amplified DNA (target) is labelled, during PCR, with the hapten digoxigenin. The
hybrids are detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase.
The enzymatic activity is monitored by using fluorosalicylphosphate as substrate. The
fluorosalicylate produced forms highly fluorescent complexes with Tb$^{3+}$-EDTA, which are
measured by time-resolved fluorometry (47, 53).

2.2 Materials

2.2.1 Cells

The LNCaP human prostate adenocarcinoma cell line, positive for PSA mRNA
(ATCC CRL 1740) and the HL-60 promyelocytic leukemia cell line, negative for PSA
mRNA (ATCC CCL 240) were both obtained from the American Type Culture Collection
(Rockville, MD).
2.2.2 Chemicals and Biochemicals

RPMI 1640 with L-glutamine, fetal bovine serum (FBS), penicillin, streptomycin, Fungizone (amphotericin B) and trypan blue used for cell culture procedures were purchased from Gibco Laboratories Life Technologies, Inc. (Gaithersburg, MD). The Trizol® LS Reagent, Moloney murine leukemia virus reverse transcriptase (M-MLV RT), dithiothreitol (DTT), oligo (dT)12-18 primer and biotin-14-dATP were also from Gibco.

Ultrapure 2'-deoxyribonucleoside 5'-triphosphates (dNTPs) were purchased from Pharmacia Biotech (Montreal, PQ).

Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIG-dUTP), terminal deoxynucleotidyl transferase (TdT), alkaline phosphatase-labelled sheep polyclonal anti-digoxigenin antibody, Fab fragments (anti-DIG-ALP), and bovine serum albumin (BSA) were obtained from Boehringer Mannheim Biochemica (Laval, PQ).

Streptavidin, ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), diethylpyrocarbonate (DEPC), mineral oil, polyoxyethylene sorbiton monolaurate (Tween-20) and Polaroid 667 film were purchased from Sigma (St. Louis, MO).

Three oligonucleotides (shown below) were designed for this work. Two of them, each consisting of 20 bases in length, were used as PCR primers. The upstream (U) primer and the downstream (D) primer are complementary to sequences in exons 2 and 3, respectively, of the PSA gene. PCR amplification of PSA cDNA using these primers yields a single 289 bp fragment. The third oligonucleotide (a 24-mer) was used as a probe and binds to a specific sequence in exon 3 of the PSA gene, a sequence within the PCR product. All oligonucleotides were synthesized by DNAgency (Aston, PA).

PSA U-Primer 5'-ATT GTG GGA GGC TGG GAG TG-3'
PSA D-Primer 5'-GGT CGT GGC TGG AGT CAT CA-3'
PSA Probe 5'-TCA GGA TGA AAC AGG CTG TGC CGA-3'

Agarose was from ICN Biomedicals, Inc. (Costa Mesa, CA).
The Bio-Ladder™ DNA molecular weight markers, used in agarose gel electrophoresis were purchased from Bio-Synthesis, Inc. (Lewisville, TX).

Bromophenol Blue was purchased from Bio-Rad Laboratories (Hercules, CA).

The phosphate ester of 5'-fluorosalicylic acid (FSAP) was from CyberFluor Division, Nordion International (Toronto, ON).

Terebin chloride hexahydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI).

The recombinant plasmid pA75 was a gift from Dr. J. Trapman, M.D., Anderson Cancer Center, Houston, Texas. This plasmid contains a 1.4 kb PSA cDNA insert (73).

Sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen orthophosphate, sodium hydroxide, hydrochloric acid, hydroxymethylaminomethane (Tris), chloroform, isopropyl alcohol, ethanol, glacial acetic acid and glycerol were all from BDH, Inc. (Toronto, ON).

2.2.3 Supplies

Polystyrene cell culture flasks (25 cm²) and polypropylene cryogenic vials (2.0 mL) were purchased from Corning (Corning, NY).

Sterile, disposable 10-mL graduated, polystyrene, serological pipettes, as well as 3 and 10 cc syringes were from Falcon® Becton and Dickinson Labware (Lincoln Park, NJ).

Nalgene™ sterile 25 mm syringe filters (0.2 μm pore size) and 6-inch glass pasteur pipettes were from Baxter Diagnostics, Corp. (Toronto, ON).

Sterile conical polypropylene centrifuge tubes (15 and 50 mL) were from VWR (West Chester, PA). Disposable Universal Fit pipette tips (10-200 μL and 200-1000 μL) were also from VWR.

Titertek Plus Microtips (0.5 μL-10 μL) were from ICN Biomedicals, Inc. (Costa Mesa, CA).

Eppendorf 1.5-mL microcentrifuge tubes were purchased from Brinkman
Instruments (Westburg, NY).

Gene Amp reaction tubes for PCR were obtained from Perkin Elmer (Norwalk, CT).

Sephadex® G-25 gel filtration columns (NAP-5 and NAP-10) were from Pharmacia Biotech (Montreal, PQ).

Opaque, 12-well polystyrene Microlite™ 2 microwell strips and Removawell strip holders were obtained from Dynatech Laboratories, Inc. (Chantilly, VA).

2.2.4 Apparatus

The CyberFluor 615 Immunoanalyzer (CyberFluor Division, Nordion International, Toronto, ON), a time-resolved fluorometer, was used to measure the fluorescence of FSAP-Tb³⁺-EDTA solutions in microtitre wells. The excitation and emission wavelengths were set at 337 and 615 nm, respectively.

The Perkin-Elmer Cetus (Norwalk, CT) 48-well DNA Thermal Cycler was used to carry out the polymerase chain reactions.

Agarose gel electrophoresis was performed using the Miniature Horizontal Gel System MLB-06 (Tyler Research Instruments Edmonton, AB) and the Fotoforce 500™ Power Supply (Fotodyne, Inc., Bio-Can Scientific, Mississauga, ON). Photographs of the gels were taken using the Model MP4 Polaroid Camera System from Polaroid Corp. (Cambridge, MA).

The Nuair AutoFlow CO₂ Water-Jacketed Incubator and the Class II Type A/B3 laminar flow hood (Nuair Biological Safety Cabinets, Plymouth, IL) were used for the culturing of cells.

Cells were counted using the Neubauer Brightline hemocytometer from Hauser Scientific (American Scientific Products, McGaw Park, IL) and a Nikon TMS Microscope (Japan).

The Shimadzu UV-160 spectrophotometer (Shimadzu Corp., Japan) was used to
take absorbance measurements of RNA solutions.

All pH measurements were made with the Corning pH Meter, Model 340 (Corning, NY). Prior to each measurement, the combination [Ag/AgCl] reference/pH electrode was calibrated using pH standard buffer solutions with pH values of 4.0, 7.0 and 10.0 (BDH, Inc., Toronto, ON).

Micropipetting was carried out using both Eppendorf pipettes (Germany) and Nichiryo Model 5000 pipettes (Japan). An Eppendorf Repeater® pipette with Eppendorf Combitips® were used for repetitive pipetting.

Centrifugations required for cell culture procedures were performed using a desktop Safety-Head Centrifuge (Canlab, Mississauga, ON). Microcentrifugations were carried out using the Eppendorf Model 5415C microcentrifuge from Baxter/Canlab Inc. (Mississauga, ON).

Other laboratory equipment included the Haake Model W19 water tank with a Haake D1 heater and thermostat (Haake, Germany), the Model EAW II microtitre plate washer from SLT Lab Instruments (Austria), the Amerlite Shaker/Incubator (Amersham Canada Ltd., Oakville, ON), a portable Model UVG-II Shortwave UV (254 nm) Mineralight® Lamp, the Genie 2 Vortex from Fischer Scientific Canada Ltd. (Toronto, ON), and a Corning Stirrer/Hotplate (Corning, NY).

2.3 Methods

2.3.1 Cell Culture

2.3.1.1 Reagents

*Phosphate-Buffered Saline (PBS).* Consisted of 0.14 mol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄ and 1.76 mmol/L KH₂PO₄, pH 7.4.

*LNCaP and HL-60 Cell Culture Media.* Consisted of 90% (v/v) RPMI 1640, 10% (v/v) FBS, 100 kU/L penicillin, 100 mg/L streptomycin and 0.25 mg/L Fungizone.

*Cell Freezing Medium.* Consisted of 80% (v/v) FBS, 15% (v/v) RPMI 1640 and
5% (v/v) DMSO.

2.3.1.2 Procedures

2.3.1.2.1 Culturing Cells

All the glassware used for cell culturing was autoclaved to avoid any bacterial contamination of the cells. Furthermore, media bottles, sterile tubes and cell culture flasks were only opened in the laminar flow hood.

LNCaP (positive for PSA mRNA) and HL-60 (negative for PSA mRNA) cell lines were both cultured in a similar manner, the only difference being the positioning of the culture flasks during the incubation of the cells. In the case of LNCaP cells, the flask was laid down horizontally such that there was a greater surface area for the loose attachment of these cells. On the other hand, HL-60 cells were grown in suspension in which the culture flask was placed in an upright position.

Frozen cells obtained from the ATCC were thawed by rapid agitation and immediately washed with PBS in a 15-mL conical tube. Cells were pelleted by centrifugation at 300 x g for 15 min and resuspended in 10 mL of the cell culture medium. The cells were then pipetted into a 25-cm² culture flask and grown at 37 °C with 5% CO₂. The culture medium was replaced with fresh media every 2-3 days depending on cell density. To replace expired medium with fresh medium, the cells were collected in a 15-mL conical tube and pelleted by centrifugation. The expired medium was aspirated and the cells were resuspended in fresh culture medium.

2.3.1.2.2 Freezing Cells

In order to store cells for future use, cell pellets were resuspended in cell freezing medium at a minimum density of 1 x 10⁶ cells/mL and then aliquotted into 2.0-mL cryogenic vials. These aliquots were immediately placed at -20 °C for 2 hours and then stored at -80 °C until needed.
2.3.1.2.3 Counting Cells

The number of cells in a particular volume of culture medium was determined by mixing a 20-µL aliquot of the cell-containing solution with an equal volume of trypan blue. Trypan blue, a dye which is able to penetrate only into "dead cells" since their cell membrane is not intact, allows for the identification of viable cells. A 10-µL aliquot of the trypan blue/cell mixture was pipetted onto each of the two grids of the hemocytometer, and covered with a glass cover slip. Each of the grids are divided into four separate squares. The number of viable cells (lacking the blue dye in their cytoplasm) in each square was counted. The average number of cells per square was divided by the volume of the square (0.0001 mL) and then multiplied by the dilution factor of 2. The final result of this calculation expresses the number of cells/mL.

2.3.2 Isolation of Total RNA using the Trizol® LS Reagent

The Trizol® LS Reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is a ready-to-use reagent for the isolation of total RNA from cells and tissues (74). During sample homogenization or lysis, the Trizol® Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components.

For the isolation of total RNA, approximately 1 mL of culture media containing ten million cells was placed in a 1.5-mL microcentrifuge tube. The cells were precipitated by centrifugation at 12,000 x g for 2 min and the supernatant was removed. For the isolation of total RNA from the lung tumour specimens, 150 mg of tissue were placed in each microcentrifuge tube. A 750-µL aliquot of the Trizol® Reagent was then added to each tube. Repetitive pipetting was performed to aid in the lysis of the sample. The sample volume was adjusted to 1 mL by the addition of DEPC-treated water. The sample was incubated at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes. Next, 200 µL of chloroform were added to the sample and the tube was vigorously shaken by hand for 15 sec. Following a 10-min incubation at room
temperature, the sample was centrifuged at 12,000 × g for 15 min at 4 °C. After centrifugation, the mixture separates into a lower, red-coloured phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phase, which contains the RNA, was transferred to a clean microcentrifuge tube. Subsequently, 500 µL of isopropyl alcohol was added to precipitate the RNA. The sample was incubated for 10 min at room temperature and then centrifuged at 12,000 × g for 10 min at 4 °C. The RNA forms a gel-like pellet on the side and bottom of the tube. Following removal of the supernatant, the RNA pellet was washed with 1 mL of 75% ethanol and then centrifuged at 7500 × g for 5 min at 4 °C. The supernatant was removed and the pellet was air-dried for 5-10 min. The RNA pellet was then dissolved in 20 µL DEPC-treated water and incubated for 10 min at 55-60 °C. The concentration of the isolated RNA was determined spectrophotometrically at 260 nm. A reverse transcription negative, which contained all reagents except for the RNA, was included in every series of reactions performed.

2.3.3 Reverse Transcription

In the presented work, an oligo(dT)12-18 primer capable of binding to the poly A tails of mRNA molecules was utilized to synthesize cDNA from all mRNA molecules. An aliquot containing 5 µg of total RNA was added to 0.5 µg of oligo(dT)12-18 primer and brought to a final volume of 12 µL with DEPC-treated water. The solution was heated to 70 °C for 10 min and immediately chilled on ice. Then, 8 µL of reverse transcription reaction buffer (125 mmol/L Tris-HCl, pH 8.3, 188 mmol/L KCl, 7.5 mmol/L MgCl₂, 25 mmol/L dithiothreitol, 2.5 mmol/L of each dNTP (dATP, dCTP, dGTP and dTTP) and 200 U of M-MLV reverse transcriptase was added. Following an incubation period of 1h at 37 °C, the reverse transcriptase was inactivated by heating the solution to 95 °C for 4 min. The synthesized cDNA was stored at -20 °C.

2.3.4 Polymerase Chain Reaction

In order to avoid DNA carryover, dedicated PCR pipettes, pipette tips, and
microcentrifuge racks were irradiated with UV light for approximately 1 h prior to setting up PCR experiments (75). These items, as well as the autoclaved glassware, microcentrifuge tubes, PCR tubes, and water used for PCR experiments, were kept in a separate room exclusively designated for the isolation of total RNA and setting up the PCR experiments. The amplification reactions and analyses of PCR products were also performed in separate rooms.

PCR was carried out in a total reaction volume of 100 µL which consisted of 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9.0), 1 mL/L Triton X-100, 2.5 mmol/L MgCl₂, 0.1 mmol/L of each dNTP (dATP, dCTP, dGTP and dUTP), 5 µmol/L of DIG-dUTP, 2.5 U of Taq DNA polymerase and 10 µL of the sample cDNA. The PCR mixture was layered with 80 µL of mineral oil to prevent evaporation. The 'hot-start' protocol was applied (76). The mixture was first heated to 95 °C for 5 min, during which time 50 pmol of both the PSA-U and PSA-D primers were added to each tube. Thirty cycles of PCR were carried out; each cycle consisted of a denaturation step at 95 °C for 30 s, primer annealing at 60 °C for 30 s, and primer extension at 72 °C for 1 min. At the end of the cycles the reaction mixture was kept at 72 °C for 10 min and then cooled to 4 °C. A PCR negative, which contained all the reagents but no template, was included in each PCR series in order to check for contamination.

2.3.5 Agarose Gel Electrophoresis

2.3.5.1 Reagents

*Ethidium Bromide Stock Solution.* Consisted of 10 g/L ethidium bromide in water.

*Tris-Acetate EDTA (TAE) Stock Buffer.* A 50x concentrated stock (2 mol/L Tris-acetate, 0.05 mol/L EDTA) was prepared by mixing 242 g Tris, 57.1 mL of glacial acetic acid and 100 mL of 0.5 mol/L EDTA, pH 8.0, in a final volume of 1 L. This solution was stored at 4 °C.

*1x TAE Electrophoresis Buffer.* This solution, which was prepared just prior to
use, consisted of 25 μL of ethidium bromide stock solution and 10 mL of the 50x TAE stock buffer in a final volume of 500 mL.

*Gel Loading Buffer.* Contained 0.25% (w/v) Bromophenol Blue and 30% (v/v) glycerol.

### 2.3.5.2 Procedure

A 2% agarose gel was prepared by adding 1 g of agarose into 50 mL of the 1x TAE electrophoresis buffer and microwaving the mixture at high power until the agarose was completely dissolved (approximately 1-2 min). After allowing the solution to cool slightly, it was poured into the gel casting tray and an 8-well slot former was positioned in the agarose. Following the solidification of the agarose, the slot former was removed and the gel was placed into the electrophoresis chamber filled with 1x TAE electrophoresis buffer.

Samples were prepared for electrophoresis by mixing 20 μL of sample and 4 μL of the gel loading buffer. A 20-μL aliquot of this mixture was loaded onto the gel. A 10-μL aliquot of the Bio-Ladder™ DNA molecular weight markers was mixed with 2 μL of gel loading buffer. Then 10 μL of this solution was loaded onto the gel. Electrophoresis was carried out at 70-80 volts for approximately 1-2 h. Existing bands in the gel were initially viewed using a portable UV light. Then, if required, photographs of UV-illuminated gels were taken using Polaroid 667 film.

### 2.3.6 Tailing of the PSA Probe with Biotin-14-dATP

The oligonucleotide which was used as a PSA-specific probe was enzymatically tailed with biotin-14-dATP using terminal deoxynucleotidyl transferase. The tailing reaction was carried out in a final volume of 20 μL containing 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl (pH 6.6), 0.25 g/L bovine serum albumin, 5 mmol/L CoCl₂, 125 μmol/L of each dNTP, 20 μmol/L biotin-14-dATP, 50 U of terminal transferase and 5 μmol/L of the probe. The reaction mixture was incubated for 30 min at 37 °C.
2.3.7 Purification of the Biotin-Tailed PSA Probe by Gel Filtration

2.3.7.1 Reagents

*Equilibration Buffer.* Consisted of 10 mmol/L sodium dihydrogen phosphate, pH 6.8.

*NAP-5 Column.* Prepacked disposable column containing Sephadex® G-25 Medium of DNA grade in distilled water containing 0.15% Kathon® CG as perservative.

2.3.7.2 Procedure

The biotin-tailed PSA probe was purified from the excess of biotin-14-dATP by gel filtration using the NAP-5 column. Just prior to use, the column was equilibrated by pouring off the excess storage buffer and running approximately 10 mL of the equilibration buffer through the column. After allowing the equilibration buffer to completely enter the gel bed, the tailed-probe solution was loaded onto the column in a maximum volume of 0.5 mL. For sample volumes less than 0.5 mL, equilibration buffer was added so that the combined volume was 0.5 mL. Once this solution had completely entered the gel bed, the purified sample was eluted with 1.0 mL of equilibration buffer into a sterile microcentrifuge tube and stored at -20 °C until required for use.

2.3.8 Microtitre Well Hybridization Assay of PCR Products using Time-Resolved Fluorescence

2.3.8.1 Reagents

*Wash Solution.* Consisted of 50 mmol/L Tris, pH 7.5, 0.15 mol/L NaCl and 0.1% (v/v) Tween-20.

*Phosphate-Buffered Saline with Tween-20 (PBST).* PBS solution (see Section 2.3.1.1) containing 0.1% (v/v) Tween-20.

*ALP Buffer.* Consisted of 0.1 mol/L Tris, pH 9.05, 0.1 mol/L NaCl and 1 mmol/L MgCl₂.
*Substrate Stock Solution.* A 0.01 mol/L stock solution of FSAP was prepared by dissolving 11.8 mg of the compound in 5 mL of 0.1 mol/L NaOH and kept at 4 °C. A 0.001 mol/L FSAP working solution was prepared just prior to use by diluting the FSAP stock solution 10-fold in ALP buffer.

*Developing Solution.* Contained 1 mol/L Tris, 0.4 mol/L NaOH, 3 mmol/L EDTA and 2 mmol/L TbCl₃.

2.3.8.2 Procedure

Opaque, polystyrene microtitre wells were coated overnight (at room temperature) with 100 μL of 1.4 mg/L solution of streptavidin in PBS. Prior to use, the wells were washed three times with wash solution. Then 100 μL of 33 nmol/L biotin-tailed PSA probe diluted in the wash solution were pipetted into each well. Following a 45-min incubation (with shaking) at room temperature, the wells were washed three times and 80 μL of PBST (preheated to 42 °C) were pipetted into each well. PCR products were denatured by heating at 95 °C for 10 min, and immediately cooled on ice. A 20-μL aliquot of the PCR product was then added into each well, already containing the 80 μL of PBST. All assays were carried out in duplicate. Hybridization was carried out for 45 min at 42 °C with shaking. The wells were then washed three times, and 100 μL of a 750 U/L alkaline phosphatase-labelled anti-digoxigenin antibody (diluted in wash solution) were added. Following a 30-min incubation at room temperature, the wells were washed three times and 100 μL of the substrate were added. The enzymatic reaction was allowed to proceed for 30 min at room temperature after which time 100 μL of developing solution were pipetted into each well and mixed for 1 min. The fluorescence was then measured with the CyberFluor 615 Immunoanalyzer in which the excitation and emission wavelengths were set at 337 nm and 615 nm, respectively. The procedure of the hybridization assay is summarized in Figure 2.1.
FIGURE 2.1
Schematic Representation of the Time-Resolved Hybridization Assay for the Detection of PSA mRNA

Legend

The biotin (B)-tailed PSA probe is immobilized onto streptavidin (SA)-coated microtitre wells. The amplified DNA (target), which is labelled during PCR with the hapten digoxigenin (D), is denatured and allowed to hybridize to the probe. The hybrids are detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase (ALP). The enzymatic activity is monitored by using fluorosalicylphosphate (FSAP) as substrate. The fluorosalicylate (FSA) produced forms highly fluorescent complexes with Tb³⁺-EDTA, which are measured by time-resolved fluorometry.
FIGURE 2.1

DIG-labelled
289 bp PSA PCR Product

Add biotin-tailed
PSA probe
Incubate at RT for 45 min
Wash

Add 20 μL denatured
PCR product
Incubate at 42 °C for 45 min
Wash

Add anti-DIG-ALP
Incubate at RT for 30 min
Wash

Add FSAP
Incubate for 30 min at RT
Wash.

Add Tb³⁺-EDTA
Developing Solution
Mix for 1 min

Measure Fluorescence using CyberFluor
λex 337.1 nm and λem 615 nm
2.4 Results and Discussion

The effect of the biotin-tailed probe concentration was studied by preparing various dilutions of the probe in the range of 1.5 to 50 nmol/L and using them for measurement of amplified DNA from 10,000 PSA cDNA molecules. The results from this study are presented in Figure 2.2. The fluorescence increases steadily with the probe concentration up to 25 nmol/L and then reaches a plateau, due to saturation of streptavidin binding sites. Thus, the maximum amount of probe which can be immobilized is 2.5 pmol/well.

The effect of the volume of PCR mixture used in the hybridization assay was also studied with amplified DNA corresponding to 10,000 and 1000 LNCaP cells (see Figure 2.3). The fluorescence increases as the volume of product, applied per well, increases. However, a 20-μL sample volume was chosen since it provides adequate sensitivity (see below) and allows for analysis of replicates.

The time required for completion of the hybridization reaction was studied in the range of 15 to 90 min and the results are presented in Figure 2.4. As the incubation time is extended, a continuous increase of the fluorescence is observed. However, the signal-to-background ratio reaches a plateau after a 45 min incubation period. With longer incubations there is a concomitant increase in the background due to nonspecific binding of the excess (unincorporated) DIG-dUTP, which is present in the PCR mixture. The short incubation period required for hybridization in microtitre wells is a significant advantage over the Southern blot technique where hybridization requires several hours followed by long and tedious washing steps.

In order to assess the sensitivity of the proposed assay, we prepared serial dilutions of a plasmid containing PSA cDNA. Then, aliquots that contained 0, 16, 160, 1600, 16,000 and 160,000 molecules were subjected to PCR and the products were assayed by hybridization. The fluorescence, as well as the signal-to-background ratio were plotted against the number of molecules (Figure 2.5). Signal to background estimates represent
FIGURE 2.2
The Effect of the Biotin-Tailed Probe Concentration

Legend

The concentration of the biotin-tailed probe used for hybridization was optimized. The probe (100 µL) was captured on streptavidin coated wells and then hybridized to denatured amplified product generated from $10^4$ PSA cDNA molecules.
FIGURE 2.3

Study of the Volume of PCR Mixture used in the Hybridization Assay

Legend

The effect of the volume of PCR mixture used for the hybridization assay was studied. The final volume in the well was always 100 μL (PBST was used as diluent). Lines 1 and 2 represent amplified PSA mRNA from 10,000 and 1000 LNCaP cells, respectively.
FIGURE 2.4
Optimization of the Time Allowed for Hybridization

Legend

The time allowed for hybridization of the immobilized probe with amplified DNA produced by PCR of mRNA corresponding to 1000 LNCaP cells was studied. The fluorescence (1) and the signal-to-background ratio (2) are plotted against the incubation periods. The background is the fluorescence obtained when no PSA cDNA is present in the PCR mixture.
FIGURE 2.5
Sensitivity of the Hybridization Assay Coupled to PCR

Legend

The sensitivity of the hybridization assay coupled to the PCR was studied by preparing serial dilutions of a plasmid containing the PSA cDNA sequence. Plots of (1) the fluorescence and (2) the signal-to-background ratio against the number of PSA cDNA molecules originally present in the PCR mixture. The assay detects 160 PSA cDNA copies with a signal-to-background ratio of 10.
FIGURE 2.5

Fluorescence (arb. units)

Signal/Background

Number of PSA cDNA molecules
the average of two determinations. A signal-to-background ratio of 10 was obtained with 160 cDNA molecules. We also compared directly the sensitivity of the proposed assay to that of agarose gel electrophoresis and ethidium bromide staining. Aliquots (20 μL) from the same PCR mixtures were loaded onto a 2% agarose gel, electrophoresed and stained with ethidium bromide. From a photograph of this gel (not shown) it was observed that only the samples corresponding to 16,000 and 160,000 PSA cDNA molecules appear as visible bands. Thus, the detection limit for electrophoresis is at least 100 times that for the time-resolved fluorometric hybridization assay.

The ability of the proposed system to detect a few PSA-expressing cells in the presence of a large excess of cells which do not express PSA, was tested by preparing and analyzing samples containing PSA-mRNA corresponding to 0, 1, 10, 100, 1000 and 10,000 LNCaP cells in the presence of one million HL-60 cells. Amplification always produced a 289 bp fragment (Figure 2.6). If genomic DNA was coamplified it would give a 1918 bp fragment (based on the positions of the primers). However such a fragment was not detected either by electrophoresis or by time-resolved fluorometry. In Figure 2.7, the fluorescence and the signal-to-background ratio are plotted versus the number of LNCaP cells. The assay can detect amplified mRNA from a single LNCaP cell with a signal-to-background ratio of 3.

Within-run precision studies for the hybridization assay were performed using amplified DNA generated from mRNA representative of 100, 1000 and 50,000 LNCaP cells in the presence of 300,000 HL-60 cells. The reproducibility of the fluorescence was 12.4, 4.9 and 6.8% for 100, 1000 and 50,000 LNCaP cells, respectively (n=12). To assess the overall reproducibility, including the PCR step, a sample containing mRNA from 1000 LNCaP cells in the presence of 200,000 HL-60 cells was amplified in 7 side-by-side reactions and the amplified products were determined by the proposed hybridization assay. The CV obtained for the signal was 8.8%.
FIGURE 2.6
Agarose Gel Electrophoresis of the PSA PCR Product

Legend

The expected size of the PSA PCR product (based on the positions of the PSA primers on the PSA mRNA) was 289 bp. To check the size and purity of the amplified product, aliquots of PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide (as described in Section 2.3.5). Lane 1 represents a PCR negative control in which all reagents (including the HL-60 cDNA) are present in the original PCR mixture except for the PSA cDNA. Lane 2 shows the DNA molecular weight marker. The first band at the top of the lane represents 1000 bp, and each consecutive band decreases by 100 bp. Lane 3 and 4 represent PCR products generated from $8 \times 10^4$ PSA cDNA molecules in the presence of water and one million HL-60 cells, respectively.
FIGURE 2.7

The Sensitivity and Linearity of the Proposed RT-PCR Method

Legend

The sensitivity and linearity of the overall method, including reverse transcription, PCR and the hybridization assay, was studied. Mixtures containing mRNA from 1 to 10,000 LNCaP cells each in the presence of one million of HL-60 cells were subjected to RT-PCR and the amplified fragments were analyzed by hybridization. The fluorescence (1) and the signal-to-background ratio (2) was plotted against the number of LNCaP cells. The background is defined as the fluorescence obtained in the presence of 1 million HL-60 cells with no LNCaP cells.
FIGURE 2.7

![Graph showing fluorescence and signal/background against the number of LNCaP cells.](image-url)
The proposed PSA RT-PCR method was also applied to the analysis of 56 lung tumour specimens. The isolation of total RNA, RT-PCR and the hybridization assay were carried out as described in Section 2.3. The PCR products were analyzed in duplicate using the hybridization assay. If the two trials varied by more than 20%, the sample was assayed a second time again in duplicate. Both negative and positive controls were included in these analyses. It was found that 9 out of the 56 (16.1%) tumour specimens gave S/B ratios greater than 3. This value increased to 19.6% if the arbitrary cutoff was decreased to a S/B ratio of 2.5.

2.5 Conclusions

Deguchi et al. (68) proposed a PCR-based assay for PSA mRNA, which employed agarose gel electrophoresis, Southern blot, membrane hybridization and immunodetection of the hybrids. The assay was able to detect a single LNCaP cell in the presence of $10^6$ PSA-negative cells. Katz et al. (70) also applied a single PCR, followed by electrophoresis and ethidium bromide staining of the amplified products. The reported sensitivity was $10 \text{ LNCaP}/10^6$ PSA-negative cells. The sensitivity was further enhanced by Southern blot and immunodetection of PCR products. Israeli et al. (69) devised a nested PCR (two rounds, 25 cycles each) and analyzed the products by electrophoresis and ethidium bromide staining to achieve a sensitivity of $1 \text{ LNCaP}/10^6$ negative cells. They also performed Southern transfer and hybridization with $^{32}$P-labelled probes but found no improvement in sensitivity. Also, Jaakkola et al. (71) performing nested PCR (two rounds, 30 cycles each) with electrophoretic analysis of the amplified DNA, achieved a sensitivity of about $2 \text{ LNCaP}/10^6$ negative cells. Compared to the above approaches, the proposed assay achieves equivalent sensitivity and specificity ($1 \text{ LNCaP}/10^6$ PSA-negative cells) without the need for nested PCR. As a consequence, the possibility of contamination associated with a second round of PCR is eliminated. Furthermore, hybridization in microtitre wells allows for confirmation of amplified product identity without Southern transfer and
membrane hybridization. Thus, the proposed assay is automatable and adaptable to the routine clinical laboratory and may be useful in the detection of metastatic cancer cells in lymph nodes and in peripheral blood of patients diagnosed with prostate cancer.

It has been shown that prostate-specific antigen is present in 30-40% of female breast tumours, more rarely in other tumours, in normal breast tissue and in biological fluids including amniotic fluid (21-25). These new findings have recently been reviewed (19). Although the physiological role of PSA in these normal as well as cancerous tissues still remains unknown, it has been suggested that PSA may act as a growth factor or growth factor regulator.
CHAPTER 3

QUANTITATIVE REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION FOR PROSTATE-SPECIFIC ANTIGEN mRNA

3.1 Introduction

In recent years the number of patients found with small localized prostate tumours has been increased as a consequence of screening that is based on digital rectal examination and serum prostate-specific antigen (PSA). And although these tests have allowed for the earlier detection of prostate tumours, still, in approximately 60% of newly diagnosed patients the disease has already spread beyond the prostate (77). Many of these metastatic tumours go undetected, and patients are treated as if they had less advanced tumours (i.e., by radical prostatectomy). Early detection of metastasis could spare many men from the morbidity of such inappropriate operations.

Unfortunately, the sensitivity of current staging modalities, including computed tomography scans, transrectal ultrasonography, and magnetic resonance imaging, is inadequate and understaging is very common. Thus, there exists an obvious need for a sensitive staging method capable of more accurately identifying patients with extraprostatic disease at the time of diagnosis (78).

Although the molecular mechanism involved in the development of metastases and the factors determining the metastatic potential of prostatic cancer cells are obscure, it is currently believed that during the early stages of the metastatic process, tumour cells penetrate the capsule and circulate through the bloodstream or lymphatic channels before attaching to a distant site (e.g., bones). Therefore the detection of prostatic cells in peripheral blood of patients with prostate cancer is considered as an early indication of metastasis.

In the last 3-4 years the PSA mRNA detected by reverse transcriptase polymerase chain reaction (RT-PCR) has been used as a specific marker of prostate epithelial cells (67-
These assays have been applied to the detection of micrometastases peripheral blood, lymph nodes and bone marrow of prostate cancer patients. RT-PCR offers excellent sensitivity and shows great promise of becoming that much-needed method for accurately assessing prostate cancer spread. Using an odds ratio analysis, Katz et al. (70), have shown that no other preoperative staging modality tested was related more strongly to extraprostatic disease or organ-confined disease than the RT-PCR method for PSA mRNA (81). The mRNA for prostate-specific membrane (PSM) antigen has also been proposed as a marker for circulating prostatic tumour cells (69, 84).

All the RT-PCR based methods for PSA (or PSM) mRNA reported so far are qualitative, that is, they detect the presence or absence of the particular mRNA without referring to its quantity. We were interested in developing a quantitative methodology where the relative changes of PSA mRNA can be monitored. A quantitative RT-PCR assay would be very beneficial in the study of prostate cancer spread because it would allow monitoring of PSA mRNA levels in patients over a period of time and correlation of these levels to types of treatment, aggressiveness of tumours and clinical outcome.

In the present work the PSA mRNA can be monitored quantitatively by simultaneous analysis of β-actin mRNA. After reverse transcription, the PSA and actin cDNA are subjected to PCR amplification using PSA-specific and actin-specific primers. The hapten digoxigenin is incorporated into the amplified sequences. The PCR products are analyzed by two (separate) hybridization assays with specific probes immobilized in microtiter wells. The hybrids are detected using alkaline phosphatase-labelled antidigoxigenin antibody. The enzymatic activity is monitored by using fluorosalicylicphosphate as substrate. The fluorosalicylate produced forms fluorescent complexes with Tb³⁺-EDTA, which are measured by time-resolved fluorometry (47, 53).
3.2 Materials

3.2.1 Chemicals and Biochemicals

The LNCaP human prostate adenocarcinoma and HL-60 promyelocytic leukemia cell lines were both obtained from the American Type Culture Collection (Rockville, MD).

RPMI 1640 with L-glutamine, fetal bovine serum (FBS), penicillin, streptomycin, Fungizone (amphotericin B) and trypan blue used for cell culture procedures were purchased from Gibco Laboratories Life Technologies, Inc. (Gaithersburg, MD). The Trizol® LS Reagent, Moloney murine leukemia virus reverse transcriptase (M-MLV RT), DTT and biotin-14-dATP were also from Gibco.

Ultrapure 2’-deoxyribonucleoside 5’-triphosphates (dNTPs) were purchased from Pharmacia Biotech (Montreal, PQ).

The oligonucleotides used in the course of this work (synthesized by DNAgency, Aston, PA) are as follows:
(a) 5’-CTCTCGTGCGAGGCAGTCT-3’, a 20mer used as the upstream PSA (Up) primer homologous to a sequence in exon II of the PSA gene;
(b) 5’-GGTCGTGGGTGGCTGATCATCA-3’, a 20mer used as the downstream PSA (Dp) primer complementary to a sequence in exon III of the PSA gene;
(c) 5’-ATCAGCTTTTGGTCTCGATGCGAG-3’, a 24mer used as the PSA probe, which spans the exonII/exonIII junction in the PSA mRNA, by having a sequence complementary to the last 12 bases of exon II plus the first 12 bases of exon III of the PSA gene. The junction probe was chosen to ensure that even if genomic DNA is amplified during PCR, it would not be detected by the hybridization assay.
(d) 5’-ACAATGAGCTGCGGTGGCT-3’, a 20mer used as the upstream actin (UA) primer homologous to a sequence in exon II of the actin gene;
(e) 5’-TCTCCTTAATGTCACGCACGA-3’, a 21mer used as the downstream actin (DA) primer complementary to a sequence in exon III of the actin gene (68, 85)
(f) 5'-TCTCAACACATGATCTGGGTACATCT-3', a 24mer used as the actin-specific probe, which was designed to span the exonII/exonIII junction of actin mRNA. It has a sequence complementary to the last 12 bases of exon II and the first 12 bases of exon III.

Yeast tRNA, digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIG-dUTP), terminal deoxynucleotidyl transferase (TdT), alkaline phosphatase-labelled sheep polyclonal anti-digoxigenin antibody, Fab fragments (anti-DIG-ALP), and bovine serum albumin (BSA) were obtained from Boehringer Mannheim Biochemica (Laval, PQ).

Streptavidin, pUC18 DNA Hae III digest (0.675 g/L), EDTA, DMSO, DEPC, mineral oil, Tween-20 and Polaroid 665 film were purchased from Sigma (St. Louis, MO).

Agarose was from ICN Biomedicals, Inc. (Costa Mesa, CA).

The phosphate ester of 5'-fluorosalicylic acid (FSAP) was from CyberFluor Division, Nordion International (Toronto, ON).

Terbium chloride hexahydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI).

The recombinant PSA cDNA-containing plasmid pA75 (described in Section 2.2.2) was a gift from Dr. J. Trapman, M.D., Anderson Cancer Center, Houston, Texas (73).

Sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen orthophosphate, sodium hydroxide, hydrochloric acid, Tris, chloroform, isopropyl alcohol, ethanol, glacial acetic acid and glycerol were all from BDH, Inc. (Toronto, ON).

3.2.2 Supplies

Polystyrene cell culture flasks (25 cm²) and polypropylene cryogenic vials (2.0 mL) were purchased from Corning (Corning, NY).

Sterile, disposable 10-mL graduated, polystyrene, serological pipettes, as well as 3- and 10-cc syringes were from Falcon® Becton and Dickinson Labware (Lincoln Park, NJ).
Nalgene™ sterile 25 mm syringe filters (0.2 µm pore size) and 6-inch glass
pasteur pipettes were from Baxter Diagnostics, Corp. (Toronto, ON).

Sterile conical polypropylene centrifuge tubes (15 and 50 mL) were from VWR
(West Chester, PA). Disposable Universal Fit pipette tips (10-200 µL and 200-1000 µL)
were also from VWR.

Tiertek Plus Microtips (0.5 µL-10 µL) were from ICN Biomedicals, Inc. (Costa
Mesa, CA).

Eppendorf 1.5-mL microcentrifuge tubes were purchased from Brinkman
Instruments (Westburg, NY).

Gene Amp reaction tubes for PCR were obtained from Perkin Elmer (Norwalk,
CT).

Sephadex® G-25 gel filtration columns (NAP-5 and NAP-10) were from
Pharmacia Biotech (Montreal, PQ).

Opaque, 12-well polystyrene Microlite™2 microwell strips and Removawell strip
holders were obtained from Dynatech Laboratories, Inc. (Chantilly, VA).

3.2.3 Apparatus

The CyberFluor 615 Immunoanalyzer (CyberFluor Division, Nordion
International, Toronto, ON), a microtitreplate time-resolved fluorometer, was used to
measure the fluorescence of FSAP-Tb³⁺-EDTA solutions in microtitre wells. The
excitation and emission wavelengths were set at 337 and 615 nm, respectively.

The Perkin-Elmer Cetus (Norwalk, CT) 48-well DNA Thermal Cycler was used to
carry out the polymerase chain reactions.

Agarose gel electrophoresis was performed using the Miniature Horizontal Gel
System MLB-06 (Tyler Research Instruments Edmonton, AB) and the Fotoforce 500™
Power Supply (Fotodyne, Inc., Bio-Can Scientific, Mississauga, ON). Photographs of
the gels were taken using the Model MP4 Polaroid Camera System from Polaroid Corp.
(Cambridge, MA).

The quantitation of DNA products, separated by electrophoresis, was performed using the Bio-Rad Model GS-670 imaging densitometer (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

The Nuaire Autoflow CO₂ Water-Jacketed Incubator and the Class II Type A/B3 laminar flow hood (Nuaire Biological Safety Cabinets, Plymouth, IL) were used for the culturing of cells.

Cells were counted using the Neubauer Brightline hemocytometer from Hauser Scientific (American Scientific Products, McGaw Park, IL) and a Nikon TMS Microscope (Japan).

The Shimadzu UV-160 spectrophotometer (Shimadzu Corp., Japan) was used to take absorbance measurements of RNA solutions.

All pH measurements were made with the Corning pH Meter, Model 340 (Corning, NY). Prior to each measurement, the combination [Ag/AgCl] reference/pH electrode was calibrated using pH standard buffer solutions with pH values of 4.0, 7.0 and 10.0 (BDH, Inc., Toronto, ON).

Micropipetting was carried out using both Eppendorf pipettes (Germany) and Nichiryo Model 5000 pipettes (Japan). An Eppendorf Repeater® pipette with Eppendorf Combitips® was used for repetitive pipetting.

Centrifugations required for cell culture procedures were performed using a desktop Safety-Head Centrifuge (Canlab, Mississauga, ON). Microcentrifugations were carried out using the Eppendorf Model 5415C microcentrifuge from Baxter/Canlab Inc. (Mississauga, ON).

Other laboratory equipment included the Haake Model W19 water tank with a Haake D1 heater and thermostat (Haake, Germany), the Model EAW II microtitre plate washer from SLT Lab Instruments (Austria), the Amerlite Shaker/Incubator (Amersham
Canada Ltd., Oakville, ON), a portable Model UVG-II Shortwave UV (254 nm) Mineralight® Lamp, the Genie 2 Vortex from Fischer Scientific Canada Ltd. (Toronto, ON), and a Corning Stirrer/Hotplate (Corning, NY).

3.3 Methods

3.3.1 Cell Culture and RNA Isolation

The LNCaP (PSA mRNA positive) and HL-60 (PSA mRNA negative) cell lines were cultured as described in Section 2.3.1. Total RNA was isolated from 10 million cells (LNCaP or HL-60) using the Trizol® LS reagent as described in Section 2.3.2. The ethanol-washed RNA pellet was air-dried and redissolved in 20 μL of DEPC treated water. A 5-μL aliquot of the RNA solution was used for the spectrophotometric determination of RNA, at 260 nm, and the remaining solution was brought to 500 μL using 2 g/L yeast tRNA dissolved in DEPC-treated water and stored at -20 °C until needed.

3.3.2 Reverse Transcription

The PSA and actin cDNAs were synthesized simultaneously (in a single reaction tube) by including both, the PSA and actin, downstream primers in the reverse transcription reaction. For each sample, a 12-μL aliquot containing 10 pmol DP primer, 10 pmol DA primer and total RNA from one million cells (1.3-1.5 μg), was heated to 70 °C for 10 min and cooled on ice. To each solution were then added 8 μL of reverse transcription mixture (125 mmol/L Tris-HCl, pH 8.3, 188 mmol/L KCl, 7.5 mmol/L MgCl₂, 25 mmol/L DTT, 2.5 mmol/L of each dNTP, and 200 units M-MLV reverse transcriptase). After a 1-h incubation at 37 °C, the reverse transcriptase was inactivated by heating the solution to 95 °C for 4 min. The synthesized cDNA was stored at -20 °C until use. A reverse transcription negative, which contained all the reagents except for the RNA, was included in every series of reactions performed.
3.3.3 Amplification of PSA and Actin cDNA by PCR

Separate polymerase chain reactions for PSA and actin cDNA were performed. The PCR mixtures consisted of 50 mmol/L KCl, 10 mmol/L Tris, pH 9.0, 0.1% (v/v) Triton X-100, 2.5 mmol/L MgCl$_2$, 20 μmol/L of each dNTP, 1 μmol/L DIG-dUTP and 2.5 units of DNA polymerase, in a total volume of 100 μL. A 16.5-μL aliquot of the reverse transcription mixture was used for PSA amplification. For the amplification of actin cDNA, the mixture was first diluted 500-fold in 1X PCR buffer (50 mmol/L KCl, 10 mmol/L Tris, pH 9.0, and 0.1% Triton X-100), and then 2 μL of this solution were pipetted into the PCR tube. Each PCR mixture was then layered with 80 μL of mineral oil. The "hot-start" protocol (76) was used, in which the PCR mixtures were heated for 5 min to 95 °C, followed by the addition of 50 pmol upstream primer and 40 pmol downstream primer. The PCR mixtures were subjected to 25 cycles of PCR, each cycle consisting of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. After completion of the cycles, the PCR mixtures were held at 72 °C for 10 min and then cooled to 4 °C. In order to test for contamination, a PCR negative (consisting of all PCR reagents except for the cDNA) was included in each PCR run.

3.3.4 Quantitation of Target DNA by Densitometry

Stock concentrations of target PSA and actin DNA solutions were determined by electrophoresing aliquots of each solution along with pUC18 DNA Hae III digest on a 2% (w/v) agarose gel containing ethidium bromide (as described in Section 2.3.5). The gel (while under UV illumination) was photographed using Polaroid 665 film. The negative produced was extensively rinsed with distilled water, dried and then scanned with the imaging densitometer. The masses of target PSA and actin DNA were calculated from the calibration curve constructed using the known quantities of pUC18 DNA fragments.
3.3.5 Microtitre Well Hybridization Assays for the Amplified PSA and Actin DNA

3.3.5.1 Reagents

*Phosphate-Buffered Saline (PBS).* Consisted of 0.14 mol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na$_2$HPO$_4$ and 1.76 mmol/L KH$_2$PO$_4$, pH 7.4.

*Wash Solution.* Consisted of 50 mmol/L Tris, pH 7.5, 0.15 mol/L NaCl and 0.1% (v/v) Tween-20.

*Blocking Solution.* Contained 1% (w/v) blocking reagent in 0.1 mol/L maleic acid and 0.15 mol/L NaCl, pH 7.5.

*ALP Buffer.* Consisted of 0.1 mol/L Tris, pH 9.05, 0.1 mol/L NaCl and 1 mmol/L MgCl$_2$.

*Substrate Stock Solution.* A 0.01 mol/L stock solution of FSAP was prepared by dissolving 11.8 mg of the compound in 5 mL of 0.1 mol/L NaOH and kept at 4°C. A 1 mmol/L FSAP working solution was prepared just prior to use by diluting the FSAP stock solution 10-fold in ALP buffer.

*Developing Solution.* Contained 1 mol/L Tris, 0.4 mol/L NaOH, 3 mmol/L EDTA and 2 mmol/L TbCl$_3$.

3.3.5.2 Procedure

The PSA and actin probes were first tailed with biotin-14-dATP using terminal transferase and purified by size-exclusion chromatography using NAP-5 columns (as per Section 2.3).

Microtiter wells were coated overnight at room temperature with 100 µL of a 1.4 mg/L solution of streptavidin in PBS. Prior to use, the wells were washed three times with wash solution. Biotinylated probes specific for PSA or actin were then immobilized on the wells by incubating 100 µL of 33.3 nmol/L probe (diluted in blocking solution) for 45 min at room temperature. The unbound probe was removed by washing the wells three times.
The PCR products were denatured by heating to 95 °C for 10 min, and immediately cooled on ice. For PSA, 20 μL of PCR mixture were pipetted into each well containing 80 μL of blocking solution that was preheated at 42 °C. For actin, 5 μL of PCR mixture were pipetted into each well containing 95 μL of blocking solution at 42 °C. The hybridization reactions were carried out at 42 °C for 45 min, with shaking. The wells were then washed, as above, and 100 μL of 750 U/L anti-DIG-ALP (diluted in blocking solution) were pipetted into each well. After a 30-min incubation period, the wells were washed and 100 μL of substrate solution were added into each well. The enzymatic reaction was allowed to proceed for 30 min at room temperature, at the end of which, were added 100 μL of developing solution. Following a 1-min shaking, the fluorescence was measured with the CyberFluor 615 time-resolved fluorometer. A summary of the hybridization assay procedures is presented in Figure 3.1.

3.4 Results and Discussion

Amplification of the PSA and β-actin mRNA using the primers designed in this work yields a 233 bp and a 372 bp product, respectively. The following equations describe the exponential amplification of PSA and actin cDNA.

\[ P_1 = (1 + E_1)^n T_1 \quad [1] \quad \text{and} \quad P_2 = (1 + E_2)^n T_2 \quad [2] \]

where 1 and 2 represent PSA and actin, respectively, T1 and T2 represent the quantities of target DNA prior to amplification, E1 and E2 are the amplification efficiencies and P1, P2 represent the amounts of accumulated products after n cycles. A small fraction of each amplification product is used in the corresponding hybridization assay. This is equal to 0.2P1 for PSA and 0.05P2 for actin. The time-resolved fluorescence values F1 and F2 are linearly related to the amount of PSA and actin DNA (equations 3 and 4, respectively).

\[ F_1 = 0.2s_1 P_1 \quad [3] \quad \text{and} \quad F_2 = 0.05s_2 P_2 \quad [4] \]

where s1 and s2 represent the slopes.
FIGURE 3.1

Schematic Representation of the PSA and Actin Hybridization Assays

Legend

The PSA and actin PCR products are analyzed, separately, using a PSA hybridization assay and an actin hybridization assay. Biotinylated probes specific for PSA or actin are immobilized on separate series of wells coated with streptavidin. Denatured PSA and actin PCR products are incubated with their respective probes. Hybrids formed are then detected via an alkaline phosphatase (ALP)-labelled anti-digoxigenin antibody. ALP cleaves the substrate fluorosalicylphosphate (FSAP) releasing fluorosalicylate (FSA) which forms fluorescent complexes with Tb$^{3+}$-EDTA. The fluorescence is measured using the CyberFluor 615 time-resolved fluorometer, with excitation and emission wavelengths set at 337.1 nm and 615 nm, respectively.
FIGURE 3.1

Add biotin-tailed PSA junction probe in streptavidin-coated well
Incubate 45 min at RT
Wash

Add 20 μL of denatured PSA PCR product (233 bp, DIG-labelled)
Incubate for 45 min at 42 °C
Wash.

Add anti-DIG-ALP
Incubate for 30 min at RT
Wash.

Add FSAP
Incubate for 30 min at RT
Wash

Add developing solution (Tb³⁺-EDTA)
Mix for 1 min
Measure fluorescence using the CyberFluor 615
λex 337.1 nm and λem 615 nm
By substituting the P₁ and P₂ from eqs. 1 and 2 into eqs. 3 and 4 and then dividing equation 3 by equation 4 we have:

$$\frac{F_1}{F_2} = \frac{4s_1(1 + E_1)^n}{s_2(1 + E_2)^n} \frac{T_1}{T_2} \quad [5]$$

Equation 5 shows that the ratio of the fluorescence values is linearly related to the ratio of the target cDNA molecules prior to amplification. In order for this relationship to be valid, however, there are two pertinent requirements: (a) the amount of PSA and actin amplification product analyzed must be in the linear range of the calibration curve of the respective hybridization assay and (b) the PCR for both PSA and actin must be carried out in the exponential phase of amplification where equations 1 and 2 are valid. In this phase, the product is linearly related to the amount of target in the mixture prior to amplification.

In order to establish the sensitivity and the linear range of each hybridization assay, DIG-labelled amplification products from several PCRs of the PSA and actin cDNA were pooled separately. The concentration of each pool was determined by scanning densitometry as described in Section 3.3.4. Various dilutions of each pool were then prepared, using the blocking solution as a diluent, and aliquots (20 μL per well) were analyzed in duplicate by hybridization. In Figure 3.2 the fluorescence, corrected for the background, is plotted against the concentration of amplified PSA and actin DNA. The background represents the fluorescence reading in the absence of amplification product, and is a measure of the nonspecific binding of the anti-digoxigenin antibody to the solid phase. The linear ranges for both hybridization assays extend from 1.4 to 110 pmol/L (0.14 to 11 fmol/well). The fluorescence value at the upper limit of the linear range is about 150,000. The curvatures observed at higher concentrations are due to substrate depletion. The signal-to-background ratios at the level of 1.4 pmol/L are 2.6 and 3.3 for the PSA and actin hybridization assays, respectively. Because the number of DIG-dUTP molecules incorporated into each amplified DNA fragment is generally proportional to its
FIGURE 3.2
Linear Ranges of the PSA and Actin Hybridization Assays

Legend

Serial dilutions of the PSA and actin DNA stock solutions (prepared in blocking solution) were analyzed in duplicate by their respective hybridization assay as described in Section 3.3.5.2. The fluorescence (corrected for the background) is plotted against the concentration of amplified DNA in the assay mixture. The background is the fluorescence obtained when no amplified DNA is added to the well. The solid and dashed lines represent the PSA and actin hybridization assays, respectively.
size, it was expected that more haptens would be incorporated into the actin amplified DNA (372 bp) than in the PSA DNA (233 bp) and thus a higher signal would be obtained from the actin hybridization assay. However, the signals observed are practically identical. This may be attributed to the fact that the same number of antibody molecules are bound to each hybrid due to steric hindrance.

In order to assess the effect of the amount of target DNA on the signal observed we amplified aliquots containing 330 to 210,000 target DNA molecules. For PSA, the plasmid pA75 containing a 1.4 kb PSA cDNA insert (73) was used as a target DNA. For actin, we prepared serial dilutions of the 372 bp amplification product and used them as target DNA in this study. In Figure 3.3, the fluorescence is plotted against the number of target DNA molecules in the mixture prior to amplification. Signal-to-background ratios of 5 and 14 are obtained with 330 PSA and actin DNA molecules, respectively.

Equations 1 and 2 give the relation between the target DNA and the accumulated amplification product. Taking the logarithm of both equations yields:

\[ \log P_i = n \log(1 + E_i) + \log T_i \quad \text{where } i=1,2 \quad [6] \]

i.e., the \( \log P_i \) is linearly related to \( \log T_i \) and the constant term of equation 6 is a function of the amplification efficiency. From the signals obtained at various target DNA levels and the calibration curve for each hybridization assay, we determined the amount of product (P) generated from each amplification of PSA and actin DNA (T). When the signal obtained was out of the linear range of the hybridization assay, the PCR product was prediluted (10 to 20 fold) prior to analysis. The \( \log P \) was then plotted against the \( \log T \). The results are presented in Figure 3.4. The linearity of the graph extends up to 200,000 and 100,000 PSA and actin target DNA molecules, respectively. Using equation 6, we determined the amplification efficiencies to be 67% and 85% for PSA and actin, respectively.

The sensitivity and linearity of the overall quantitative assay for PSA mRNA (including RT-PCR coupled to time-resolved fluorometry) was tested by preparing
FIGURE 3.3
Sensitivity of the PSA and Actin Hybridization Assays
Coupled to PCR

Legend

The sensitivity of the PSA and actin hybridization assays coupled to their respective PCRs were studied by preparing serial dilutions of the PSA and actin cDNAs. The fluorescence (corrected for the background) is plotted against the number of target DNA molecules originally present in the PCR mixtures prior to amplification. The background is defined as the signal obtained from a PCR mixture containing no target DNA. The solid and dashed lines represent the PSA and actin assays, respectively.
FIGURE 3.4
The Exponential Ranges of PSA and Actin DNA Amplification

Legend

The effect of the number of target DNA molecules (prior to amplification) on the amount of generated PCR product was studied. The amount of amplified DNA in the PCR mixture is plotted against the number of target DNA molecules present in the PCR mixture prior to amplification. The solid and dashed lines represent the PSA and actin amplification, respectively.
mixtures containing total RNA corresponding to 0, 20, 100, 300, 1000, 3000 and 10,000 PSA-expressing cells (LNCaP cells) in the presence of RNA from one million cells that do not express PSA (HL-60 cells). Following reverse transcription, aliquots of the synthesized cDNA were used directly for PSA PCR. The reverse transcription mixture, however, was diluted appropriately before amplification of the actin cDNA. This ensured that amplification of actin cDNA was well within the exponential phase. Subsequently, aliquots of each PCR product were analyzed by hybridization to PSA and actin specific probes. The ratio, F1/F2, of the fluorescence values obtained for PSA and actin was plotted against the number of LNCaP cells. The linear range of the proposed assay extends from 20 to 3000 LNCaP cells (see Figure 3.5). The sensitivity and range of the proposed assay may further be varied at will by simply changing the number of PCR cycles n and/or the volume of the PCR mixture used in the hybridization assay. In all cases, however, two requirements must be fulfilled. The PCR should not enter in the plateau phase and the amount of PCR product used in each hybridization assay should fall in the linear range of the assay. It was found that using 25 PCR cycles and assaying 20 µL of the PSA PCR products gave enough sensitivity to allow determination of low numbers of PSA-expressing cells in a range extending over two orders of magnitude. For actin, only 5 µL of PCR product was used for analysis, so that the signal remains within the linear range of the hybridization assay.

The overall reproducibility of the proposed system was studied by preparing three pools containing total RNA from LNCaP cells in the presence of one million HL-60 cells and analyzing them four times over a period of four days. Separate runs of reverse transcription reactions, PCR and hybridization assays were performed each time. From the F1/F2 ratio for each sample and the graph of Figure 3.5, it was determined that the pools contained 152, 307 and 1604 cells with %CVs of 22.7, 16.7 and 17.7, respectively. The %CVs of the F1/F2 ratios were 14.7, 11.8 and 12.2, respectively.
FIGURE 3.5
The Sensitivity and Linearity of the Overall Quantitative RT-PCR Method

Legend

The sensitivity and linearity of the overall quantitative assay for PSA mRNA (including RT-PCR coupled to time-resolved fluorometry) was tested by preparing mixtures containing total RNA corresponding to 0, 20, 100, 300, 1000, 3000 and 10,000 PSA-expressing cells (LNCaP cells) in the presence of RNA from one million cells that do not express PSA (HL-60 cells). Following reverse transcription and PCR (as per Section 3.3), aliquots of each PCR product were analyzed by hybridization to PSA and actin specific probes. The ratio, $F_1/F_2$, of the fluorescence values obtained from the hybridization assays of the amplified PSA and actin mRNA, is plotted against the number of LNCaP cells used in the initial RNA mixtures.
FIGURE 3.5

Number of LNCaP cells vs. $F_1/F_2$.
3.5 Conclusions

The necessity for a quantitative approach to the RT-PCR of PSA mRNA has already been addressed (86). Recent studies by Katz et al. (81) show that the rates of prostate cancer relapse were significantly higher in patients who were RT-PCR positive prior to prostatectomy than those who were negative. We expect that the proposed quantitative PCR assay will aid these studies and provide much more information by correlating the number of circulating PSA-expressing cells in the postoperative period with the progress of the disease, the time of relapse and the survival time of the patient.

Prostate cancer patients with metastatic disease are currently treated by androgen deprivation. Serum PSA is measured in these patients as an indicator of the success of therapy. However, Ghossein et al. (82) using RT-PCR for PSA mRNA have detected circulating prostatic cells in patients who were undergoing hormone therapy and had undetectable PSA. Monitoring of the relative amount of PSA mRNA after a therapeutic intervention by quantitative PCR may be a better index of the efficacy of therapy than PSA, by directly providing the rate of elimination of the circulating prostatic cancer cells.

In addition to its significance in the analysis of PSA mRNA, the present work contributes to the quantitative PCR methodology in general. Quantification of a target mRNA by amplifying, in parallel, the mRNA of a housekeeping gene and then relating the amounts of the PCR products has been already reported (87, 88). However, in these approaches the analysis of PCR products is accomplished mainly by gel electrophoresis ethidium bromide staining and scanning densitometry. Alternatively, radiolabelled primers or nucleotides are incorporated in the amplification product and, after electrophoretic separation, the band is excised from the gel and the radioactivity is measured in the liquid scintillation counter. Recently, an improved nonradioactive methodology was proposed that involves a direct analysis of amplified sequences from target mRNA and the housekeeping gene by HPLC using an anion-exchange column and spectrophotometric
detection (88). The system proposed here is advantageous over HPLC in that it detects the PCR products by hybridization, thus confirming the sequence of the product. As a consequence, nonspecific amplification products with sizes similar to the desirable sequence (such as a smear around the band of interest) may cause errors in HPLC analysis but are not detected by hybridization. Moreover, the proposed hybridization assays are performed in microtiter wells with assay configurations that greatly enhance the practicality and facilitate automation of the system.
CHAPTER 4

BIOLUMINESCENCE HYBRIDIZATION ASSAYS USING
RECOMBINANT AEQUORIN. APPLICATION TO THE DETECTION OF
PROSTATE-SPECIFIC ANTIGEN mRNA

4.1 Introduction

Aequorin is a photoprotein present in the hydromedusan *Aequorea victoria* (jellyfish). It is a complex composed of apoaequorin (a single polypeptide chain of 189 amino acids), the cofactor coelenterazine and molecular oxygen. Apoaequorin has three Ca\(^{2+}\)-binding sites. Upon Ca\(^{2+}\) binding to aequorin, a luminescent reaction is triggered which yields apoaequorin, coelenteramide, CO\(_2\) and light at 469 nm with a quantum yield of 15% (63, 89-91).

Aequorin has been used widely as a reagent for the specific and sensitive monitoring of intracellular Ca\(^{2+}\) concentrations by microinjecting the protein into the cells. In more recent studies the DNA encoding apoaequorin is introduced into the cell instead of the protein (62, 92). The DNA is expressed in the cell and the apoaequorin produced binds to coelenterazine (which permeates the cell membrane easily) to form a fully functional aequorin. The advantage of this approach is that a Ca\(^{2+}\)-specific bioluminescent reagent is generated intracellularly without disrupting the cell membrane.

Another direction in the development of aequorin-based analytical methodology involves the use of aequorin as a label that can be determined with high sensitivity (down to 1-2 amoles) in the presence of excess Ca\(^{2+}\). The preparation of recombinant aequorin (60, 93-95) has greatly facilitated research in this direction. The detection of proteins or nucleic acids on Western or Southern blots was also reported by using biotinylated specific antibodies or probes (59). Moreover, a bioluminescence, competitive, homogeneous assay for biotin was developed using biotinylated recombinant aequorin (96). Recently, conjugates of aequorin with monoclonal or polyclonal antibodies have been used in the
development of noncompetitive, 'two-site' immunoassays for peptide hormones (97, 98). Furthermore, the gene for apoaequorin has been fused with the IgG heavy chain gene (99) or with the gene for protein A (100) to produce (after expression) fusion molecules that retain the functions of the two proteins. These engineered reagents may be promising in the field of immunoassays.

In the present work, we designed and studied microtitre well-based hybridization assays using recombinant aequorin as a reporter molecule. The target DNA is hybridized, simultaneously, with two oligonucleotide probes. One probe is immobilized in microtitre wells and the other is biotinylated. Three approaches for determination of the hybrids were studied: (a) reaction with streptavidin that is covalently attached to aequorin; (b) sequential reaction of the hybrids with streptavidin and biotinylated aequorin and (c) a one-step reaction of the hybrids with preformed complexes of biotinylated aequorin and streptavidin. The proposed assays were applied to the determination of prostate-specific antigen (PSA) mRNA amplified by the polymerase chain reaction (PCR). The sensitivity and specificity of the combined procedure (PCR coupled to the bioluminescence hybridization assay) was studied by detecting mRNA from a few PSA-expressing cells in the presence of a large excess of PSA-negative cells.

4.2 Materials

4.2.1 Chemicals and Biochemicals

The human metastatic prostate adenocarcinoma (LNCaP) and the human promyelocytic leukemia (HL-60) cell lines (see Section 2.2.1) were obtained from the ATCC (Rockville, MD). RPMI 1640 with L-glutamine, fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B (Fungizone) and trypan blue were purchased from Gibco Laboratories Life Technologies, Inc. (Gaithersburg, MD). The Trizol® LS Reagent, Moloney murine leukemia virus reverse transcriptase (M-MLV RT), DTT and biotin-14-dATP were also from Gibco.
Ultrapure 2’-deoxyribonucleoside 5’-triphosphates (dNTPs) were purchased from Pharmacia Biotech (Montreal, PQ).

Blocking reagent, polyclonal (sheep) anti-digoxigenin antibody, digoxigenin-11-2’-deoxy-uridine-5’-triphosphate (DIG-dUTP), terminal deoxy-nucleotidyl transferase (TdT), alkaline phosphatase-labelled sheep polyclonal anti-digoxigenin antibody, Fab fragments (anti-DIG-ALP), and bovine serum albumin (BSA) were obtained from Boehringer Mannheim Biochemica (Laval, PQ).

Calcium chloride, pUC18 DNA Hae III digest (0.675 g/L), ethidium bromide, streptavidin, EDTA, EGTA, DMSO, DEPC, mineral oil, Tween-20 and Polaroid 665 film were purchased from Sigma (St. Louis, MO).

The streptavidin and biotin conjugates of recombinant aequorin were obtained from Molecular Probes, Inc. (Eugene, OR) in lyophilized forms.

Four oligonucleotides, specific for the PSA mRNA, were used in this work as PCR primers and probes (all synthesized by DNAgency, Aston, PA): (a) 5’-CTCTCGTGCGACGGCAGTCT-3’, a 20-mer homologous to exon II of the PSA gene, which serves as the upstream primer for PCR; (b) 5’-GTGCTTTTGGCCCGGCTGATCCA-3’, a 20-mer complementary to exon IV of the PSA gene, which serves as the downstream PCR primer; (c) 5’-ATCACGCTTTTGGTTCTGATGCAG-3’, a 24-mer complementary to the exon II/exon III junction in the PSA mRNA, which is used as a capture probe and (d) 5’-GGTCGTTGGCTGGAGTCATCA-3’, a 20-mer complementary to exon III of the PSA gene and used as a detection probe. The relative positions of the oligonucleotides on the PSA mRNA are shown in Figure 4.1.

Agarose was from ICN Biomedicals, Inc. (Costa Mesa, CA).

The recombinant PSA cDNA-containing plasmid pA75 (described in Section 2.2.2) was a gift from Dr. J. Trapman, M.D., Anderson Cancer Center, Houston, Texas.

Sodium chloride, potassium chloride, disodium hydrogen phosphate, sodium
FIGURE 4.1
Location of the Designed Primers and Probes on PSA mRNA

Legend

Schematic representation of prostate-specific antigen (PSA) mRNA and the relative positions of the primers and probes used in this work. The roman numerals correspond to the exons of the PSA mRNA. Oligonucleotides a and b are the upstream and downstream PCR primers, respectively. Oligonucleotides c and d are the capture and the detection probes, respectively.
dihydrogen phosphate, potassium dihydrogen orthophosphate, sodium hydroxide, hydrochloric acid, Tris, maleic acid, chloroform, isopropyl alcohol, ethanol, glacial acetic acid and glycerol were all from BDH, Inc. (Toronto, ON).

4.2.2 Supplies

Polystyrene cell culture flasks (25 cm²) and polypropylene cryogenic vials (2.0 mL) were purchased from Corning (Corning, NY).

Sterile, disposable 10-mL graduated, polystyrene, serological pipettes, as well as 3- and 10-cc syringes were from Falcon® Becton and Dickinson Labware (Lincoln Park, NJ).

Nalgene™ sterile 25-mm syringe filters (0.2 μm pore size) and 6-inch glass pasteur pipettes were from Baxter Diagnostics, Corp. (Toronto, ON).

Sterile conical polypropylene centrifuge tubes (15 and 50 mL) were from VWR (West Chester, PA). Disposable Universal Fit pipette tips (10-200 μL and 200-1000 μL) were also from VWR. Titertek Plus Microtips (0.5 μL-10 μL) were from ICN Biomedicals, Inc. (Costa Mesa, CA).

Eppendorf 1.5-mL microcentrifuge tubes were purchased from Brinkman Instruments (Westburg, NY).

Gene Amp reaction tubes for PCR were obtained from Perkin Elmer (Norwalk, CT).

Sephadex® G-25 gel filtration columns (NAP-5 and NAP-10) were from Pharmacia Biotech (Montreal, PQ).

Opaque, 12-well polystyrene Microlite™ 2 microwell strips and Removawell strip holders were obtained from Dynatech Laboratories, Inc. (Chantilly, VA).

4.2.3 Apparatus

Luminescence measurements were carried out on the Luminoskan RS microplate luminometer from Labsystems (Fisher Scientific, Toronto, ON).
The Perkin-Elmer Cetus (Norwalk, CT) 48-well DNA Thermal Cycler was used to carry out the polymerase chain reactions.

Agarose gel electrophoresis was performed using the Miniature Horizontal Gel System MLB-06 (Tyler Research Instruments, Edmonton, AB) and the Fotoforce 500™ Power Supply (Fotodyne, Inc., Bio-Can Scientific, Mississauga, ON). Photographs of the gels were taken using the Model MP4 Polaroid Camera System from Polaroid Corp. (Cambridge, MA).

The Bio-Rad Model GS-670 imaging densitometer (Bio-Rad Laboratories Ltd., Mississauga, ON) was used to quantitate DNA separated by electrophoresis.

The eight-well microtitre plate washer (Model EAW II) was from SLT-Lab Instruments (Austria).

The Amerlite shaker/incubator was from Amersham Canada Ltd. (Oakville, ON).

High-performance liquid chromatography (HPLC) was performed using the Shimadzu system (Shimadzu Corp., Kyoto, Japan) with the Bio-Sil Sec 400-5, 300 x 7.8 mm size exclusion column from Bio-Rad Laboratories Ltd. (Mississauga, ON).

The Nuaire Autoflow CO₂ Water-Jacketed Incubator and the Class II Type A/B3 laminar flow hood (Nuaire Biological Safety Cabinets, Plymouth, IL) were used for the culturing of cells. Cells were counted using the Neubauer Brightline hemocytometer from Hausser Scientific (American Scientific Products, McGaw Park, IL) and a Nikon TMS Microscope (Japan).

The Shimadzu UV-160 spectrophotometer (Shimadzu Corp., Japan) was used to take absorbance measurements of RNA solutions.

All pH measurements were made with the Corning pH Meter, Model 340 (Corning, NY). Prior to each measurement, the combination [Ag/AgCl] reference/pH electrode was calibrated using pH standard buffer solutions with pH values of 4.0, 7.0 and 10.0 (BDH, Inc., Toronto, ON).
Micropipetting was carried out using both Eppendorf pipettes (Germany) and Nichiryo Model 5000 pipettes (Japan). An Eppendorf Repeater® pipette with Eppendorf Combitips® were used for repetitive pipetting.

Centrifugations required for cell culture procedures were performed using a desktop Safety-Head Centrifuge (Canlab, Mississauga, ON). Microcentrifugations were carried out using the Eppendorf Model 5415C microcentrifuge from Baxter/Canlab Inc. (Mississauga, ON).

Other laboratory equipment included the Haake Model W19 water tank with a Haake D1 heater and thermostat (Haake, Germany), the portable Model UVG-II Shortwave UV (254 nm) Mineralight® Lamp, the Genie 2 Vortex from Fischer Scientific Canada Ltd. (Toronto, ON) and a Corning Stirrer/Hotplate (Coming, NY).

4.3 Methods

4.3.1 Aequorin Calibration Curve

4.3.1.1 Reagents

Buffer 1. Contained 10 mmol/L Tris (pH 7.5), 10 mmol/L EGTA, 1 mol/L KCl, 10 mmol/L MgCl₂, 1 g/L bovine serum albumin and 1 g/L NaN₃.

Buffer 3. Consisted of 0.15 mol/L NaCl, 2 mmol/L EDTA and 10 mmol/L Tris, pH 8.0.

4.3.1.2 Procedure

A stock solution of biotinylated aequorin was prepared by dissolving 25 µg of biotinylated aequorin (MW 22,000 Da) in 1 mL of buffer 1. Serial dilutions were made from this 1.14 x 10⁻⁶ mol/L biotinylated aequorin stock solution using buffer 1 as a diluent. To prepare the calibration curve, 100 µL of buffer 3 was combined with 50 µL of the varying concentrations of biotinylated aequorin in microtitre wells. The bioluminescence of the solutions were measured (for 3 s) using the Luminoskan microtitre luminometer after the injection of 50 µL of luminescence triggering solution.
4.3.2 Cell Culture and Isolation of Total RNA

Total RNA was isolated from both LNCaP and HL-60 cells. The procedures followed for the culturing of the cell lines and isolation of total RNA are described in Section 2.3.1.

4.3.3 Reverse Transcription

Reverse transcription reactions were carried out in a total volume of 20 μL containing 50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl$_2$, 10 mmol/L dithiothreitol, 1 mmol/L of each dNTP, 10 pmol of primer b, 200 U of M-MLV reverse transcriptase and total RNA from one million cells (1.3-1.5 μg). The mixture was incubated at 37 °C for 1 h after which the transcriptase was inactivated by heating at 95 °C for 4 min. The synthesized cDNA was stored at -20 °C.

4.3.4 Polymerase Chain Reaction

The PCR mixtures contained 50 mmol/L KCl, 10 mmol/L Tris (pH 9.0), 0.1 % Triton X-100, 2.5 mmol/L MgCl$_2$, 0.1 mmol/L of each dNTP, 5 μL of the cDNA and 2.5 U DNA polymerase. Each mixture was layered with 80 μL of mineral oil and PCR was initiated using the 'hot-start' protocol, in which the reaction mixtures were heated to 95 °C for 5 min. Then 50 pmol of primer a and 40 pmol of primer b were added to each tube. Each amplification cycle consisted of a denaturation step at 95 °C for 30s, primer annealing at 60 °C for 30s, and extension at 72 °C for 1 min. After completion of the cycles, the mixtures were kept at 72 °C for 10 min and then cooled to 4 °C.

4.3.5 Tailing of the Capture and Detection Probes

The capture probe c was enzymatically tailed with DIG-dUTP. The tailing reaction was performed in a total volume of 20 μL containing 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl (pH 6.6), 0.25 g/L bovine serum albumin, 5 mmol/L CoCl$_2$, 0.5 mmol/L dATP, 50 μmol/L DIG-dUTP, 25 U terminal transferase, and 100 pmol probe. After a 1-h incubation at 37 °C, the DIG-tailed probe was purified twice by size-exclusion
chromatography using NAP-5 columns (as described in Section 2.3.7). The final concentration of the probe was 60 nmol/L.

The detection probe d was tailed with biotin-14-dATP as described above, except that 50 µmol/L biotin-14-dATP was included in the reaction mixture instead of DIG-dUTP. Purification of the biotinylated probe was not necessary.

4.3.6 Microtitre Well-Based Hybridization Assays

4.3.6.1 Reagents

Carbonate Buffer. Consisted of 0.1 mol/L sodium hydrogen carbonate, pH 9.6.

Wash Solution. Consisted of 50 mmol/L Tris, 0.15 mol/L NaCl, 2 mmol/L EDTA and 0.05% (v/v) Tween-20, pH 7.5.

Maleic Acid Buffer. Consisted of 0.1 mol/L maleic acid and 0.15 mol/L sodium chloride, pH 7.5.

Diluent A. Contained 1% (w/v) blocking reagent in 0.1 mol/L maleic acid, 0.15 mol/L NaCl, 2 mmol/L EDTA, pH 7.5.

Diluent B. Consisted of 1% (w/v) blocking reagent in 60 mmol/L sodium citrate (pH 7.5), 0.6 mol/L NaCl and 2 mmol/L EDTA.

Diluent C. Contained 1% (w/v) blocking reagent in 2 mmol/L EDTA.

Luminescence-Triggering Solution. Contained 100 mmol/L CaCl2 in 100 mmol/L Tris-HCl, pH 7.5.

4.3.6.2 Procedures

4.3.6.2.1 Protocol A

Opaque, polystyrene microtitre wells were coated (overnight at room temperature) with 50 µL of 5 mg/L anti-digoxigenin antibody diluted in carbonate buffer. Prior to use, the wells were washed three times with wash solution. Then, 50 µL of 2 nmol/L DIG-tailed capture probe (diluted in diluent A) were added into each well. Following a 1-h incubation, the wells were washed three times. A 40-µL aliquot of a 2.5 nmol/L
biotinylated detection probe (diluted in diluent B and preheated to 42 °C) was pipetted into each well. The PCR products were denatured by heating to 95 °C for 10 min and cooled on ice. Subsequently, 10 μL of denatured PCR product were added to each well and allowed to hybridize with the two probes for 1 h at 42 °C. The wells were then washed as above and 50 μL of 10 U/L streptavidin-aequorin conjugate, diluted in diluent A, were added per well. Following a 30 min incubation, the wells were washed and 50 μL of luminescence-triggering solution were dispensed into each well. The luminescence was integrated over a 3 s period. A sample containing no PCR product (assay blank) was included in every assay in order to determine the background luminescence. All samples were analyzed in duplicate. A summary of this assay is presented in Figure 4.2.

4.3.6.2.2 Protocol B

The initial steps (including hybridization) for protocol B were as described under protocol A. However, following hybridization, the wells were washed three times and 50 μL of 1.4 mg/L streptavidin (diluted in diluent A) were pipetted into each well and allowed to bind to the hybrids for 25 min. The wells were then washed and 50 μL of 11 nmol/L biotinylated aequorin were added to each well. After a 25-min incubation and washing, the luminescence was measured as described in protocol A. A schematic representation of the procedure for this assay is given in Figure 4.3.

4.3.6.2.3 Protocol C

Complexes of streptavidin with biotinylated aequorin were prepared by mixing the two components at equimolar concentrations (55 nmol/L each, diluted in diluent A). Following a 30 min incubation at room temperature, the mixture was diluted 10 fold in diluent A and used directly in the hybridization assay. The procedure for protocol C was as described under protocol A up to the hybridization step. Following hybridization, the wells were washed and 50 μL of preformed streptavidin-biotinylated aequorin complex were added per well. After a 25-min incubation, the wells were washed and the luminescence
FIGURE 4.2
Bioluminescence Hybridization Assay for the Detection of PSA mRNA Using Streptavidin-Aequorin Conjugate
(Protocol A)

Legend

The PSA digoxigenin (D)-tailed junction probe is captured onto anti-digoxigenin antibody-coated microtitre wells. The PSA PCR product (495 bp) is allowed to hybridize to the bound PSA probe and, simultaneously, to a second PSA-specific, biotin (B)-tailed probe. The hybrids formed are detected using a streptavidin-aequorin (SA-AQ) conjugate that binds to biotin moieties of the second PSA probe. Immediately upon the addition of the luminescence-triggering solution, containing CaCl$_2$, the luminescence is measured for 3 s using a microtitre plate luminometer.
Coat microtitre wells with anti-DIG antibody
Incubate overnight at RT
Wash

Add DIG-tailed PSA probe
Incubate 1 h at RT
Wash

Add 10 µL PCR product (495 bp) and biotin-tailed PSA probe
Incubate 1 h at 42 °C
Wash

Add streptavidin-AQ conjugate
Incubate 30 min at RT
Wash

Add CaCl₂ luminescence-triggering solution
Immediately measure luminescence for 3 s
FIGURE 4.3

Bioluminescence Hybridization Assay for the Detection of PSA mRNA

Using Biotinylated Aequorin

(Protocol B)

Legend

The PSA PCR product is captured in a sandwich-type format between an immobilized digoxigenin (D)-tailed PSA probe and a biotin (B)-tailed PSA probe (as in protocol B). Following the binding of streptavidin to the biotin moieties of the detection PSA probe, biotinylated aequorin (B-AQ) is added to the wells. The CaCl$_2$ luminescence-triggering solution is then injected and the luminescence is immediately measured for 3 s using a microtitre plate luminometer.
FIGURE 4.3

Coat microtitre wells with anti-DIG antibody
Incubate overnight at RT
Wash

Add DIG-tailed PSA probe
Incubate 1 h at RT
Wash

Add 10 μL PCR product and biotin-tailed PSA probe
Incubate 1 h at 42 °C
Wash

Add 10-fold diluted streptavidin/biotinylated AQ preformed complexes
Incubate 25 min at RT
Wash

Add CaCl₂ luminescence-triggering solution
Immediately measure luminescence for 3 s
was measured as in protocol A. The assay procedure is summarized in Figure 4.4.

4.3.7 Analysis of Aequorin Complexes by Size Exclusion Chromatography

4.3.7.1 Reagent

HPLC Buffer (Mobile Phase). Consisted of 50 mmol/L Na$_2$HPO$_4$, 50 mmol/L NaH$_2$PO$_4$, 0.15 mol/L NaCl and 2 mmol/L EDTA, pH 6.8.

4.3.7.2 Procedures

4.3.7.2.1 Biotinylated Aequorin

A 50-µL aliquot of 27.5 nmol/L biotinylated aequorin (diluted in HPLC buffer) was injected onto the Bio-Sil Sec 400 size exclusion column. The flow rate was set at 0.5 mL/min and the UV detector at 280 nm. Upon injection of the sample 0.5 mL (1 min) fractions were collected. A 50-µL aliquot of each fraction was pipetted into empty, noncoated, microtitre wells and the luminescence was measured as described above.

4.3.7.2.2 Streptavidin-Aequorin Conjugates

With the HPLC conditions set as described for the biotinylated aequorin run, a 50-µL aliquot of 0.11 U/mL of the streptavidin-aequorin conjugate solution (diluted in HPLC buffer) was injected onto the size exclusion column. Again, 0.5-mL fractions were collected from which 50-µL aliquots were measured for luminescence.

4.3.7.2.3 Preformed Streptavidin/Biotinylated Aequorin Complexes

Such that proteins included in the blocking reagent would not interfere with the analysis of the preformed streptavidin/biotinylated aequorin complexes, HPLC buffer was used, in place of diluent A, in the formation of these complexes described in protocol C. Following the 30 min incubation, a 50-µL aliquot of this solution was directly injected onto the size exclusion column and analyzed as described in the HPLC runs above.
FIGURE 4.4

Bioluminescence Hybridization Assay for the Detection of PSA mRNA

Using Preformed Streptavidin/Biotinylated Aequorin Complexes

(Protocol C)

Legend

As in protocols A and B, the PSA PCR product is captured between an immobilized digoxigenin (D)-tailed PSA probe and a biotin-tailed PSA probe. Then preformed complexes of streptavidin and biotinylated aequorin (prepared as per Section 4.3.6.2.3) are used to detect the hybrids formed. The luminescence is measured (for 3 s) immediately upon the addition of CaCl$_2$. 
FIGURE 4.4

1. Coat microtiter wells with anti-DIG antibody
   Incubate overnight at RT
   Wash

2. Add DIG-tailed PSA probe
   Incubate 1 h at RT
   Wash

3. Add 10 μL PCR product and biotin-tailed PSA probe
   Incubate 1 h at 42 °C
   Wash

4. Add 10-fold diluted streptavidin/biotinylated AQ preformed complexes
   Incubate 25 min at RT
   Wash

5. Add CaCl₂ luminescence-triggering solution
   Immediately measure luminescence for 3 s

   Light
4.4 Results and Discussion

A calibration curve was constructed to determine the intensity of the luminescent signal over a range of aequorin concentrations, in our lab. The results of this study, presented in Figure 4.5, showed that the luminescence intensity produced by biotinylated aequorin was linear over a wide range of biotinylated aequorin concentrations (approximately 10 to 56,000 amol). In addition, 0.6 amol of biotinylated aequorin was detected with a signal-to-background ratio of 26, showing a very high sensitivity.

The amplification of PSA mRNA by reverse transcriptase-polymerase chain reaction, using primers a and b, generated a 495 bp DNA fragment. Optimization studies for hybridization assay protocols A-C were carried out by preparing pools of amplification products. The concentration of the specific, 495 bp, DNA fragment (target DNA) in each pool was determined as follows. A 20-μL aliquot was electrophoresed on a 2% agarose mini-gel followed by ethidium bromide staining. A lane containing the pUC18 DNA restriction fragments was also included for construction of a calibration curve. A picture of the stained gel was taken under UV excitation and the negative was produced. The target DNA was then determined by scanning densitometry. Solutions of target DNA at various concentrations were prepared by diluting the pool in diluent C.

We first optimized the concentration of streptavidin-aequorin conjugate used in assay protocol A. Various dilutions of the conjugate were prepared (in diluent A) in the range of 0.4 to 100 U/L and used to analyze target DNA at the level of 7.5 fmol/well. In Figure 4.6, the luminescence (corrected for the background) as well as the signal-to-background (S/B) ratio are plotted against the streptavidin-aequorin concentration. The background is defined as the luminescence obtained when no target DNA is present in the well and is a measure of the nonspecific binding of the biotinylated detection probe and the streptavidin-aequorin conjugate to the solid phase. We observe that the luminescence increases with the concentration of the conjugate up to 12 U/L and then a plateau is
FIGURE 4.5

Calibration Curve for Biotinylated Aequorin

Legend

The luminescence (solid line) and the signal-to-background ratio, S/B (dashed line) are plotted against the concentration of the biotinylated aequorin in the microtitre well (50 μL). The luminescence was measured over a period of 3 s immediately upon the injection of 50 μL of the luminescence triggering solution containing calcium chloride.
FIGURE 4.6

Study of the Streptavidin-Aequorin Conjugate Concentration

Legend

The effect of the concentration of streptavidin-aequorin conjugate used in hybridization assay protocol A was studied. The luminescence, corrected for background (solid line) and the signal-to-background ratio, S/B (dashed line) were plotted against the concentration of the conjugate used in the analysis of 7.5-fmol target DNA (as per Section 4.3.6.2.1). The background is defined as the luminescence obtained when no PCR product is present in the well and is a measure of the nonspecific binding of the biotinylated detection probe and the streptavidin-aequorin conjugate to the solid phase.
reached. However, a peak in S/B ratio occurs in the range of 3 to 12 U/L. At higher concentrations, the nonspecific binding of the conjugate increases and the S/B ratio drops.

The sensitivity and linearity of assay protocol A were established by analyzing serial dilutions of target DNA. In Figure 4.7A, the luminescence is plotted as a function of the concentration of target DNA. The assay is linear in the range of 0.1 to 200 pmol/L (5 amol-10 fmol per well). The S/B ratio at the level of 5 amol/well is 5.3.

The assay protocol B involves determination of the hybrids by sequential reactions first with an excess of streptavidin and then with biotinylated aequorin. A 1.4 mg/L streptavidin solution was chosen because it corresponds to a 10-fold molar excess compared to the highest amount of target DNA used for analysis. The effect of biotinylated aequorin concentration was studied in the range of 3.4 to 110 nmol/L. The luminescence reached a plateau at 13 nmol/L. At higher concentrations the S/B ratio dropped due to an increased nonspecific binding of the biotinylated aequorin to the solid phase. Data pertaining to the sensitivity and linearity of the optimized protocol B are presented in Figure 4.7B. The linearity extends from 1 to 200 pmol/L and the S/B ratio of 3.0 was obtained at the level of 137 amol/well.

In protocol C, streptavidin was first complexed to biotinylated aequorin and then the complexes were allowed to bind to the hybrids. The streptavidin to biotinylated aequorin ratio required for maximum signal was optimized. Solutions containing a constant concentration of biotinylated aequorin (55 nmol/L) and various concentrations of streptavidin, ranging from 27 to 550 nmol/L, were prepared and incubated for 30 min at room temperature. The complexes were then used directly in the assay of 7.5-fmol target DNA. The results are presented in Figure 4.8. The luminescence reaches a maximum at 55 nmol/L streptavidin, which corresponds to a streptavidin/biotinylated aequorin molar ratio of 1. The signal drops dramatically at lower or higher concentrations of streptavidin. In the case of excess biotinylated aequorin, all the biotin binding sites on streptavidin are
FIGURE 4.7

Sensitivity and Linearity of the Bioluminescence Hybridization Assays

Legend

The sensitivity and linearity of the hybridization assays were determined and compared by plotting the measured corrected luminescence against the number of femtomoles of target DNA applied per well (50 µL). For each assay protocol, the background corresponds to samples containing no PCR product. The lines A, B and C correspond to hybridization assay protocols A, B and C, respectively. The assays were performed as described in Section 4.3.6.2.
FIGURE 4.8
Optimization of the Streptavidin and Biotinylated Aequorin Concentrations for the Formation of Complexes

Legend

The streptavidin to biotinylated aequorin molar ratio required for preparation of preformed complexes was optimized. Preformed complexes were prepared by incubating 55 nmol/L biotinylated aequorin with various concentrations of streptavidin. The complexes were then used to analyze 7.5 fmol target DNA, as per Section 4.3.6.2.3. The resultant luminescence was plotted against the concentration of streptavidin that was mixed with 55 nmol/L biotinylated aequorin for the formation of the complexes.
FIGURE 4.8

[Graph showing luminescence (RLU) against Streptavidin (nmol/L).]
occupied and the complexes cannot bind to the hybrids. On the other hand, when streptavidin is in excess, uncomplexed streptavidin competes with the streptavidin-aequorin complexes for binding to the hybrids.

The formation of streptavidin biotinylated aequorin complexes was further studied by HPLC. Solutions containing biotinylated aequorin or aequorin covalently attached to streptavidin or preformed complexes of streptavidin with biotinylated aequorin were analyzed by HPLC using a size exclusion column. Fractions (0.5 mL) were collected and 50 μL aliquots were mixed with luminescence-triggering solution and the luminescence was measured. The results are presented in Figure 4.9. A considerable shift to higher molecular weights is observed when biotinylated aequorin is mixed with streptavidin, which proves the formation of larger complexes. The molecular weights range from 80,000 - 800,000 Da. Streptavidin acts as a link between two or more biotinylated aequorin molecules, thus leading to the formation of complexes that contain multiple aequorin and streptavidin molecules and retain their ability to interact with the biotinylated detection probe. The data in Figure 4.9 also suggest that a heterogeneous population of complexes with various sizes is produced.

The concentration of preformed complexes required for the hybridization assay was optimized by preparing various dilutions of the streptavidin/biotinylated aequorin mixture in diluent A, just prior to its use in the assay. A 50-μL aliquot of each solution was then used in the analysis of target DNA. A sample containing no target DNA was also assayed in order to determine the background luminescence at each concentration of the complex. In Figure 4.10 the luminescence, as well as the S/B ratio are plotted against the concentration of the complexes. It is observed that there is a continuous increase in the signal as the complex concentration increases. The S/B ratio, however, reaches a maximum at 5.5 nmol/L and then it drops because of the higher nonspecific binding of the complexes to the solid phase.
FIGURE 4.9

Analysis of the Aequorin Conjugates by Size Exclusion Chromatography

Legend

The biotinylated aequorin (circles) and streptavidin-aequorin conjugates (diamonds), as well as the streptavidin/biotinylated aequorin complexes (squares) were analyzed by HPLC using a size-exclusion column. The mobile phase consisted of 50 mmol/L Na₂HPO₄, 50 mmol/L NaH₂PO₄, 0.15 mol/L NaCl and 2 mmol/L EDTA, pH 6.8. The flow rate was set at 0.5 mL/min and 0.5 mL fractions were collected. The luminescence of a 50-μL aliquot from each fraction was then analyzed as described in Section 4.3.6. The left axis shows the data from the analysis of streptavidin/biotinylated aequorin. The right axis corresponds to the analysis of streptavidin-aequorin conjugates and biotinylated aequorin.
FIGURE 4.10

Study of the Dilution of Preformed Complex Solutions

Legend

The concentration of preformed complexes required for the hybridization assay was optimized by preparing various dilutions of the streptavidin/biotinylated aequorin equimolar mixture in diluent A, just prior to its use in the assay. These solutions were then used to analyze 7.5-fmol target DNA, as described in Section 4.3.6.2.3. The corrected luminescence (solid line) and the signal-to-background ratio, S/B (dashed line) obtained from the hybridization assay were plotted against the dilution factor. The background represents the signal obtained from a sample containing no PCR product.
The sensitivity and linearity of the optimized assay protocol C was studied by analyzing various amounts of target DNA. The results are presented in Figure 4.7C. The linearity extends from 0.1 to 100 pmol/L. The S/B ratio at the level of 5 amol/well is 2.5.

Comparison of protocols B and C (see Figure 4.7) shows that there is a 3-4 times improvement in the signal when preformed complexes are used instead of the sequential addition of streptavidin and biotinylated aequorin. On the other hand, the optimized protocols A and C (aequorin covalently attached to streptavidin versus preformed complexes) show equivalent assay performance in terms of signal and sensitivity. It should be noted that when we used undiluted preformed complexes, the signal was found 2.5 times higher than that obtained with the streptavidin-aequorin conjugate. However, the nonspecific binding of the complexes was also high and the S/B ratio dropped dramatically, even below the ratios obtained with protocol A. Finally, a 10-fold dilution of the complex caused a decrease in the signal but the S/B ratio improved. Consequently, the high nonspecific binding of the preformed complexes to the solid phase is the main sensitivity-limiting factor in protocol C. Nevertheless, an advantage of using preformed complexes is that they are prepared conveniently by simply mixing the two components, thus providing a practical alternative to the use of streptavidin conjugates prepared by covalent crosslinking techniques.

The sensitivity of the overall system, including PCR amplification coupled to the bioluminescence hybridization assay of the products, was assessed by preparing serial dilutions of the plasmid pA75 which contains the cDNA of PSA gene (73). Aliquots containing various numbers of plasmid molecules were then subjected to PCR (25 cycles). The amplification products were diluted 4 times in diluent B and 10-µL aliquots were analyzed by hybridization using protocol A. In Figure 4.11, the luminescence and the S/B ratio are plotted against the number of plasmid molecules present in the original mixture.
FIGURE 4.11

Sensitivity of the Bioluminescence Hybridization Assay Coupled to PCR

Legend

The sensitivity the bioluminescence hybridization assay (protocol A) coupled to PCR amplification was assessed by preparing serial dilutions of the plasmid pA75 which contains the cDNA of PSA gene. The corrected luminescence (solid line) and the signal-to-background ratio, S/B (dashed line) was plotted as a function of the number of PSA cDNA molecules present in the original PCR mixture (prior to amplification). The background represents the signal obtained from a PCR product which contained no target cDNA (the PCR negative).
prior to amplification. A S/B ratio of 29 was observed when 330 plasmid molecules were present.

In order to test the ability of the proposed method to detect a few PSA-expressing cells in the presence of a large excess of cells that do not express PSA, we prepared mixtures containing total RNA from various numbers of LNCaP cells in the presence of one million HL-60 cells. Following reverse transcription, the cDNA was used directly for PCR (30 cycles). Then 10 μL of each amplification product were analyzed by the hybridization assay (protocol A). In Figure 4.12, the luminescence is plotted against the number of LNCaP cells present in the original mixture. PSA mRNA from a single cell in the presence of one million HL-60 cells was detected with a S/B ratio of 2.5. The background here is the luminescence obtained from a sample containing RNA from one million HL-60 cells and no LNCaP cells present. The response is linear up to 10,000 LNCaP cells.

The reproducibility of the hybridization assay was tested by analyzing amplification products from 5, 100 and 3000 LNCaP cells in the presence of one million HL-60 cells. The %CVs were 5.3, 6.4 and 6.3, respectively (n=7). In addition, the reproducibility of the combined PCR and hybridization steps was studied. Seven separate amplifications (30 cycles) were set up using a sample containing 1000 LNCaP cells and one million HL-60 cells. Subsequently, a 10-μL aliquot from each PCR product was analyzed in duplicate by hybridization. The CV of the means of the duplicates was 3.7%.

4.5 Conclusions

Several methods have been proposed for the determination of PCR products. The classical approach involves gel electrophoresis followed by ethidium bromide staining. Confirmation of the products is then accomplished by Southern transfer and membrane hybridization. In more recent reports, various 'affinity molecules' or 'detection molecules' are incorporated in the amplification products by using modified deoxynucleotides and/or
FIGURE 4.12

Sensitivity and Linearity of the Overall RT-PCR Method

Legend

The ability of the proposed method (including RT-PCR and the hybridization assay) to detect a few PSA-expressing cells in the presence of a large excess of PSA negative cells was determined by preparing mixtures containing RNA from 1 to 10,000 LNCaP cells in the presence of one million HL-60 cells. These mixtures were subjected to reverse transcriptase-polymerase chain reaction and the products were analyzed by the proposed bioluminescence hybridization assay (protocol A). The corrected luminescence was plotted against the number of LNCaP cells present in the original PCR mixture. The background corresponds to the luminescence obtained from a sample representing one million HL-60 cells with no LNCaP cells.
primers labelled at the 5' end (101-103). Subsequently, the amplified DNA can be captured on a solid phase and then linked to a nonisotopic detection system. The assay configuration proposed here does not require labeling of the PCR products during amplification. The amplified DNA is determined by simultaneous hybridization with capture and detection probes. Thus, the applicability of the assay is not limited to PCR products but extends to any target DNA for which two specific oligonucleotide probes can be synthesized. The fact that the capture probe is immobilized to the solid phase through the digoxigenin/anti-digoxigenin interaction, enhances the practicality of the system because the same solid phase can be used for the assay of different target DNA molecules. Microtitre well hybridization is easily automatable and avoids long hybridization and washing steps associated with Southern blots. Furthermore, the detection of photoprotein aequorin is practically instantaneous, as opposed to the long substrate incubation periods required for detection of streptavidin conjugated to alkaline phosphatase or horseradish peroxidase.

In the last 3-4 years, PSA-mRNA has been used as a specific marker for prostate epithelial cells (67-71). It is believed that during the early stages of metastatic process, cancer cells penetrate the capsule and circulate in the blood stream prior to their deposition to the bones. Because metastatic prostate cancer calls for a therapeutic intervention that is different than that applied to organ-confined disease, the detection of circulating PSA-expressing cells becomes a unique test for correct staging of the disease (71, 81, 83). The present work describes a powerful analytical methodology that may facilitate clinical studies in this area.
Part II

FLUOROMETRIC AND TIME-RESOLVED IMMUNOFLUOROMETRIC
ASSAYS FOR PROTEIN-TYROSINE PHOSPHATASE AND
KINASE ACTIVITY
CHAPTER 5
GENERAL INTRODUCTION

5.1 Protein Phosphorylation

Protein phosphorylation is one of the most common post-translational modifications to occur in eukaryotic cells. This reversible modification is regulated by the actions of specific protein kinases and phosphatases. Protein kinases are enzymes that catalyze the transfer of a phosphate group from a nucleoside triphosphate (usually ATP) to an amino acid side chain of a substrate protein. Protein phosphatases reverse the action of protein kinases by hydrolyzing phosphoryl groups from phosphoproteins (104). Protein phosphorylation is recognized as a fundamental mechanism for the transduction of extracellular signals (105, 106). Many hormones, as well as growth factors and neurotransmitters, bind to specific membrane-spanning receptors on target cells, converting the dormant receptor to an active state. Subsequently, the receptor stimulates intracellular biochemical pathways leading to a diverse array of cellular responses including growth, proliferation and differentiation (104, 107, 108). Although these pathways are not clearly defined, in many cases the internal signals act on kinases or phosphatases thereby initiating a cascade of events which results in the amplification of the external signal.

To date several hundred protein kinases have been reported (109). Protein kinases are classified by the amino acid residue that they phosphorylate. The vast majority of phosphorylation events in eukaryotic cells occur on serine (Ser) and threonine (Thr) residues (by protein-Ser/Thr kinases) or tyrosine (Tyr) residues (by protein-Tyr kinases) (110). Although it was initially believed that kinases were specific either for Ser/Thr or for Tyr residues, it has been demonstrated that a few kinases utilize both types of side chains as substrates (111, 112). Many kinases also catalyze self-phosphorylation (autophosphorylation). As a result there can be multiple phosphoamino acids at different positions in one polypeptide, transferred onto the protein by one or more different kinases.
or by a combination of intermolecular and intramolecular (autophosphorylation) reactions (104). Phosphorylation groups at certain positions will control reactivity of kinases with other kinases, i.e., some kinases recognize phosphoproteins as substrates (113). These mechanisms allow separate extracellular signals to reinforce or negate one another by changing the phosphorylation state of a particular kinase in the transduction pathway. Cellular responses will depend on this type of combinatorial effect on kinases in the transduction pathways (104).

5.1.1 Protein-Tyrosine Kinases

Until 1980, only phosphoserine and phosphothreonine had been identified as naturally occurring phosphoamino acids. However, tyrosine phosphorylation began to receive greater attention when it was determined that pp60⁵-src, the transforming principle of the Rous sarcoma virus, had increased tyrosine kinase activity. Analysis of the phosphoamino acid content of a normal cell revealed that 0.01-0.05% was present as phosphotyrosine. Upon transformation by v-src, phosphotyrosine content increased to 1-3% (114). It is now known that approximately one third of known oncogenes encode for PTKs. Three of the best known oncogene-encoded PTKs associated with human cancers include; abl in chronic myelogenous leukemia, src in colon carcinomas, and neu in breast cancer (115, 116). Very little is known about the biochemical pathway by which protein products of oncogenes send signals from the cell surface to the nucleus. However, PTK activity has been implicated in the early steps of the cascade of events that occur during this signal transduction which leads to the transformation of normal cells to cancer cells. With the increasing numbers of identified tyrosine kinases, both intracellular and receptor-linked, it has become clear that tyrosine phosphorylation is an important regulator of cellular function (109, 117).
5.1.1.1 The Receptor PTKs

The receptor PTKs possess a large glycosylated extracellular ligand binding domain, a single hydrophobic transmembrane region, and a cytoplasmic domain that contains a highly conserved tyrosine kinase catalytic domain (118). A great deal of research has been performed to study the structure and function of the PTK domains. Ligand binding and the subsequent conformational alteration of the extracellular domain has been reported to induce receptor oligomerization, which stabilizes interactions between adjacent cytoplasmic domains and leads to activation of the kinase activity. The main function of the transmembrane domain is to anchor the receptor in the plane of the plasma membrane, thereby connecting the extracellular environment with internal compartments of the cell. The tyrosine kinase or catalytic domain, consisting of about 260 amino acids, is the most highly conserved portion of all receptor tyrosine kinase molecules. A glycine-rich region and followed by a lysine residue (approximately 15-20 residues to the carboxy-terminal side of this region) is found in this conserved portion of all PTKs (119). The Lys residue has been found to be essential for PTK activity. The carboxy-terminal tail sequences, which are among the most divergent between all known receptor tyrosine kinases, have been shown to possess several autophosphorylation sites. Examples of receptor PTKs include the receptors for the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (110, 118).

5.1.1.2 The Nonreceptor PTKs

The nonreceptor (or cytoplasmic) PTKs, for which the src and abl proteins are prototypes, represent a collection of cellular enzymes that are grouped together because of their lack of extracellular sequences (120). This group of PTKs range in size from around 50 kDa for the c-src kinase family to approximately 150 kDa for the Abl kinase family. The catalytic domain of nonreceptor PTKs shows homology with that of the receptor PTKs. And as with the receptor PTKs, this domain is the major area of sequence
homology between the nonreceptor PTKs and presumably the region of greatest shared structural identity. The catalytic domains for the nonreceptor PTKs are considered the SH1 (src homology 1) domains since this is the region between the kinases that shares the greatest sequence similarity with the c-src catalytic domain. A few of these kinases seem to have two distinct catalytic domains, although it appears that the PTK activity is a function of the most amino-terminal catalytic domain. The role of the second catalytic domain has not been determined (121).

Almost all nonreceptor PTKs also possess one or more SH2 domains and/or an SH3 domain amino-terminal to the catalytic domains (120). The SH2 domain is a sequence of approximately 100 amino acids, and is capable of high affinity binding to selected phosphotyrosine-containing proteins (108). It has been suggested that the SH2 domains regulate protein-protein interactions by recognizing peptide sequences that encompass tyrosine phosphorylation sites (122). The function of the SH3 domain (approximately 60 amino acids) is not clear, however it has been suggested that it may play a role in the subcellular localization of the PTK (123).

5.1.1.2.1 pp60\textsuperscript{c-src}

pp60\textsuperscript{c-src}, the normal cellular homologue of the retroviral oncogene product, pp60\textsuperscript{v-src}, was used as a model PTK for a portion of the work presented in this dissertation. pp60\textsuperscript{c-src} is a 60 kDa nonreceptor PTK associated with the cytoplasmic face of several cellular membranes including the plasma membrane (119). Although this PTK is found in the tissues of virtually all higher eukaryotes, pp60\textsuperscript{c-src} PTK activity is especially high in neurons, lymphocytes and platelets (124). In spite of numerous investigations, the physiological role of pp60\textsuperscript{c-src} remains unknown.

The size difference in pp60\textsuperscript{c-src} (composed of 533 amino acids) and pp60\textsuperscript{v-src} (526 amino acids) is primarily due to the replacement of the carboxy-terminal 19 amino acids of pp60\textsuperscript{c-src} by 12 completely different amino acids in pp60\textsuperscript{v-src} (125). Due to this
alteration in structure, the major phosphorylation sites of tyrosine residues of these two proteins are different: \( \text{pp60}^{\text{v-src}} \) is phosphorylated at Tyr\(^{416} \) while \( \text{pp60}^{\text{c-src}} \) is phosphorylated at Tyr\(^{527} \). Dephosphorylation at Tyr\(^{527} \) of the \( \text{pp60}^{\text{c-src}} \) was shown to result in the activation of its tyrosine kinase activity towards exogenous substrates. Furthermore, deletion of the carboxy-terminal tail of \( \text{pp60}^{\text{c-src}} \), including Tyr\(^{527} \) and substitution of Tyr\(^{527} \) with Phe both produce highly transforming variants of c-src (125). These findings suggested that tyrosine phosphorylation at this site might be an important mechanism for c-src repression, and illustrates that not only kinases but also PTPs are involved in bringing about increased phosphorylation of proteins. A complete understanding of the physiological role of tyrosine phosphorylation and its potential as a mechanism for the reversible modulation of protein function must thus encompass the characterization of the PTPs in addition to the PTKs.

5.1.2 Protein-Tyrosine Phosphatases

While tyrosine phosphorylation was being avidly pursued as a central theme of many cellular processes during the 1980s, studies into tyrosine dephosphorylation seemed to lag behind. The lack of an isolated phosphatase, and hence the absence of protein and nucleic acid sequences, limited such studies. It was not until 1988, when the partial amino acid sequence of a PTP (PTP 1B) was obtained, that tyrosine phosphatases began to attract wider attention (126). The sequence of this PTP had no similarity to known Ser/Thr phosphatases thus immediately defined a new family of phosphatases. Since the isolation of PTP 1B, more than 30 different PTPs have been isolated (122). The majority of the PTPs are derived from human or rodent sources, but the presence of PTP genes in insects and yeast indicate that these enzymes are probably ubiquitous among eukaryotes (127).

A comparison of the PTP and PTK families reveals an interesting similarity. The general structure of the two tyrosine enzymes parallel each other, in that there are both transmembrane receptor proteins and proteins that are totally intracellular. In addition, the
intracellular PTPs and PTKs also share at least one functional motif, namely the SH2 domain.

Most PTPs are multidomain proteins (composed of approximately 200-2000 amino acids) that contain at least one catalytic domain as well as additional unrelated sequences of variable size. The combination of catalytic domains with a wide variety of structural motifs accounts for the diversity among the PTPs. Nonreceptor isoforms possess noncatalytic sequences, such as SH2 domains, which confer distinct modes of regulation and target the PTPs to specific subcellular compartments. It has been presumed that the fusion of PTP domains with a transmembrane segment and a variety of extracellular domains has created receptor-like PTPs that each have the ability to respond to distinct ligands, however, very little is known about PTP substrate specificity in vivo (127).

Despite exceptional diversity in size and structural organization, a common evolutionary origin for the PTP family is demonstrated by the presence of at least one conserved segment of 240 amino acid residues (105, 128). This segment has been shown to contain the active site of these enzymes. Of particular interest is a segment of 11 residues located near the carboxy-terminus of the domain with the consensus sequence (I/V)HCXAGXXR(S/T)G, where X is an unconserved amino acid. The cysteine within this motif is found in all PTPs except the carboxy-terminal PTP domains of the receptor-like PTPs containing two PTP domains. In these second domains (closer to the carboxy-terminus) the cysteine is occasionally replaced by aspartate (127).

The functional significance of the two-domain arrangement that characterizes the receptor-like PTPs is not yet understood. It is known, however, that mutation of the conserved cysteine residue in the active site of the first domain completely eliminates the PTP activity toward a number of synthetic substrates (129). Conversely, the identical mutation in the second domain was reported to have no effect on PTP activity. Although the role of the second domain remains unclear, it has been proposed that it may regulate the
activity of the first domain in terms of substrate specificity, as well as have some catalytic activity (122).

Direct involvement of the cysteine residue in the catalytic mechanism of PTPs has been further supported by the absolute requirement of thiol-reducing agents (e.g., dithiothreitol and β-mercaptoethanol) for PTP activity. Based on these observations it has been proposed that a cysteine-phosphate intermediate is involved in the catalytic mechanism of PTPs (122).

5.1.2.1 T-Cell ΔC11 PTP

The T-cell ΔC11 PTP was utilized as a model PTP for the development of PTP assays described in this work. This PTP is a 37 kDa human T-cell PTP missing an 11 kDa carboxy-terminal segment of the full-length 48 kDa T-cell PTP. T-cell PTP cDNA was first isolated from a human T-cell library in 1989 (130), and later expressed in baby hamster kidney (BHK) cells in order to study its structure and function (131). It was found that this 415-amino acid intracellular PTP was restricted to the particular fraction of the cells, localizing with the endoplasmic reticulum (ER). As with many other PTPs and PTKs, the physiological substrate for the T-cell PTP has not been identified thus far.

The T-cell ΔC11 PTP was synthesized (by introducing a premature stop codon after the catalytic domain) in order to study the function of a segment of 19 hydrophobic amino acids in the 11 kDa carboxy-terminus tail of the T-cell PTP (131). The T-cell ΔC11 PTP was found to be readily soluble in aqueous solutions and to localize in the soluble fraction of the cellular extracts. An enhancement in tumourigenicity by overexpression of the full-length phosphatase is observed when BHK cells are injected into mice. On the other hand, tumour formation is greatly reduced if suppressed with BHK cells containing the truncated form (104). Although the mechanism of this cell transformation has not been elucidated, it does suggest that the subcellular localization of the PTP plays an essential role. In addition to being responsible for the protein's localization, the 11 kDa tail also
seems to affect the PTP's specificity toward artificial substrates.

Progress in the characterization of PTKs and PTPs has been hindered by two major factors: (1) the unknown nature of the physiological substrates for these enzymes; and (2) the lack of sufficient homogeneously purified enzymes for physical, chemical, and kinetic characterizations. The characterization of the T-cell PTPs has been greatly facilitated by the expression and purification of full-length and truncated T-cell PTPs from a high-level baculovirus expression system (132).

5.1.2.1.1 The Baculovirus Expression Vector System

The baculovirus system has emerged as the method of choice for high-yield production (1-100 mg/L of culture) of recombinant proteins in eukaryotic cells. The most commonly used baculovirus species for expression is the Autographica californica nuclear polyhedrosis virus (AcNPV). The AcNPV infects the insect tissue culture line Sf9 derived from the fall armyworm, Spodoptera frugiperda, ovary. The baculovirus system is very desirable since it correctly performs post-translational modifications on the recombinant proteins it expresses. Furthermore, the expression system is safe because the AcNPV does not infect vertebrate cells (133).

During AcNPV infection, two forms of viral progeny are produced: extracellular virus (EV) and occluded virus particles (OV). The latter are embedded in proteinaceous viral occlusions, called polyhedra. A polyhedrin protein with a molecular weight of 29,000 Da is the major structural component of the viral occlusions. In infected Sf9 cell cultures, the polyhedrin accumulates to very high levels, routinely 1 mg/mL per 1-2 x 10⁶ infected cells. This accounts for 50-75% of the total stainable protein of the cell detected on SDS-polyacrylamide gels (133).

The viral occlusions are an important part of the natural virus life cycle, providing the means for transmission of the virus. When infected larvae die, a large number of polyhedra are left in the decomposing tissue. The viral occlusions protect the embedded
virus particles from inactivation by environmental factors that would otherwise rapidly inactivate EV. When larvae feed on contaminated plants, they ingest the polyhedra. The occlusions dissolve in the alkaline environment of the insect gut, releasing viral particles which invade and replicate in the cells of the midgut tissue (133).

The polyhedrin gene of AcNPV, which has been mapped and sequenced, has proved to be nonessential for infection or replication of the virus (133). Therefore, substitution of a foreign gene for this viral gene results in high yields of the protein of interest. Deletion or insertional inactivation of the polyhedrin gene results in the production of occlusion negative viruses, which form plaques (Occ⁻) that are distinctly different from those of wild-type, occlusion positive (Occ⁺) viruses. These distinctive plaque morphologies provides a simple method to screen for recombinant viruses in which the wild-type AcNPV polyhedrin gene has been replaced with a hybrid gene of choice. The nonessential nature and high levels of expression of the polyhedrin gene make this gene particularly suitable for its use as an expression vector.

5.2 Research Proposal

The phosphorylation of proteins on tyrosyl residues is considered one of the most important mechanisms by which extracellular signals produce their biological responses in cells. Protein-tyrosine kinases (PTKs) together with protein-tyrosine phosphatases (PTPs) are responsible for regulating the cellular level of protein tyrosine phosphorylation. The observation that many oncogenes encode for proteins with increased PTK activities is one of the main reasons for much of the attention recently placed on protein-tyrosine phosphorylation.

The rate of identification of new protein-tyrosine kinases and phosphatases has greatly overtaken their characterization. This reflects, in part, the low levels of expression of these regulatory enzymes, which necessitates the development of highly sensitive techniques for their assay. Several methods have been described for the measurement of
PTK and PTP activities. The most commonly used assay for PTK utilizes the transfer of radioactive phosphate from ATP to synthetic substrates. For PTP, the release of radioactive phosphate from radiolabelled synthetic substrates is measured. These methods are very laborious, time-consuming and difficult to apply to a high throughput screening program.

The purpose of my research, presented in the second part of this DISSERTATION, was to develop sensitive, nonisotopic assays for the determination of PTK and PTP activity. These assays offer simple and time-efficient methods for the determination of PTP and PTK activities in sample preparations, as well as for the study of substances that might inhibit or stimulate these enzymes.

Chapter 6 focuses on the highly sensitive time-resolved fluorometric immunoassay developed for the determination of PTK activity. The pp60c-src is used as a model PTK for the development of this assay, however the method can be adapted to other PTKs. The assay is based on the immobilization of poly (Glu, Tyr), a synthetic PTK substrate, on microtitre wells. Following incubation with the PTK, the tyrosine residues that were phosphorylated by the PTK are detected using a anti-phosphotyrosine (anti-P-Tyr) antibody and a second antibody, GAMIg-ALP, which is conjugated to alkaline phosphatase (ALP) and recognizes the anti-P-Tyr. The ALP cleaves the substrate, fluorosalicyl-phosphate (FSAP), producing fluorosalicyliclate which forms highly fluorescent complexes with Tb³⁺-EDTA. A time-resolved fluorometer is used to measure these long-lived fluorescent complexes.

In Chapter 7, two types of assays for the determination of PTP activity are described using the T-cell ΔC11 PTP as a model PTP. The first assay, which uses conventional fluorometry, is based on the previously reported finding that Tb³⁺ forms fluorescent complexes with phosphotyrosine (P-Tyr) but not tyrosine (Tyr). Thus Tb³⁺ is used as a specific label for P-Tyr residues. The activity of the PTP (dephosphorylation of
P-Tyr) is followed by a decrease in fluorescence. This fluorometric assay is very simple and fast, requiring only a 30-min incubation step. Although these characteristics render the assay ideal for quick determinations of pure PTP preparations, its homogeneous nature combined with conventional fluorometric detection predisposes it to interferences when other proteins are present in the sample.

The second method developed for the determination of PTP activity involves a microtitre well-based immunoassay using time-resolved fluorometric detection. In this assay, synthetic polypeptides containing tyrosine residues are first phosphorylated using a PTK, and then immobilized onto microtitre wells. After incubation with the PTP, the remaining unconverted phosphosubstrate is determined using anti-P-Tyr, GAM Ig-ALP and the FSAP substrate. The utilization of a time-resolved fluorometric detection system in combination with the heterogeneous microtitre well format allows this assay to be very sensitive and essentially free of interferences.
CHAPTER 6
ENZYME-AMPLIFIED TIME-RESOLVED IMMUNOFLUOROMETRIC DETERMINATION OF PROTEIN-TYROSINE KINASE ACTIVITY

6.1 Introduction

Protein-tyrosine kinases (PTKs) are implicated in the mechanisms by which extracellular signals produce their biological responses in cells (108). PTK activity has been shown to be associated with the activation of growth factor receptors, proto-oncogene products and normal cells (107, 134). The rate of identification of new PTKs, as well as protein-tyrosine phosphatases (PTPs), has greatly overtaken their enzymological characterization. This reflects, in part, the low levels of expression of these regulatory enzymes which necessitates the development of highly sensitive methods for their assay (135).

Several methods have been described for the measurement of PTK activity. The most commonly used assay involves the transfer of radioactive phosphate ($^{33}$P or $^{32}$P) from ATP to a synthetic peptide substrate (136, 137). Separation of the labelled phosphopeptide from excess radiolabelled ATP is achieved by adsorption to a phosphocellulose paper followed by numerous washings. This procedure is very laborious and time-consuming. The extensive washes, furthermore, generate a large volume of liquid radioactive waste which requires special disposal and presents environmental concerns. Alternatively, nonisotopic, ELISA-type assays have been developed using detection enzymes (alkaline phosphatase- or horseradish peroxidase-labelled) which produce a measurable colorimetric reaction (138, 139). Babcook et al. (135) have reported a particle concentration fluorescence immunoassay (PCFIA) for PTK and PTP activities using fluorescein as a label. Most recently, a solid-phase PTK assay using [$\gamma$-$^{33}$P]ATP and scintillating microtitration plates coated with a PTK substrate [poly(Glu, Tyr) 4:1] has been reported (140). In contrast to the traditional radioactive-based assays, this solid-phase
technology has dramatically simplified procedures making it adaptable to high-volume work. Unfortunately, the disadvantages of working with radioactivity still apply.

In this work, we present a highly sensitive time-resolved immunofluorometric assay for the determination of PTK activity. Tyrosyl groups phosphorylated by PTK are detected using a specific anti-phosphotyrosine monoclonal antibody and a secondary, alkaline phosphatase-labelled goat anti-mouse antibody. The enzymatic activity is monitored by using fluorosalicylphosphate as substrate. The fluorosalicylate produced forms fluorescent complexes with Tb$^{3+}$-EDTA, which are measured by time-resolved fluorometry (47, 53).

6.2 Materials

6.2.1 Chemicals and Biochemicals

Bovine serum albumin (BSA), monoclonal anti-phosphotyrosine IgG antibody (anti-P-Tyr) and adenosine triphosphate (ATP) were obtained from Boehringer Mannheim (Laval, Quebec).

Alkaline phosphatase-labeled goat anti-mouse antibody (GAM Ig-ALP) was from Jackson Immunoresearch Lab. Inc. (West Grove, PA).

Dithiothreitol (DTT) was purchased from Gibco Laboratories Life Technologies, Inc. (Gaithersburg, MD).

Streptavidin, EDTA, Tween-20, poly (Glu, Tyr) 4:1 (PGT) and N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES) were purchased from Sigma (St. Louis, MO).

The phosphate ester of 5'-fluorosalicylic acid (FSAP) was from CyberFluor Division, Nordion International (Toronto, ON).

Terbium chloride hexahydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Zinc chloride, magnesium chloride, sodium chloride, potassium chloride, disodium
hydrogen phosphate, potassium dihydrogen orthophosphate, sodium hydroxide, hydrochloric acid, hydroxymethylaminomethane (Tris), and glycine were from BDH, Inc. (Toronto, ON).

The baculovirus expressed, wild-type mouse protein-tyrosine kinase, pp60c-src, was a gift from Martin Broome (Signal transduction laboratory, The Salk Institute, San Diego, CA).

6.2.2 Supplies

Sterile conical polypropylene centrifuge tubes (15 and 50 mL) were from VWR (West Chester, PA). Disposable Universal Fit pipette tips (10-200 µL and 200-1000 µL) were also from VWR.

Titertek Plus Microtips (0.5 µL-10 µL) were from ICN Biomedicals, Inc. (Costa Mesa, CA).

Eppendorf 1.5-mL microcentrifuge tubes were purchased from Brinkman Instruments (Westburg, NY).

Opaque, 12-well polystyrene Microlite™ 2 microwell strips and Removowell strip holders were obtained from Dynatech Laboratories, Inc. (Chantilly, VA).

6.2.3 Apparatus

The CyberFluor 615 Immunoanalyzer (CyberFluor Division, Nordion International, Toronto, ON), a time-resolved fluorometer, was used to measure the fluorescence of FSAP-Tb³⁺-EDTA solutions in microtitre wells. The excitation and emission wavelengths were set at 337 and 615 nm, respectively.

All pH measurements were made with the Corning pH Meter, Model 340 (Corning, NY). Prior to each measurement, the combination [Ag/AgCl] reference/pH electrode was calibrated using pH standard buffer solutions with pH values of 4.0, 7.0 and 10.0 (BDH, Inc., Toronto, ON).

Micropipetting was carried out using both Eppendorf pipettes (Germany) and
Nichiryo Model 5000 pipettes (Japan). An Eppendorf Repeater® pipette with Eppendorf Combitips® were used for repetitive pipetting.

Microcentrifugations were carried out using the Eppendorf Model 5415C microcentrifuge from Baxter/Canlab Inc. (Mississauga, ON).

Other laboratory equipment included the Haake Model W19 water tank with a Haake D1 heater and thermostat (Haake, Germany), the Model EAW II microtitre plate washer from SLT Lab Instruments (Austria), the Amerlite Shaker/Incubator (Amersham Canada Ltd., Oakville, ON), the Genie 2 Vortex from Fischer Scientific Canada Ltd. (Toronto, ON), and a Corning Stirrer/Hotplate (Corning, NY).

6.3 Methods

6.3.1 Protein-Tyrosine Kinase Assay

6.3.1.1 Reagents

Phosphate-Buffered Saline (PBS). Consisted of 0.14 mol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄ and 1.76 mmol/L KH₂PO₄, pH 7.4.

Wash Solution. Contained 25 mmol/L Tris, pH 7.8, 150 mmol/L NaCl and 0.1% Tween-20 and 0.05% (w/v) sodium azide.

Blocking Buffer. Contained 3% (w/v) BSA, 25 mmol/L Tris, pH 7.8, 150 mmol/L NaCl, 0.2% (v/v) Tween-20 and 0.05% (w/v) sodium azide.

Kinase Buffer. Consisted of 20 mmol/L HEPES pH 7.4, 10 mM MgCl₂, 1 mmol/L DTT and 0.1% BSA

ALP Buffer. Consisted of 0.1 mol/L Tris, pH 9.05, 0.1 mol/L NaCl and 1 mmol/L MgCl₂.

Substrate Stock Solution. A 0.01 mol/L stock solution of FSAP was prepared by dissolving 11.8 mg of the compound in 5 mL of 0.1 mol/L NaOH and kept at 4 °C. A 0.001 mol/L FSAP working solution was prepared just prior to use by diluting the FSAP stock solution 10-fold in ALP buffer.
Developing Solution. Contained 1 mol/L Tris, 0.4 mol/L NaOH, 3 mmol/L EDTA and 2 mmol/L TbCl₃.

6.3.1.2 Procedure

The microtitre wells were coated overnight at room temperature with 100 µL/well of a solution containing 3 µg/mL poly (Glu, Tyr) 4:1 (PGT) in PBS buffer. Just prior to use, the wells were washed three times with wash solution. In order to block sites on the wells which were not covered with PGT, the wells were incubated with 250 µL/well of blocking solution for 30 min at room temperature with mechanical shaking. After washing the wells as above, 100 µL of the pp60⁵²⁻src PTK, diluted in kinase buffer containing 0.4 mmol/L ATP, were pipetted into the wells, in duplicate. Following a 45-min incubation at room temperature, the wells were washed and 100 µL of 50 ng/mL anti-phosphotyrosine (anti-P-Tyr) antibody diluted in blocking solution were pipetted into each well and incubated for 30 min at room temperature. Subsequently, 100 µL of 200 ng/mL goat anti-mouse IgG conjugated to alkaline phosphatase (GAMlg-ALP), diluted in blocking solution, were pipetted into each well and incubated for 45 min at room temperature. The wells were then washed and 100 µL of the 10⁻³ mol/L fluorosalicylphosphate (FSAP) substrate solution were added to each well. After a 30-min incubation at room temperature, 100 µL of developing solution were added to each well and mixed for 1 min. The fluorescence was measured using the CyberFluor 615 Immunoanalyzer at excitation and emission wavelengths set at 337.1 nm and 615 nm, respectively. A sample containing no pp60⁵²⁻src was included in every assay in order to determine the background fluorescence. A schematic representation of the assay procedure is shown in Figure 6.1.

6.4 Results and Discussion

In the absence of clear physiological PTK target substrates, a number of exogenous substrates have been used for in vitro PTK assays. The random polymer, poly (Glu, Tyr) 4:1 (PGT), used in the proposed time-resolved immunofluorometric assay, is a general
FIGURE 6.1

Schematic Representation of the Time-Resolved Immunofluorometric PTK Assay

Legend

The pp60c-src, a model PTK, is incubated with ATP in poly (Glu, Tyr) 4:1 (PGT)-coated microtitre wells. Tyrosine residues which have been phosphorylated by pp60c-src are allowed to react with a monoclonal antibody specific for phosphotyrosine (anti-P-Tyr) residues. The immunocomplexes are then detected with a goat anti-mouse IgG conjugated to alkaline phosphatase (GAM Ig-ALP). The ALP cleaves the substrate fluorosalicyl-phosphate (FSAP) releasing fluorosalicylate (FSA) which forms fluorescent complexes with Tb³⁺-EDTA. The fluorescence was measured using the CyberFluor 615 Immuno-analyzer, a microplate time-resolved fluorometer, with excitation and emission wavelengths set at 337.1 nm and 615 nm, respectively.
FIGURE 6.1

Poly (Glu, Tyr) 4:1 coated microtitre wells

Add PTK and ATP
Incubate 45 min at RT
Wash

Add anti-P-Tyr antibody
Incubate 30 min at RT
Wash

Add GAM Ig-ALP
Incubate 45 min at RT
Wash

Add FSAP
Incubate 30 min at RT
Wash

Add Tb³⁺-EDTA developing solution
Mix 1 min

Measure fluorescence using the CyberFluor 615
substrate recently introduced for many PTKs (141, 142). In order to determine the optimal concentration of PGT for plate coating, serial dilutions of the PGT were made in PBS buffer. The wells were coated with 100 µL of the PGT solutions and incubated overnight at room temperature. The PTK assay was carried out using these wells as described in Section 6.3.1.2 with a constant concentration of pp60c-src (15 ng/mL). An increase in fluorescence was observed with increasing PGT concentrations from 0.125 to 2 µg/mL (corresponding to 12.5-200 ng/well). However, at higher concentrations of PGT the fluorescence decreased (see Figure 6.2). Other investigators (140) who have also observed this biphasic profile for the PGT coating, have suggested that perhaps at higher concentrations the polymer might be less tightly attached and subsequently washed away during the assay. In accordance with the previous suggestion, another possible explanation for this observation is that at higher concentrations the PGT forms layers (perhaps loosely bound to the wells) which cover exposed Tyr residues rendering these sites unavailable for phosphorylation by the PTK.

The effect of the ATP concentration available for the PTK reaction was studied at two pp60c-src concentrations (see Figure 6.3). In both cases, the fluorescence increases with increasing ATP concentrations from 0 to approximately 200 µmol/L. However at higher concentrations the fluorescence reaches a plateau. For subsequent assays, an ATP concentration of 400 µmol/L was chosen in order to ensure that ATP would be in excess and would not limit the phosphorylation reaction.

The PTK reaction as a function of time was also evaluated. The pp60c-src was incubated in PGT coated wells for 15, 30, 45 and 60 min. The fluorescence, representative of the phosphorylation reaction, continuously increased in a linear fashion within this time frame. A 45-min incubation period was chosen for our assay conditions. In situations where higher signals are required, for example with low concentrations of PTK, a longer incubation period may be useful.
FIGURE 6.2

Study of the PGT Concentration used for the Coating of Microtitre Wells

Legend

Serial dilutions of the poly (Glu, Tyr) 4:1 (PGT), used for the coating of microtitre wells, were prepared in PBS buffer. The wells were coated with 100 µL of the PGT solutions and incubated overnight at room temperature. The PTK assay was carried out using these wells as described in Section 6.3.1.2 with a constant concentration of pp60\textsuperscript{c-src} (15 ng/mL).
FIGURE 6.2

Fluorescence (arb. units)

Poly (Glu, Tyr) 4:1 (μg/mL)
FIGURE 6.3
Optimization of the ATP Concentration

Legend

The concentration of ATP used in the pp60c-src reaction was optimized at two pp60c-src concentrations: 10 ng/mL (solid line) and 2.5 ng/mL (dashed line). The PTK assay was carried out as per Section 6.3.1.2 with serial dilutions of ATP prepared in kinase buffer.
FIGURE 6.3

Fluorescence (arb. units) vs. ATP concentration (μmol/L)
The optimal anti-P-Tyr antibody concentration was determined by performing the assay as described under Section 6.3.1.2 but varying the amount of anti-P-Tyr antibody. In Figure 6.4, both the fluorescence, corrected for background, and the signal-to-background (S/B) ratios were plotted against the anti-P-Tyr antibody concentrations. The background represents the signal obtained from a sample containing no pp60<sup>c-src</sup>, and is a measure of the nonspecific binding of the reagents, namely the antibodies. Although the fluorescence increased with increasing anti-P-Tyr concentration, there was no net gain in the S/B ratio at concentrations greater than approximately 30 ng/mL. We also studied the effect of varying the anti-P-Tyr incubation time. Incubation periods of 15, 30, 45, 60 and 90 min were used. The highest S/B ratio was obtained with a 30-min incubation (data not shown).

The concentration and incubation period of the GAMIg-ALP were also optimized following procedures similar to those used for the anti-P-Tyr antibody. In these experiments the anti-P-Tyr and pp60<sup>c-src</sup> concentrations were kept constant at 50 ng/mL and 0.5 ng/mL, respectively. The fluorescence, corrected for background, and the S/B ratios were plotted against the GAMIg-ALP concentrations (see Figure 6.5). The highest S/B ratios were obtained with a GAMIg-ALP concentration of 200 ng/mL and a 45-min incubation period.

The linearity and sensitivity of the proposed assay were determined by constructing a PTK calibration curve. Serial dilutions of the pp60<sup>c-src</sup> were prepared in kinase buffer and used in the assay under optimized conditions. In Figure 6.6, the fluorescence, corrected for background, was plotted against the pp60<sup>c-src</sup> concentration. The linearity of the assay ranges from 0.01 to 1 ng/mL pp60<sup>c-src</sup>. This corresponds to 1-100 pg of pp60<sup>c-src</sup> per well. At higher concentrations of the PTK, the PGT becomes exhaustively phosphorylated and thus a plateau in the fluorescence is observed. The assay is very sensitive, capable of detecting 1 pg/well of pp60<sup>c-src</sup> with a S/B ratio of 1.8.
FIGURE 6.4
Optimization of the Anti-Phosphotyrosine Antibody Concentration

Legend

In order to determine the optimal concentration of anti-phosphotyrosine (anti-P-Tyr) antibody, the PTK assay was performed (as per Section 6.3.1.2) using serial dilutions of anti-P-Tyr diluted in blocking solution. The fluorescence, corrected for background and the signal-to-background ratios are represented by the solid and dashed lines, respectively. The background represents the fluorescence obtained with a sample containing no pp60csrc.
FIGURE 6.4

![Graph showing fluorescence and signal/background against Anti-P-Tyr concentration in ng/mL.](image)

- Fluorescence (arb. units)
- Signal/Background
- Anti-P-Tyr (ng/mL)
FIGURE 6.5
Optimization of the Alkaline Phosphatase-Labelled Goat Anti-Mouse IgG Concentration

Legend

The optimization of the alkaline phosphatase-labelled goat anti-mouse IgG (GAMlg-ALP) was performed using serial dilutions of this antibody in blocking solution. The assay was performed as described in Section 6.3.1.2. The fluorescence, corrected for background (solid line) and the signal-to-background ratio (dashed line) were plotted against the GAMlg-ALP concentration.
FIGURE 6.6

Linearity and Sensitivity of the Proposed PTK Assay

Legend

The linearity and sensitivity of the proposed time-resolved immunofluorometric PTK assay was studied by preparing serial dilutions of the model PTK, pp60<sup>C-src</sup> in kinase buffer and performing the assay as per Section 6.3.1.2.
FIGURE 6.6

Fluorescence (arb. units)

PTK (ng/mL)
6.5 Conclusions

In this work, we have described a highly sensitive, nonisotopic, microtitre well-based immunoassay for the determination of pp60c-src activity. Although pp60c-src was used as a model PTK for the development of the proposed assay, the technology is applicable to many other protein-tryrosine kinases. By employing a monoclonal antibody specific for phosphotyrosine residues, in combination with time-resolved fluorometry, the assay provides both specificity and sensitivity as well as a number of other significant advantages over existing methodologies.

In this assay the fluorosalicylate (produced from the hydrolysis of fluorosalicylphosphate by alkaline phosphatase) forms long-lived fluorescence complexes with Tb³⁺-EDTA which are measured by time-resolved fluorometry. The delay in fluorescence measurement after a flash excitation of the sample, excludes the background short-lived fluorescence, thereby improving the signal-to-background ratio. Therefore the sensitivities of assays using time-resolved fluorometric detection systems are, in general, superior to those of colorimetric and conventional fluorometric assays.

The PCFIA developed for the measurement of PTK activity utilizes a monoclonal anti-P-Tyr antibody and fluoresceinated anti-mouse antibodies (135). Samples of unknown PTK activity were tested at several dilutions due to a nonlinear dose-response resulting from a quenching of the fluorescent signal at high levels of fluorescence. This fluorescence quenching effect is eliminated in the proposed assay, by the use of the fluorescent FSA-Tb³⁺-EDTA complex which possesses a characteristically large Stokes shift (approximately 290 nm) (49).

The recently reported solid-phase radioactive method for PTK activity involves the measurement of ³²P on PGT-coated scintillating microtitration wells (140). Although this methodology is very simple, measuring PTK activity with a 3-step protocol, it is more time-consuming than the proposed time-resolved fluorometric assay. Furthermore, the
time-resolved immunofluorometric assay is approximately 100 times more sensitive than the radioactive method, without involving the health hazards and environmental concerns associated with the use of radioisotopes.

As with other ELISA-based PTK assays, the major drawback of this assay is its nonquantitative nature with respect to the determination of phosphate incorporated into the substrate. However, because of its superior sensitivity, simplicity to perform, and lack of radioisotopic labels, the proposed time-resolved immunofluorometric assay should prove especially beneficial for the determination of low PTK activities, and the study of various PTK inhibitors as therapeutic agents.
CHAPTER 7

FLUOROMETRIC AND TIME-RESOLVED IMMUNOFUOROMETRIC ASSAYS FOR PROTEIN-TYROSINE PHOSPHATASE ACTIVITY

7.1 Introduction

Phosphorylation of proteins on tyrosyl residues is a key mechanism for signal transduction and regulation of cell growth and proliferation (106). The extent and duration of tyrosine phosphorylation depend on the relative activities of specific protein-tyrosine kinases (PTK) and protein-tyrosine phosphatases (PTP). The highly diverse family of PTP has been separated into two categories; the receptor-like (transmembrane) PTP and the nonreceptor (intracellular) PTP (105, 122).

The physiological substrates for most PTP have not yet been identified. Thus, artificial substrates are used for determination of their catalytic activities (143). Proteins such as myelin basic protein (144), reduced carboxyamidomethylated and maleylated lysozyme (RCML) (126), casein (145) and histones (146) have been utilized. As well, random copolymers, such as poly (Glu, Tyr), and synthetic polypeptides corresponding to autophosphorylation sites of various proteins (e.g., epidermal growth factor receptor) have been employed (147, 148).

The most widely used assay for determination of PTP activity involves phosphorylation of the substrate on tyrosine with a protein-tyrosine kinase in the presence of [γ-32P]ATP. The labelled protein is then purified from the excess [γ-32P]ATP and used as a substrate for PTP. The enzymatically released [32P]P_i is measured after acid precipitation of the remaining radioactive phosphoprotein. Recently, some nonisotopic assays for PTP activity have been reported. For instance, in a spectrophotometric assay for PTP, the malachite green reaction (149) can be used to measure the inorganic phosphate.
released during PTP-catalyzed dephosphorylation of the substrate. p-Nitrophenylphosphate has also been used in a spectrophotometric PTP assay, but it is not specific for tyrosine phosphatases (150). In another assay, the change in absorbance and fluorescence spectra of phosphotyrosine (P-Tyr), upon dephosphorylation, was used for the continuous monitoring of PTP activity (151). Indeed, the rate of P-Tyr hydrolysis by PTP can be followed by the increase in absorbance (282 nm) or the fluorescence (excitation 285 and emission 305 nm). Furthermore, it was observed (152) that synthetic, tyrosine-phosphorylated peptides, when dephosphorylated by PTP, show similar changes in their absorbance and fluorescence spectra to those observed for phosphotyrosine and tyrosine. Thus, these peptides can be used in the continuous monitoring of PTP activity. Alternatively, the PTP catalyzed dephosphorylation can be followed by separating the phosphorylated and dephosphorylated peptides by HPLC (153). As well, anti-phosphotyrosine antibodies may be used to measure the remaining P-Tyr residues on the substrate after a PTP-catalyzed reaction (153).

In this work we report a fluorometric and a time-resolved immunofluorometric assay for PTP activity. The fluorometric assay is based on the recent observation (154) that Tb$^{3+}$ forms fluorescent complexes with P-Tyr but not with tyrosine. Therefore, Tb$^{3+}$ is used here as a probe to detect the dephosphorylation event. The time-resolved immunofluorometric assay uses substrates which have been phosphorylated on tyrosine, by a protein tyrosine kinase, and then immobilized on polystyrene microtitre wells. Monoclonal anti-phosphotyrosine antibodies are used to detect the remaining P-Tyr on the substrate after the PTP reaction. The immunocomplexes formed are measured by time-resolved immunofluorometry.

7.2 Materials

7.2.1 Cells

The Sf9 (fall armyworm ovary, Spodoptera frugiperda) cell line was obtained from
7.2.2 Chemicals and Biochemicals

Grace's Antheraea medium, yeastolate, lactalbumin, heat-inactivated fetal bovine serum (HI-FBS), penicillin, streptomycin, amphotericin B (Fungizone) and trypan blue used for cell culture procedures were purchased from Gibco Laboratories Life Technologies, Inc. (Gaithersburg, MD). Dithiothreitol (DTT) was also from Gibco.

3-(N-morpholino)propanesulfonic acid (MOPS) was from USB (Cleveland, OH).

Bovine serum albumin, monoclonal anti-phosphotyrosine (anti-P-Tyr) antibody and adenosine triphosphate (ATP) were obtained from Boehringer Mannheim (Laval, Quebec). Also purchased from Boehringer was the acid phosphatase from potato (PAP) with a specific activity of 2 U/mg (where 1 unit is defined as the amount of enzyme that releases 1 μmol of phosphate in 1 min using p-nitrophenylphosphate as substrate).

Alkaline phosphatase-labelled goat anti-mouse antibody (GAM Ig-ALP) was from Jackson Immunoresearch Lab. Inc. (West Grove, PA).

Sodium dodecyl sulfate (SDS), SDS molecular weight markers, streptavidin, EDTA, DMSO, Tween-20, imidazole, aprotinin, leupeptin, phenylmethanesulfonyl fluoride (PMSF), benzamidine, β-mercaptoethanol, Coomassie Brilliant Blue R-250, o-phospho-L-tyrosine (P-Tyr), poly (Glu, Tyr) 4:1 (PGT) and N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid (HEPES) were purchased from Sigma (St. Louis, MO). Reduced carboxyamidomethylated and maleylated lysozyme (RCML) was also from Sigma.

Bromophenol Blue, the protein assay dye reagent concentrate and 40% acrylamide/bis solution were purchased from Bio-Rad Laboratories (Hercules, CA).

The phosphate ester of 5'-fluorosalicylic acid (FSAP) was from CyberFluor Division, Nordion International (Toronto, ON).

Terbium chloride hexahydrate was purchased from Aldrich Chemical Co.
Sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen orthophosphate, sodium hydroxide, hydrochloric acid, magnesium chloride, hydroxymethylaminomethane (Tris), methanol, glacial acetic acid, glycine, sodium acetate and glycerol were all from BDH, Inc. (Toronto, ON).

The human T-cell protein tyrosine phosphatase (130), which is truncated at the carboxy-terminal (TC AC11 PTP) was a gift from Dr. Zhizhuang Zhao (Dept. of Biochemistry, University of Washington, Seattle, WA). The protein had been purified from Sf9 cells after infection by a recombinant baculovirus (132). The specific activity was 73,000 U/mg (where 1 unit of PTP is defined as the amount of enzyme that releases 1 nmol of phosphate per minute).

The recombinant baculovirus containing the TC AC11 PTP cDNA in place of the polyhedrin gene was a gift from Dr. Martin Page (Cell Signalling Section, The Wellcome Research Laboratories, UK)

The protein-tyrosine kinase pp60c-src (wild type from mouse) was a gift from Martin Broome (Signal transduction laboratory, The Salk Institute, San Diego, CA).

7.2.3 Supplies

Polystyrene cell culture flasks (25 cm²) and polypropylene cryogenic vials (2.0 mL) were purchased from Corning (Corning, NY).

Sterile, 10-mL graduated, polystyrene, serological pipettes, 175-cm² polystyrene cell culture flasks, 14-mL polypropylene tubes as well as 3- and 10-cc syringes were from Falcon® Becton and Dickinson Labware (Lincoln Park, NJ).

Nalgene™ sterile 25-mm syringe filters (0.2-µm pore size), 100-mL polypropylene wide mouth bottles and 6-inch glass pasteur pipettes were from Baxter Diagnostics, Corp. (Toronto, ON).

Sterile conical polypropylene centrifuge tubes (15 and 50 mL) were from VWR
(West Chester, PA). Disposable Universal Fit pipette tips (10-200 μL and 200-1000 μL) were also from VWR.

Titertek Plus Microtips (0.5 μL-10 μL) were from ICN Biomedicals, Inc. (Costa Mesa, CA).

Eppendorf 1.5-mL microcentrifuge tubes were purchased from Brinkman Instruments (Westburg, NY).

Opaque, 12-well polystyrene Microlite™ 2 microwell strips and Removawell strip holders were obtained from Dynatech Laboratories, Inc. (Chantilly, VA).

7.2.4 Apparatus

The CyberFluor 615 Immunoanalyzer (CyberFluor Division, Nordion International, Toronto, ON), a time-resolved fluorometer, was used to measure the fluorescence of FSAP-Tb³⁺-EDTA solutions in microtitre wells. The excitation and emission wavelengths were set at 337 and 615 nm, respectively.

The Hitachi F-3010 spectrofluorometer (Hitachi Ltd, Tokyo, Japan) was used for conventional fluorescence measurements.

SDS-polyacrylamide gel electrophoresis was carried out using the SVG-14 Vertical Gel Electrophoresis System (Tyler Research Instruments Edmonton, AB) and the Fotoforce 500™ Power Supply (Fotodyne, Inc., Bio-Can Scientific, Mississauga, ON).

The Class II Type A/B3 laminar flow hood (Nuaire Biological Safety Cabinets, Plymouth, IL) was used for the culturing of cells.

Cells were counted using the Neubauer Brightline hemocytometer from Hausser Scientific (American Scientific Products, McGaw Park, IL) and a Nikon TMS Microscope (Japan).

All pH measurements were made with the Corning pH Meter, Model 340 (Corning, NY). Prior to each measurement, the combination [Ag/AgCl] reference/pH electrode was calibrated using pH standard buffer solutions with pH values of 4.0, 7.0 and 10.0 (BDH,
Inc., Toronto, ON).

Micropipetting was carried out using both Eppendorf pipettes (Germany) and Nichiryo Model 5000 pipettes (Japan). An Eppendorf Repeater® pipette with Eppendorf Combitips® were used for repetitive pipetting.

Centrifugations performed at speeds lower than 10,000 x g were performed using the Sorvall® Superspeed RC2-B automatic refrigerated centrifuge (Sorvall Inc., Newton, CT). Centrifugations requiring speeds at 10,000 x g or greater, were carried out using the Sorvall® UltraPro 80 centrifuge and the TH641 Rotor (DuPont Co., Wilmington, DE). Microcentrifugations were carried out using the Eppendorf Model 5415C microcentrifuge from Baxter/Canlab Inc. (Mississauga, ON).

The purification of TC ΔC11 PTP by size exclusion chromatography was performed using a Pharmacia C 26 column (Pharmacia, Uppsala, Sweden), a Gilson Minipuls 2 (Mandel Scientific Co., Guelph, ON), a Gilson UV Detector Model 111B and a Frac-100 fraction collector from Pharmacia.

Other laboratory equipment included the Haake Model W19 water tank with a Haake D1 heater and thermostat (Haake, Germany), the Model EAW II microtitre plate washer from SLT Lab Instruments (Austria), the Amerlite Shaker/Incubator (Amersham Canada Ltd., Oakville, ON), a Shimadzu UV-160 spectrophotometer (Shimadzu Corp., Japan), the Genie 2 Vortex from Fischer Scientific Canada Ltd. (Toronto, ON), and a Corning Stirrer/Hotplate (Corning, NY).

7.3 Methods

7.3.1 Cell Culture

7.3.1.1 Reagents

Cell Culture Medium. Consisted of 90% Grace’s Antheraea medium, pH 6.3, 3.3 g/L yeastolate, 3.3 g/L lactalbumin, 100 kU/L penicillin, 100 mg/L streptomycin and 0.25 mg/L Fungizone.
Freezing Medium. Consisted of 90% cell culture medium and 20% DMSO.

7.3.1.2 Procedures

7.3.1.2.1 Cell Culturing

Frozen Sf9 cells received from the ATCC were thawed by rapid agitation, suspended in 10 mL of cell culture medium and pipetted into a 25-cm² culture flask. Cells were grown as monolayer cultures at 27 °C. The old culture medium was replaced with fresh culture medium approximately three times per week. To replace the medium, cells were first resuspended in the old medium (by forceful pipetting of medium across the cell monolayer). The cell-containing medium was then transferred to a 15-mL sterile tube and centrifuged at 1000 x g for 10 min. The supernatant was aspirated and the cell pellet was resuspended in 10 mL of fresh culture medium. Upon reaching confluency, the cells were collected in 2 mL of fresh medium and pipetted into a 175-cm² culture flask containing 10 mL of fresh medium. When the cell density reached about 2-2.5 x 10⁶ cells/mL, the cells were subcultured into 175-cm² flasks.

7.3.1.2.2 Freezing Cells

Excess Sf9 cells growing in monolayer were suspended in the culture medium, transferred to a 15-mL centrifuge tube, pelleted by centrifugation (1000 x g for 10 min) and resuspended in fresh culture medium to a density of at least 4 x 10⁶ cells/mL. The cell suspension was then diluted with an equal volume of freezing medium and aliquotted into 2.0-mL cryogenic vials. The cells were frozen slowly by placing the vials at -20 °C for the first hour, and then at -80 °C for storage.

7.3.2 Expression and Purification of TC ΔC11 PTP

7.3.2.1 Reagents

Lysis Buffer. Consisted of 25 mmol/L imidazole, pH 7.2, 2 mmol/L EDTA, 0.1% (v/v) β-mercaptoethanol, 1 mmol/L benzamidine, 0.002% (w/v) PMSF, 2 mg/L leupeptin and 1 mg/L aprotinin.
G-75 Buffer. Consisted of Lysis Buffer with 50 mmol/L NaCl.

Sample Loading Buffer. Contained 125 mmol/L Tris, pH 6.8, 5% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 4% (w/v) SDS and 0.1% Bromophenol Blue

7.3.2.2 Procedures

7.3.2.2.1 Infection of Sf9 Cells and Purification of TC ΔC11 PTP

The infection of Sf9 cells with the recombinant baculovirus containing the TC ΔC11 PTP cDNA insert was performed as described by Zander et al. (132). In this procedure, 15 culture flasks (175 cm²) of 50% confluent Sf9 cells were used for the infection. To each flask was added 35 mL of fresh cell culture medium and 5 mL of recombinant baculovirus stock solution. Following a 36-h incubation at 27 °C, the cells remaining attached to the culture flasks were gently suspended into the medium by pipetting the medium across the floor of the flask. The cell suspensions were collected into 100-mL wide mouth bottles and centrifuged for 10 min at 2000 x g. The supernatants were stored at 4 °C for future infections. The cell pellets were pooled in 5 mL of extraction buffer and disrupted by 30 strokes in a Dounce homogenizer on ice. The homogenate was then centrifuged for 10 min at 10,000 x g. The supernatant was loaded onto a Sephadex G75 Superfine column (2.5 x 90 cm). The flow rate was set at 20 mL/h and 20 min fractions were collected into glass test tubes.

7.3.2.2.2 SDS-Polyacrylamide Gel Electrophoresis

Based on previously reported results, the elution of the TC ΔC11 PTP was expected around fractions 54-67. These fractions were therefore subjected to SDS-PAGE to check for the presence of this 37 kDa protein.

A gel consisting of a 12% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel was prepared as described previously (155). In microcentrifuge tubes, 60-μL aliquots of the fractions were mixed with 40 μL of the sample loading buffer. These
samples were boiled for 5 min and then cooled. The entire 100-μL sample was loaded onto the gel. In addition, 10 μL of SDS molecular weight markers (carbonic anhydrase: 29 kDa; ovalbumin: 45 kDa; albumin: 66 kDa; phosphorylase B: 97.4 kDa; β-galactosidase: 116 kDa; myosin: 205 kDa) were loaded onto the gel. Following electrophoresis at 50 V for 13 h, the gel was stained in 0.2% (w/v) Coomassie Brilliant Blue R-250 containing 40% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained with a 40% methanol and 10% acetic acid solution.

The fractions which showed the 37 kDa TC ΔC11 PTP band by SDS-PAGE (i.e., fractions 59-64, see Figure 7.1) were pooled. An aliquot of the pooled TC ΔC11 solution was used to determine the protein concentration with the Bio-Rad protein assay (156). The protein concentration, calculated from a standard curve generated using BSA standard solutions (0-1 mg/mL), was determined to be 1 mg/mL. The remainder of the TC ΔC11 PTP solution was dialyzed against G-75 buffer containing 50% glycerol, divided into aliquots, and stored at -80 °C. This TC ΔC11 PTP preparation was used, in addition to that obtained from Dr. Zhao (see Section 7.2), for the development of the proposed PTP assays.

7.3.3 Fluorometric Determination of Acid Phosphatase Activity

The reaction mixture (0.9 mL) contained 0.1 mmol/L phosphotyrosine, 10 mmol/L acetate buffer, pH 4.5-5.0, and the enzyme at final concentrations ranging from 23-1500 U/L. The reaction was carried out at 30 °C for 30 min. Subsequently, 100 μL of 1 mol/L MOPS buffer, pH 6.5, and 20 μL of 25 mmol/L Tb3+ were added and the fluorescence of the solution was measured at excitation and emission wavelengths set at 268 and 488 nm, respectively. A reaction mixture, in which the enzyme was replaced by buffer, was also prepared and measured as above (blank). The decrease in fluorescence was directly related to the phosphatase activity in the sample.
FIGURE 7.1
SDS-Polyacrylamide Gel Electrophoretogram of TC ΔC11 PTP

Legend
Following the homogenization of TC ΔC11 PTP recombinant baculovirus-infected cells and subsequent centrifugation, the resultant supernatant was loaded onto a Sephadex G75 Superfine column (2.5 x 90 cm). The flow rate was set at 20 mL/h and 20-min fractions were collected into glass test tubes. Based on previously reported results (132), the elution of the TC ΔC11 PTP was expected around fractions 54-67. These fractions were therefore subjected to SDS-PAGE to check for the presence of this 37 kDa protein.

A gel consisting of a 12% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel was prepared as described previously (155). In microcentrifuge tubes, 60-μL aliquots of the fractions were mixed with 40 μL of the sample loading buffer. After boiling the samples for 5 min and subsequent cooling, the entire 100-μL sample was loaded onto the gel. In addition, 10 μL of protein molecular weight markers, prepared in a similar manner, were loaded onto the gel. Electrophoresis was performed at 50 V for 13 h. The gel was then stained in 0.2% (w/v) Coomassie Brilliant Blue R-250 containing 40% (v/v) methanol and 10% (v/v) acetic acid, and destained with a 40% methanol and 10% acetic acid solution.

Lanes 1 through 7 and 9 through 15 correspond to fractions 54-60 and 61-67, respectively. Lane 8 represents the molecular weight markers which, from the top to the bottom of the lane, correspond to myosin: 205 kDa; β-galactosidase: 116 kDa; phosphorylase B: 97.4 kDa; albumin: 66 kDa; ovalbumin: 45 kDa; and carbonic anhydrase: 29 kDa. A pure 37 kDa band believed to be the TC ΔC11 PTP was observed in fractions 59-64. These fractions were shown to have PTP activity using the proposed fluorometric PTP assay.
7.3.4 Fluorometric Determination of TC ΔC11 PTP Activity

Protein tyrosine phosphatase reactions were carried out in polypropylene microcentrifuge tubes, which were blocked with a 1% Tween-20 solution (overnight) and rinsed extensively with distilled water. The reaction mixtures (0.9 mL) consisted of 0.2 mmol/L phosphotyrosine, 0.70 mmol/L DTT, 25 mmol/L acetate buffer, pH 5.0-5.5 and TC ΔC11 PTP at final concentrations ranging from 1100-36,500 U/L. The mixtures were incubated for 30 min at 30 °C. Then, 20 μL of a 25 mmol/L terbium chloride solution and 100 μL of 1 mol/L MOPS buffer pH 6.5 were added, and the fluorescence was measured as above.

7.3.5 Enzymatic Phosphorylation of RCML and PGT

7.3.5.1 Reagent

*Kinase Buffer.* Consisted of 0.2 mmol/L ATP, 10 mmol/L MgCl₂, 1 mmol/L DTT, 20 mmol/L HEPES, pH 7.4.

7.3.5.2 Procedure

One mg of RCML or PGT was dissolved in 1 mL of kinase buffer. Then, 1 μL of a 0.5 μg/mL pp60-src solution was added, followed by an overnight incubation at 37 °C.

7.3.6 Coating Microtitre Wells with Phosphorylated Substrates (P-PGT and P-RCML)

7.3.6.1 Reagents

*Phosphate-Buffered Saline (PBS).* Consisted of 0.14 mol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄ and 1.76 mmol/L KH₂PO₄, pH 7.4.

*Wash Solution.* Contained 25 mmol/L Tris HCl, 150 mmol/L NaCl and 0.2% (v/v) Tween-20, pH 7.8.

*Blocking Buffer.* Contained 3% (w/v) BSA, 25 mmol/L Tris HCl, 150 mmol/L NaCl, 0.2% (v/v) Tween-20 and 0.05% (w/v) sodium azide, pH 7.8.
7.3.6.2 Procedure

Coating solutions were prepared by diluting P-RCML to 2 µg/mL and P-PGT to 20 µg/mL in PBS buffer. The wells were coated overnight at room temperature with 100 µL of coating solution per well. The wells were then washed twice with wash solution and blocked with 250 µL/well of blocking buffer for 30 min at room temperature. The wells were washed two more times just before use.

7.3.7 Time-Resolved Immunofluorometric Determination of Protein-Tyrosine Phosphatase Activity

7.3.7.1 Reagents

Assay Buffer. Consisted of 25 mmol/L MOPS pH 6.0, 1 mmol/L DTT, 1 mmol/L EDTA and 1 g/L BSA.

Substrate Solution. Consisted of 1 mmol/L FSAP, 0.1 mol/L NaCl, 1 mmol/L MgCl₂ and 0.1 mol/L Tris, pH 9.5.

Developing Solution. Contained 1 mol/L Tris, 0.4 mol/L NaOH, 3 mmol/L EDTA and 2 mmol/L TbCl₃.

7.3.7.2 Procedure

A 100-µL aliquot of TC ΔC11 PTP diluted in assay buffer was pipetted in duplicate in wells coated with a phosphorylated substrate (P-RCML or P-PGT). The wells were incubated with mechanical shaking for 30 min at room temperature, after which, the reaction was terminated by washing three times. Subsequently, 100 µL/well of a 50 µg/L anti-phosphotyrosine antibody, diluted in blocking buffer, were added and incubated for 30 min, as above. The wells were washed three times and 100 µL/well of 200 µg/L GAMIg-ALP were added. After a 45-min incubation, the wells were washed three times and 100 µL of substrate solution were pipetted into each well and incubated for 30 min at room temperature. Subsequently, 100 µL of a developing solution were added to each well and shaken for 1 min. The fluorescence of the solution was measured with a time-resolved
fluorometer. Excitation and emission wavelengths were set at 337 nm and 615 nm, respectively. A reaction mixture, in which the sample was replaced by buffer, was also prepared and measured as above (blank). The decrease in fluorescence was directly related to the phosphatase activity in the sample. A summary of the assay is schematically represented in Figure 7.2.

7.4 Results and Discussion

The proposed fluorometric assay is based on the observation that Tb$^{3+}$ forms fluorescent complexes with phosphotyrosine (154). In these complexes, the energy is absorbed by P-Tyr (268 nm) and, through an intramolecular energy transfer process, is transferred to Tb$^{3+}$, which subsequently fluoresces (488 nm). The fluorescence intensity is linearly related to phosphotyrosine concentration. It has also been shown (154) that tyrosine and other amino acids, as well as, phosphoserine, phosphothreonine and nucleotides (except GMP) do not enhance Tb$^{3+}$ fluorescence significantly. Thus, the enzymatic hydrolysis of phosphotyrosine can be monitored selectively by adding an excess of Tb$^{3+}$ at the end of the reaction and measuring the decrease in fluorescence. Moreover, from the plots of fluorescence against P-Tyr, the decrease in P-Tyr concentration and the moles of phosphate released can be calculated at various PTP levels.

The specificity of the Tb$^{3+}$-P-Tyr interaction has been studied in detail (154). Here we examined the effect of pH on the fluorescence intensity of this complex. The results, presented in Figure 7.3, show that the fluorescence increases with pH in the range of 3.5 to 6.5. At pH values higher than 7.0, a precipitate was observed due to the formation of insoluble terbium hydroxides. Thus, a pH of 6.5 was chosen for further studies. In the presence of a constant Tb$^{3+}$ concentration (0.45 mmol/L), a plot of the logarithm of fluorescence intensity versus logarithm of P-Tyr concentration was found linear in the range of 3 to 300 μmol/L phosphotyrosine.

The assay was applied to the measurement of the activity of two purified enzymes,
FIGURE 7.2
Schematic Representation of the Time-Resolved Immunofluorometric Assay for PTP Activity

Legend

The time-resolved immunofluorometric assay employs tyrosine-phosphorylated substrates (P-PGT and P-RCML), immobilized on microtitre wells. After incubation with PTP, the remaining phosphotyrosine residues are reacted with a specific antiphosphotyrosine antibody. The immunocomplexes formed are detected with an alkaline phosphatase (ALP)-labelled goat anti-mouse antibody. The phosphate ester of 5' fluorosalicylate (FSAP) is used as substrate. The fluorosalicylate produced forms highly fluorescent complexes with Tb$^{3+}$-EDTA in alkaline solution. The fluorescence is measured using the CyberFluor 615 (a time-resolved fluorometer) with excitation and emission wavelengths set at 337.1 nm and 615 nm, respectively.
FIGURE 7.2

1. Microtitre wells coated with phosphorylated peptide
   \[ \text{TYR}^{\text{P}} \]
   \[ \text{TYR}^{\text{P}} \]

2. Add PTP
   \[ \text{TYR}^{\text{P}} \]
   \[ \text{TYR}^{\text{P}} \]
   + PTP

3. Add anti-P-Tyr antibody
   \[ \text{TYR}^{\text{P}} \]
   \[ \text{TYR} \]

4. Add GAM Ig-ALP
   \[ \text{TYR}^{\text{P}} \]
   \[ \text{TYR} \]
   + FSAP
   \[ \text{FSAP} \]
   \[ \text{FSA} \]

5. Add FSAP
   \[ \text{TYR}^{\text{P}} \]
   \[ \text{TYR} \]

6. Add Tb$^{3+}$-EDTA developing solution
   \[ \text{Mix 1 min} \]

Measure fluorescence using the CyberFluor 615
FIGURE 7.3

The Effect of pH on the Fluorescence of Tb$^{3+}$-P-Tyr Complexes

Legend

The effect of pH on the fluorescence intensity of the Tb$^{3+}$-P-Tyr complex was studied, using the proposed fluorometric PTP assay, at two P-Tyr concentrations: (1) 0.3 mmol/L and (2) 0.15 mmol/L. All solutions contained a constant Tb$^{3+}$ concentration of 0.3 mmol/L. Acetate buffers were used for pH 3.0-5.5 and MOPS for pH 6.0-8.0.
FIGURE 7.3

Fluorescence (arb. units)

pH

(1)

(2)
namely, an acid phosphatase (PAP, a nonspecific phosphatase) and the TC ΔC11 PTP. The assay protocol involves two steps. First the enzyme is incubated with an excess of P-Tyr and subsequently the remaining P-Tyr is measured by adding a solution of Tb$^{3+}$. This configuration allows the enzymatic reaction to proceed at its own pH optimum. Whereas the final pH is the optimum for Tb$^{3+}$-P-Tyr fluorescence measurements.

The effect of pH on PAP and TC ΔC11 PTP activity was studied in the range from 3.5 to 8.0. Acetate buffers were used for pH 3.0-5.5 and MOPS for pH 6.0-8.0. The observed decrease in fluorescence was converted to decrease of P-Tyr concentration (D[P-Tyr]) by using calibration curves obtained at different pH values. The results are presented in Figure 7.4. The pH optima were found to be 4.5-5.0 for PAP and 5.0-5.5 for TC ΔC11 PTP.

In order to study the effect of P-Tyr concentration on PTP activity as well as its effect on the sensitivity of our assay, we constructed calibration curves, that is, plots of the decrease in fluorescence against enzymatic activity, at P-Tyr concentrations ranging from 50 to 400 μmol/L. It was found that the reaction rate increased as the initial concentration of P-Tyr increased. However, a lower sensitivity was observed at P-Tyr levels higher than 200 μmol/L. This was because small decreases in fluorescence were not distinguishable from the high blank readings associated with elevated P-Tyr concentrations. Similarly, an optimum P-Tyr concentration of 100 μmol/L was found for PAP assay.

Calibration curves generated for PAP and TC ΔC11 PTP under optimized conditions, are presented in Figure 7.5 A and B, respectively. PAP activities in the range of 23-1500 U/L can be measured. TC ΔC11 PTP can be determined in the range of 1100-36,500 U/L. At higher phosphatase activities a plateau is observed due to substrate depletion. Samples with higher PTP activities can still be measured by increasing the initial P-Tyr concentration or decreasing the incubation time.

A significant characteristic of this assay is the large Stokes shift (greater than 200
FIGURE 7.4
The Effect of pH on the Activities of TC ΔC11 PTP and PAP

Legend
The effect of pH on the activities of TC ΔC11 PTP and PAP was studied in the pH range from 3.0-8.0 using (1) 0.5 μg/mL TC ΔC11 PTP and (2) 0.25 μg/mL PAP. Acetate buffers were used for pH 3.0-5.5 and MOPS for pH 6.0-8.0. The observed decrease in fluorescence was converted to a decrease of P-Tyr concentration ([P-Tyr]) by using calibration curves of fluorescence vs P-Tyr concentration obtained at for the different pH values.
FIGURE 7.5

TC ΔC11 PTP and PAP Calibration Curves for the Fluorometric Assay

Legend

The linearity and sensitivity of the fluorometric TC ΔC11 PTP and PAP assays were determined by constructing calibration curves for each enzyme under optimized conditions. Serial dilutions of the TC ΔC11 PTP and PAP were made in 25 mmol/L MOPS, pH 6.0, with 1 mmol/L DTT, and distilled deionized water, respectively. A sample containing no enzyme was included for each curve. The decrease in fluorescence (DF) which represents the fluorescence in the absence of enzyme minus the fluorescence in the presence of enzyme, was plotted against the final enzyme concentration in each solution.
FIGURE 7.5

A

Log(DF)

PAP (U/L)

B

Log(DF)

PTP (U/L)
nm) of the fluorescent Tb$^{3+}$-P-Tyr complexes. Also, the emission wavelength of these complexes is far beyond the emission maxima of most proteins. On the other hand, when the fluorescence of tyrosine (produced from hydrolysis of phosphotyrosine) is monitored as a measure of PTP activity (151, 152), then the Stokes shift is only 20 nm (excitation and emission wavelengths of 285 and 305 nm, respectively). The short Stokes shift results in a high "inner filter effect" and nonlinearity of fluorescence intensity with substrate concentration.

A drawback of the homogeneous fluorometric assay is that the PTP-catalyzed hydrolysis of P-Tyr cannot be followed continuously since Tb$^{3+}$ interferes when added at the beginning of the reaction. This is probably due to the fact that formation of Tb$^{3+}$-P-Tyr complexes prevents the P-Tyr from binding to the catalytic site of the enzyme.

A noncompetitive immunoassay for PTP is also proposed here, where microtitre wells are coated with polypeptides which have been previously phosphorylated on tyrosine using a protein-tyrosine kinase. The PTP-containing mixture is incubated with the solid phase and the reaction is terminated by washing the wells. Subsequently, the remaining phosphotyrosyl groups are reacted with a monoclonal anti-phosphotyrosine antibody. An ALP-labelled goat anti-mouse antibody is then used for detection of the immunocomplexes. ALP hydrolyzes the phosphate ester of fluorosalicylate and the free fluorosalicylate produced forms long-lived fluorescent complexes with Tb$^{3+}$-EDTA at pH 12.5 (47, 53) which are measured with a time-resolved fluorometer. Time-resolved fluorometry is used as a means to eliminate the background in fluorescence measurements thus, improving the signal to noise ratio (51).

Coating-optimization experiments were performed, where microtitre wells were coated overnight with various concentrations of phosphorylated RCML and PGT. The results are presented in Figure 7.6. The solid phase becomes saturated with concentrations of P-RCML and P-PGT higher than 5 and 20 $\mu$g/mL, respectively. The effect of time and
FIGURE 7.6
Optimization of P-PGT and P-RCML Concentrations
used for Coating of Microtitre Wells

Legend

The artificial substrates, poly (Glu:Tyr) 4:1 (PGT) and reduced carboxyamidomethylated and maleylated lysozyme (RCML) were phosphorylated using PTK as described in Section 7.3.5. Serial dilutions of the stock P-PGT and P-RCML solutions were made in PBS buffer. The microtitre wells were then coated with 100 µL of the various solutions and incubated for 3 h at 42 °C. P-Tyr was detected using the anti-P-Tyr antibody, GAMlG-ALP and FSAP as described in Section 7.3.7. The fluorescence, measured by time-resolved fluorometry, was plotted against the concentration of the P-PGT (1) or P-RCML (2) preparations used in coating the wells.
temperature on the coating was also studied. Wells were incubated with saturating P-PGT and P-RCML solutions for periods of 1, 2, 3, 4, 5, 6, and 16 h (overnight) at both room temperature and 42 °C. Incubating the wells for 3 h at 42 °C gave similar fluorescence readings as an overnight incubation at room temperature.

The activity of TC ΔC11 PTP on the immobilized substrates was studied at pH values ranging from 3.5 to 8.5, at two enzyme concentrations, 0.5 and 1 ng/mL. The highest activities were observed at pH values between 5.5 and 6.5 (see Figure 7.7).

In order to assess the sensitivity of the assay, we prepared serial dilutions of the enzyme in the assay buffer and plotted the decrease of fluorescence intensity against the PTP activity for each sample. Experiments were performed with both the P-RCML and P-PGT substrates and the results are presented in Figure 7.8. TC ΔC11 PTP from as low as 36 to 7300 U/L (0.5 to 100 ng/mL) can be measured using P-RCML as substrate. This corresponds to 50 pg-10 ng of enzyme in the reaction mixture. The calibration curve reaches a plateau when the immobilized substrate is dephosphorylated completely. The assay with P-PGT was about four times less sensitive.

The precision of the proposed immunofluorometric assay was estimated by repeatedly measuring the activity of three TC ΔC11 PTP samples containing 0.5, 2.0 and 8.0 ng/mL. The coefficients of variation were 2.6, 1.2 and 0.7 %, respectively (n=12).

7.5 Conclusions

Comparing the two assays proposed here we find that the time-resolved immunofluorometric assay is about 30 times more sensitive than the fluorometric one (see Figures 7.5B and 7.8). Furthermore, the immunofluorometric assay has the unique advantage that it is practically free from interferences. This is because the enzymatic reaction can be performed under optimized conditions for PTP and then the solid phase is washed prior to the determination of the remaining phosphotyrosine. The main disadvantage of the immuno fluorometric assay is that it does not provide information on
FIGURE 7.7
The Effect of pH on TC ΔC11 PTP Activity on Immobilized P-RCML

Legend

The effect of pH on the activity of TC ΔC11 PTP on an immobilized substrate was studied using P-RCML-coated microtitre wells. The time-resolved immunofluorometric assay was carried out as described in Section 7.3.7 with PTP concentrations of (1) 1.0 μg/mL and (2) 0.5 μg/mL, each in assay buffers of pH values ranging from 3.5 to 8.5. Acetate buffers were again used for pH 3.5-5.5 and MOPS for pH 6.0-8.5. At each pH, a sample containing no PTP was also included. The decrease in fluorescence (DF), representing the fluorescence in the absence of PTP minus the fluorescence in the presence of PTP, was plotted against the pH of the assay buffer utilized.
FIGURE 7.8
Sensitivity and Linearity of the Time-Resolved Immunofluorometric Assay
for TC ΔC11 PTP

Legend

The sensitivity and linearity of the time-resolved immunofluorometric assay for TC ΔC11 PTP was studied using both P-RCML and P-PGT. Serial dilutions of the PTP were prepared in the assay buffer, and pipetted into (1) P-RCML- and (2) P-PGT-coated microtitre wells. The assay was carried out as described in Section 7.3.7. A sample containing no PTP was also included for each calibration curve. The decrease in fluorescence, DF (fluorescence in the absence of enzyme minus the fluorescence in the presence of enzyme) was plotted against the TC ΔC11 PTP concentration present in the well. The DF on the left axis corresponds to the P-RCML-based calibration curve, and the DF on the right axis corresponds to the P-PGT-based calibration curve.
the kinetics of the enzymatic reaction. The phosphorylated substrate is immobilized and the moles of phosphate released cannot be estimated directly. Nevertheless, the assay can be very useful for comparing PTP activities of various cell extracts, as well as, for the screening of potential inhibitors or activators. The two nonisotopic assays described here should prove beneficial for the determination and study of various PTP. Tb$^{3+}$ is a useful probe for following the tyrosine-phosphorylation/dephosphorylation reaction directly and time-resolved immuno-fluorometry, in combination with anti-P-Tyr antibodies, provides a highly sensitive method for determination of PTP.
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**Presentations:**

July 1996  
Quantitative RT-PCR for Prostate-Specific Antigen (PSA) mRNA. A poster presented at the AACC/CSCC 1996 Annual Meeting, Chicago, Illinois, USA.

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