# INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

**The quality of this reproduction is dependent upon the quality of the copy submitted.** Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

**UMI**

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

# Identification and Characterization of a Novel Testis-Specific Gene, *Pom-1*, Transcriptionally Regulated During Spermatogenesis

by

Rhonda L. Schwartz

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy



Department of Medicine
Division of Experimental Medicine
McGill University
Montreal, Quebec

## Abstract

The gene designated *Pom*-1 is a new mouse gene identified in the region of the Gin-1 common proviral integration site. The origin of the *Gin*-1 region has been described elsewhere (Villemur et al., 1987). The novel Pom-1 gene is testis-specific and encodes two major RNA species of 1.0 and 1.2 kbp. Our data have revealed that there is no clear evidence to suggest that *Pom*-1 is involved in the tumorigenic pathway that resulted in the Gross Passage A Murine leukemia virus-induced thymomas. The pattern of expression of *Pom*-1 was studied by a time course Northern blot, the STAPUT cell separation technique and *in situ* hybridization. Analysis of mRNA from enriched populations of spermatogenic cells from adult testes and localization by *in situ* hybridization revealed that *Pom*-1 transcripts are most abundant in the round and elongated spermatids, although there is a weak expression in pachytene spermatocytes. Immunocytochemistry data have shown protein expression to be localized in the Golgi apparatus of pachytene spermatocytes (stages IX-XII), round spermatids (steps 5-8) and elongated spermatids (steps 9-15).

The *Pom*-1 gene is not homologous to any sequences present in the Genebank. Sequence analysis predicts a 6 kilodalton protein which is basic, lysine and arginine rich (~12%). It is also relatively rich in potential phosphoacceptor amino acids (~20%), mainly threonine and serine, several of which are located in phosphorylation consensus sequences. These results suggest a role for the novel *Pom*-1 gene in spermatogenesis.

## Résumé

Le gène, *Pom*-1, est un nouveau gène (de souris) isolé dans la region commune d'insertion provirale appelée *Gin*-1. L'origine de la région *Gin*-1 a déjà été ecrite auparavant (Villemur et al., 1987). Le gène *Pom*-1 est spècifiquement exprimé dans les testicules et code pour deux ARN messagers de 1.0 et 1.2 kbp.

Nous n'avons pas pu obtenir à ce jour d'évidence que le gène *Pom*-1 soit impliqué dans le processus de l'oncongenèse thymique induite par insertion provirale du virus leucémique de la souris Gross Passage A.

Le patron d'expression de *Pom*-1 a été étudié au cours du temps par la technique du Northern, la technique de STAPUT qui sépare les cellules des testicules, et l'hybridation *in situ*. L'analyse des ARN messagers des populations de cellules spermatogéniques enrichies de testicules adultes a démontré que *Pom*-1 est exprimé fortement dans les spermatides ronds et allongés. Il y aussi une faible expression de *Pom*-1 dans les spermatocytes pachytènes. Ces données ont été aussi confirmées par la technique d'hybridation *in situ*.

Par immunocytochimie, la protéine *Pom*-1 est exprimée dans l'appareil de Golgi des spermatocytes pachytènes aux stages IX-XII, dans les spermatides ronds aux étapes 5-8 et dans les spermatides allongés aux étapes 9-15.

Aucune séquence homologue au gène *Pom*-1 n'est à ce jour présente dans les banques de données habituelles (Gene Bank, Swiss Prot,...etc). L'ARN messager peut potentiellement être traduit en une protéine basique de 6 kilodaltons très riche en arginine et lysine (12%).La protéine *Pom*-1 pourrait être aussi potentiellement phosphorylée (elle

contient une accepteur sérine et thréonine (20%)). Certains sont dans des séquences consensus connues pour des kinases.

L'ensemble de ces données suggère un rôle de *Pom*-1 dans la spermatogénèse.

## Acknowledgments

I would like to thank my supervisor, Dr. Paul Jolicoeur, for the opportunity to do my Ph.D. studies in a stimulating environment.

I thank the members of the Laboratory of Molecular Biology for their support, help, and of course, the great times we've had together - Philippe Dupras, Louis Gaboury, Guy Goudreau, Jean-François Houle, Xiaoyan Jiang, Denis Kay, Steven Klein, and Yves Paquette. I would also like to thank the technicians for their help - Normand Beaulieu, Benôit Laganière, and Ginette Massé.

I would to thank Dr. Jacquetta Trasler for help with the STAPUT technique, and for valuable discussions and advice. Thanks to Dr. Solomon Igdura for help with immunocytochemistry.

I would like to thank my husband, Michael Tessler, for his patience through the highs and lows of a graduate student, for his understanding and support. I am deeply grateful to my parents, Anne and Sydney Schwartz, for their constant encouragements, belief in me, and generosity throughout the years. To my brothers, Howard and Jeffrey Schwartz, for their encouragements and for not asking me a hundred questions!

Special thanks to F.C.A.R. for their financial support during my doctoral research.

This thesis is dedicated to my son, Joshua, who always inspires me.

## Table of Contents

## List of Figures

Chapter 1



Chapter3

# List of Tables

Chapter 1



Chapter 3

## List of Abbreviations

| | |
|---|---|
| aa: | amino acid |
| AEV: | avian erythroblastosis virus |
| ALL: | acute lymphoblastic leukemia |
| ALV: | avian leukosis virus |
| A-MuLV: | Abelson murine leukemia virus |
| bp: | base pair |
| CA: | capsid protein |
| cDNA: | complemetary deoxyribonucleic acid |
| CML: | chronic myelogenous leukemia |
| c-terminal: | carboxy-terminal |
| DNA: | deoxyribonucleic acid |
| EDTA: | ethylenediamine tetraacetate |
| EGF: | epidermal growth factor |
| env: | envelope |
| FeLV: | feline leukemia virus |
| FFU: | focus forming unit |
| gag: | group antigens |
| gly: | glycine |
| gp: | glycoprotein |
| h: | hour |
| IAP: | intracisternal A-particle |
| IN: | integrase |
| kbp: | kilobase pair |
| kD: | kilodalton |
| LTR: | long terminal repeat |
| MA: | matrix protein |

| | |
|---|---|
| MCF: | mink cell focus-forming |
| mg: | milligram |
| min: | minute |
| ml: | milliliter |
| MMTV: | mouse mammary tumor virus |
| M-MuLV: | moloney murine leukemia virus |
| mRNA: | messenger ribonucleic acid |
| MuLV: | murine leukemia virus |
| NC: | nucleocapsid protein |
| N-terminal: | amino terminal |
| PCR: | polymerase chain reaction |
| PFU: | plaque forming unit |
| pol: | polymerase |
| PR: | protease |
| R: | repeated sequences |
| RNA: | ribonucleic acid |
| SDS: | sodium dodecyl sulfate |
| SSC: | salt sodium citrate solution |
| SU: | surface protein |
| TM: | transmembrane protein |
| U3: | unique sequences 3' end |
| U5: | unique sequences 5' end |
| UV: | ultraviolet light |
| μg: | microgram |
| μl: | microliter |

# CHAPTER 1

# LITERATURE REVIEW

# 1.Retrovirus structure and life cycle

## 1.1 Classification of retroviruses

The retroviridae virus family encompasses all viruses containing an RNA genome and an RNA-dependent DNA polymerase (reverse transcriptase) (Weiss et al., 1984). The family is divided into three subfamilies: (1) Oncovirinae, encompassing the majority of retroviruses, including all the oncogenic members and many closely related nononcogenic viruses; (2) Lentivirinae, the "slow" viruses; (3) Spumavirinae, the "foamy" viruses that induce persistant infections without any clinical disease (Weiss et al., 1984).

Retroviruses cause a large range of diseases including malignancies, immunodeficiency states, and a number of organ-specific syndromes. Included in malignancies are leukemias, lymphomas, sarcomas (tumors of connective tissue) and carcinomas (tumors of epithelial origin). Retroviruses exist in the most diverse species, from fish to humans. They can be transmitted both horizontally and vertically (exogenous retroviruses). Occassionally, retroviruses are incorporated in the host DNA sequences, carried stably and often in high copy numbers in the germ line of all vertebrates and are passed on only genetically. These endogenous retroviruses are often defective, transcriptionally silent and non-pathogenic, but can be activated under certain conditions (chemical, radiation) (reviewed in Coffin, 1990).

The structure of retroviruses has been investigated by electron microscopists and are grouped into A-, B-, C-, and D-type particles (Weiss et al., 1984; Coffin, 1990). The particles are generally 60-90 nm in diameter, with an electron-luscent center surrounded by a double shell. The location of

these particles leads to a further subdivision: intracisternal and intracytoplasmic. A-type particles are intracytoplasmic and non-infectious precursors of B- and D-type. B-type particles, represented by Mouse Mammary Tumor Virus (MMTV), assemble in the cytoplasm, rather than at the plasma membrane. The nucleoprotein cores are located eccentrically within the envelope and they carry prominent spikes on their surface. The C-type, the most common form, assemble their nucleocapsid just beneath the plasma membrane, at the site of budding. They have a centrally located nucleoid and the envelope glycoproteins appear as small spikes at the electron microscope (EM) level. D-type particles are associated with both intracellular and extracellular forms having characteristics of both the B- and C-type viruses. This last group is represented by the Mason-Pfizer Monkey Virus (MPMV).

## 1.2 Structure of the virus particles and genomes

The retroviral particle is composed of an external envelope derived from the host-cell membrane but studded with viral glycoproteins. The envelope surrounds an icosahedral viral core made up solely from viral proteins, within which the viral genome is found. Also present inside the core are a set of virally-encoded enzymes that are later used for viral replication and integration of the genome into the host DNA. Retroviruses consist of ~1% RNA, ~5% carbohydrate, ~3% lipid, and ~60% protein.

The genomic RNA is a 60s - 70s dimer complex composed of two identical subunits and is approximately 9 kbp. It resembles mRNA molecules in that there is a methylated cap structure at the 5' end and a polyadenylate tract at the 3' end. The structure of the genome is described, starting at the 5' end (Fig. 1) (reviewed in Varmus and Brown, 1989; Coffin, 1990). The 5'

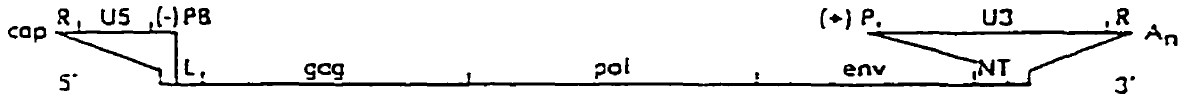



**Figure 1. Anatomy of an RNA subunit of a replication-competent retrovirus.** The important structural and genetic domains of a typical single-stranded RNA subunit (8-9 kb) of a replication-competent retrovirus are indicated by the following notations: cap, 5' "cap" nucleotide; R, terminal redundancy; U5, sequence unique to 5' terminus, present twice in viral DNA; (-) PB, binding site for (-) DNA strand primer, a host tRNA; L, leader region; gag, coding region for nucleocapsid proteins; pol, coding region for DNA polymerase; env, coding region for envelope glycoproteins; (+) P, polypurine tract that is the probable primer for the (+) DNA strand; U3, sequence unique to the 3' terminus, present twice in viral DNA; $A_n$, tract of poly(A) at the 3' terminus. Lengths of all regions are approximate and vary among retroviruses (adapted from Varmus and Brown, 1989).

end itself consists of a 10 to 230 bp long repeat called R, which is also present at the 3' end and has a crucial function during reverse transcription (see below). This is followed by the U5 sequence, 80 to 100 bp in length, which encodes no proteins, but contains at its 3' end the *att* site necessary for integration. After reverse transcription U5 becomes the 3' end of the long terminal repeat (LTR). This 3' end is determined by the primer binding site (PBS), 18 bp in length, where the previously mentioned host derived tRNA primer binds to initiate synthesis of viral DNA. Between the PBS and the start of the first structural gene, *gag*, there is a several hundred bp long leader sequence, which contains a donor site for the splicing of all subgenomic mRNA species and also codes for the $\psi$ signal that facilitates packaging of viral RNA into virions. In the Moloney Murine Leukemia Virus (Mo-MuLV) this sequence extends into the *gag* gene. All replication-competent retroviruses code for three structural proteins: *gag*, codes for the internal structural proteins, *pol* , codes for the reverse transcriptase, and *env*, encodes the envelope proteins. The order of these genes is as follows: 5' - *gag* - *pol* - *env* - 3'.

The 5' most *gag* gene, 2.0 kbp, is translated from full length RNA and encodes a precursor protein of 70 - 80 kD, which is cleaved into 3 - 5 capsid proteins that make up the core of the virus particle. In the order of their translation these include the myristylated matrix protein (MA, 15 to 20 kD) found in the matrix between the nucleocapsid and the envelope, the capsid protein (CA, 24 to 30 kD), the dominant component of viral capsid which is important for virus assembly, and the nucleic acid binding protein (NC, 10 to 15 kD).

The second gene is *pol*, (2.9 kbp). It codes for the reverse transcriptase (RT, 50 to 95 kD) with an amino-terminal RNA-dependent DNA

polymerase activity and a carboxy-terminal RNase H activity, and the integrase (IN, 32 to 45 kD).

The primary product of the *env* gene (1.8 to 4.0 kbp) is translated from a single spliced RNA, is heavily glycosylated and cleaved to form the larger amino-terminal SU (surface polypeptide) protein (46 to120 kD) on the surface of the virion where its spikes can be seen by electron microscopy. It is linked by disulfide bonds to the second, smaller, carboxy-terminal TM protein (15 to 41 kD), which contains a transmembrane region and cytoplasmic tail. TM may mediate fusion of viral and host membranes during entry of the virus into a cell.

Following *env* , there is a noncoding region of variable length which contains a polypurine tract that is important during reverse transcription.

U3 sequences are 170 to 1200 bp in length and contain multiple elements responsible for the synthesis and processing of viral RNA (reviewed in Majors, 1990). Among these are the TATA element which determines the site of transcription initiation, and other promoter and enhancer elements. In some retroviruses the polyadenylation signal is induced in U3. It also contains another *att* site at its 5' end which plays a role in retroviral integration and is an inverted repeat of its homologue in U5.

Finally, the 3' end of the genome contains another copy of the R region which can have the polyadenylation signal.

## 1.3 The retrovirus life cycle

### 1.3.1 Attachment and penetration

The retroviral life cycle begins with the entry of extracellular particles into susceptible host cells, a process that requires the attachment of virus particles to a permissive host cell via sequence cellular receptors, situated

on the cell surface and recognized by the viral envelope glycoprotein, gp70 or SU. Infection of a cell by a retrovirus generally results in interference, i.e. resistance to infection by a virus that uses the same receptor, while it remains susceptible to infection by viruses that bind to other receptors.

The receptor for the ecotropic MuLV is a basic amino acid transporter consisting of a 622 amino acid protein with multiple membrane spanning domains (Albritton et al., 1989; Wang et al., 1991). After binding the surface protein to its receptor, the virus is endocytosed into the cell, where decapsidation of the particle occurs, thus liberating the viral nucleic acid and viral enzymes (present in the viral core) into the cytoplasm, and forms a nucleoprotein complex.

1.3.2 Reverse transcription

In dividing cells, viral DNA synthesis takes place within 4 - 8 hours post-infection, while it may be incomplete in quiescent cells. This requires the presence of the plus strand RNA template, the tRNA, the activities of DNA polymerase and RNase H, and occurs in the cytoplasm within a nucleoprotein complex. It converts the single stranded RNA molecule which starts and ends with the R repeats into a double stranded DNA molecule, in which the 3' U3 region is duplicated into a 5' most position and the 5' U5 region is duplicated into a 3' terminal position. Reverse transcription is dependent on the ability of the polymerase to jump, i.e. to transfer the growing DNA chain from one R region to the other and continue replication. This reaction can be summarized in Fig 2 (reviewed in Dahlberg, 1988; Varmus and Brown, 1989; Coffin, 1990).

Reverse transcription begins in a 5' direction at the PBS close to the 5' end of the RNA, where the polymerse starts to generate a minus strand

DNA copy by using the bound tRNA as a primer; it extends this copy to the end of the genome thus replicating the U5 and R region. At the 5' end of the genome it reaches the first strong stop. At this point, the newly made short DNA molecule complexed to the polymerse transfers or jumps to the other end of the genome, where it can base-pair to the second R region. The RNase activity of RT facilitates this step by removing the already copied RNA molecule.

Following the jump, synthesis of the minus strand DNA continues to the 5' end of U3 where the RNase H activity generates a primer by cleaving the RNA template, and proceeds towards the 5' end of the genome through U3, R, and U5, until it copies the tRNA primer from which the minus strand DNA was elongated. There the nascent plus strand DNA reaches a second strong stop and jumps to the PBS of the minus strand DNA. Synthesis can now be brought to completion resulting in a full length, double stranded DNA molecule in which 5' and 3' copies of the U3, R, and U5 regions constitute the two LTRs.

1.3.3 Nuclear migration and integration

Within hours after infection, the DNA migrates to the nucleus of the cell complexed to integrase (Goff, 1992). The signals for this migration are still unknown. However, much is known about the integration process which is summarized in Fig 3. IN removes two bases from the 3' end of each strand and cleaves the host DNA target to produce staggered nicks that generate four to six base 5' overhangs. It then joins the 3' end of the viral DNA to these 5' overhangs. The remaining gaps are repaired by host DNA polymerase. Thus, two bases are lost from each end of the viral DNA and four to six bases of the host DNA are duplicated. The integrated viral

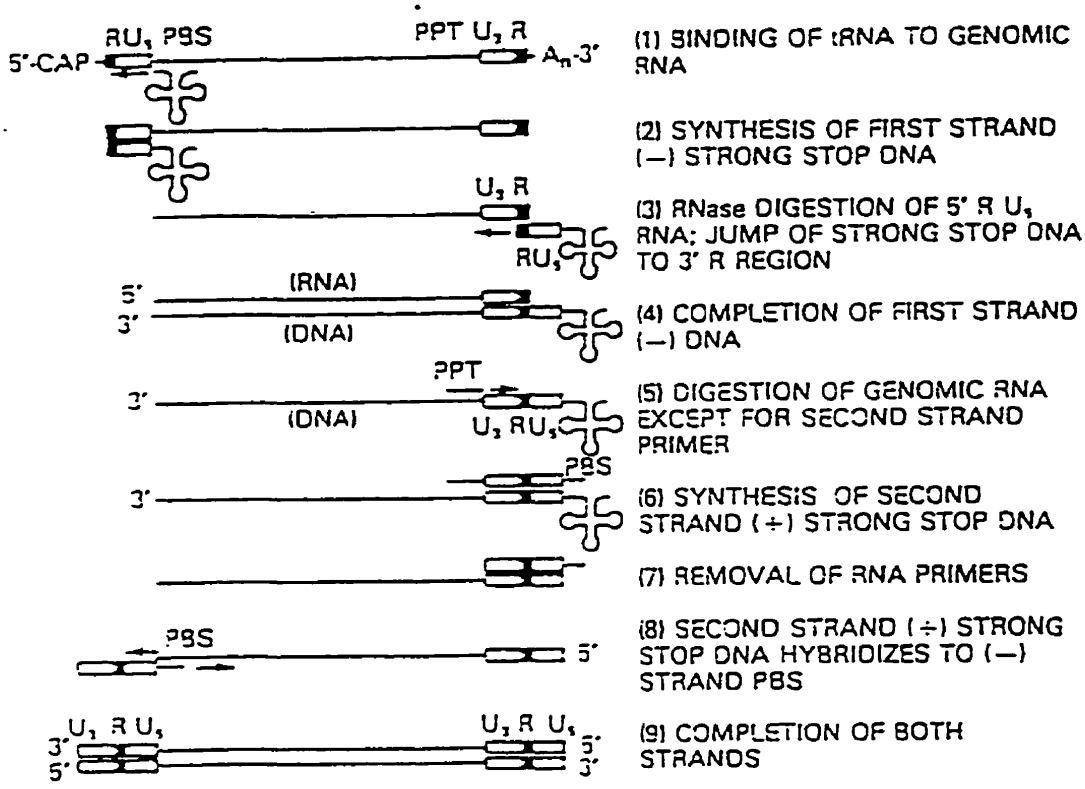



**Figure 2. Synthesis of double-stranded proviral DNA from the retroviral RNA genome.** The comments in the figure explain the sequence of events involved. Of particular importance is the production of both first strand (-) and second (+) strong stop DNA. The digestion of RNA by RNase H is followed in both cases by the movement of the strong stop DNAs to the opposite end of the genome, followed by completion of the DNA strand. This mechanism is critical to the production of the LTRs of the proviral DNA. Completion of the proviral DNA is followed by movement of the DNA to the nucleus, circularization (mediated by inverted repeats at each end of the LTR), and integration into host DNA. Typically, only a small fraction of the proviral DNA integrates, while the majority of these molecules are degraded (adapted from Dahlberg, 1988).

genome is called the provirus and is stably maintained and transmitted to daughter cells by the conventional Mendelian model. The provirus is not excised, are there is no mechanism by which it is efficiently eliminated. While target sites for retroviral integration do not share any common sequences, they are frequently found in the proximity of genomic regions that are DNase I hypersensitive and actively transcribed. Such sites are used $10^6$ times more often than expected, if integration was a random event (Varmus and Brown, 1989). Alternatively, there is also a small number of "hot spots", highly preferred sites of insertion, that may represent a significant minority of all the insertion events (Shih et al., 1988). The basis for these hot spots by viruses is uncertain.

### 1.3.4 Assembly, budding and maturation of virions

While C-type retroviruses assemble at the plasma membrane, the B- and D-type forms A-type intermediates in the cytoplasm which associate with the membrane. The assembly leads to a core consisting of two strands of full length viral RNA, host tRNA and the gene products of *gag* and *pol*. The cores associate with membrane regions containing a high density of SU and TM proteins. By budding through the membrane, they acquire an outer membrane studded with envelope proteins. The particles undergo a maturation process, in which the two RNA molecules dimerize, the *gag* and *pol* polyproteins are cleaved, and the core becomes electron dense.

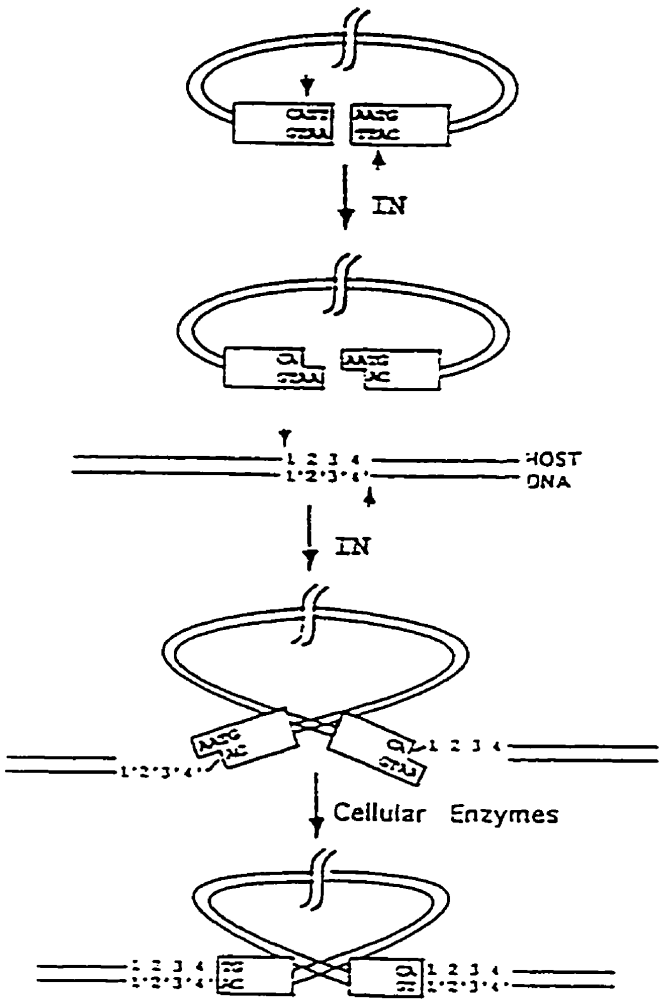



**Figure 3. The process of integration of the retroviral DNA.** The ends of the linear DNA are brought in proximity and nicked in a staggered break by the endonucleolytic action of the viral IN function. The recessed 3' ends for the viral DNA are joined to the 5' ends of the nicks. The resulting intermediate contains gaps in the target DNA flanking the provirus and the two protruding unpaired bases (AA) of the virus. Host enzymes remove the unpaired bases, fill in the gaps, and close the nicks (adapted from Goff, 1992).

## 1.4 Murine Leukemia Viruses (MuLV)

### 1.4.1 Slow-transforming Murine leukemia viruses

Slow-transforming MuLVs, also called replication-competent MuLVs, have a complete genome sructure containing all the necessary genes required for autonomous replication and propagation. Once an animal has been infected with a replication-competent retrovirus, or an endogenous provirus has been induced spontaneously, neoplasias develop with a lag of about 6 months.

Exogenous MuLVs were discovered in mice and chickens because they induce various forms of leukemias after a long latent period, but do not transform cells *in vitro*. These neoplasias do not result from the activity of viral genes, but from the activation of proto-oncogenes present in the cell. However, some MuLVs can produce changes in the host (Dulbecco and Gingsberg, 1988).

Endogenous MuLVs were recognized through observations of inbred mouse strains. Some strains were selected for a high incidence of leukemia at a young age (eg. AKR), others developed the disease infrequently and at an older age (BALB/c, C57/BL6, C3H/He); some (NIH Swiss) were essentially leukemia-free. Gross (1957) found that filtered extracts of AKR leukemic cells could transmit leukemia to newborn low-leukemia (C3H/He) animals. The agent was identified as the Gross virus. The majority of endogenous MuLVs are nonpathogenic (Weiss et al., 1984) as they are defective and not expressed.

Endogenous oncoviruses have important consequences for cells:

(1) generation of leukemia or lymphoma;

(2) contributing genetic information to other viruses by recombination;

(3) replication help for defective viruses in the form of virion proteins or polymerase;

(4) inactivation of host genes inside which they are integrated;

(5) insertion of the proviral glycoprotein into the cell membrane may confer new antigenic specificity on the cells, or resistance to infection by related retroviruses by blocking their receptors (interference). This is the mechanism of a mouse mutation (Fv-4) that renders cells resistant to ecotropic MuLVs;

(6) evolutionary spreading of the provirus through the host genome by reverse transcription like other eukaryotic transposons.

MuLVs can be classified into four groups according to how they grow in cell lines (reviewed in Dahlberg, 1988):

(1) ecotropic strains infect mouse cells, but not cell lines from other species;

(2) xenotropic viruses do not infect mouse cells, but cells of other species. The difference is in the *env* gene and the LTRs;

(3) amphotropic viruses infect murine and nonmurine cells;

(4) dualtropic MCF (Mink Cell Focus) viruses are thought to be generated by recombination between ecotropic and endogenous xenotropic sequences, thus altering host range specificity (Kelly et al., 1983; Evans and Cloyd, 1984). The tropism is due to the use of different cell recepors by the viruses.

The ecotropic MuLVs have also been classified according to whether they will or will not grow in cells derived from a particular mouse strain. This depends upon a particular allele (the n- or b-allele) of the Fv-1 gene on chromosome 4 expressed by those cells (reviewed in Jolicoeur, 1979). Viruses that grow well on NIH-3T3 cells, but poorly on BALB/c cells are called N-tropic, while viruses with the opposite growth pattern are called B-tropic. Viruses that grow in both cells are NB-tropic. Since during the infectious cycle the linear viral DNA that accumulates in the nucleus fails to

integrate when N-tropic viruses are grown on BALB/c cells, this restriction appears to be linked to integration and seems to reflect the interaction of the capsid protein (CA), and the *gag* protein, with host factors (DesGroseillers and Jolicoeur, 1983). However, the gene product of the Fv-1 gene remains unknown, but the gene has been identified and cloned.

### 1.4.2 Acute-transforming Murine Leukemia Viruses

Upon injection into a suitable host, acute-transforming MuLVs (AT-MuLVs) produce tumors within a few weeks. They are also replication-defective MuLVs but they transform fibroblastic cells *in vitro*, producing characteristic foci. This is due to the presence of an activated oncogene in the viral genome. Peyton Rous isolated the first strain of acutely transforming viruses, from a chicken with a spontaneous sarcoma, which is known as the Rous Sarcoma Virus (RSV). Many other strains were subsequently isolated in various species (Bishop, 1985).

Acute-transforming viruses are defective and all contain one or more oncogenes in replacement for normal viral sequences. The oncogenes were captured by rare recombination events with the normal proto-oncogenes of the cells, in which both viral genes and proto-oncogenes were altered. Deletions of the viral genome explain the defectiveness of these viruses, alteration of the proto-oncogenes explain its activation. The deleted genomes of AT-MuLVs, being replication-defective, require a helper for their multiplication, ATVs are then generated as pseudotypes with helper envelope.

One exception exists to this rule, and that is the Rous Sarcoma Virus, which harbors all viral structural gene (*gag*, *pol*, and *env*,) as well as additional sequence coding for the gene *src* (Weiss et al., 1985). As such,

this viruse is capable of both rapid replication and transformation, having all the gene products necessary for replication and transformation encoded within its genome.

## 1.5 Gross Passage A Murine Leukemia Virus

In 1951 Gross demonstrated that cell-free extracts from spontaneous AKR thymic lymphomas (thymomas) gave rise to similar thymomas when injected into newborn C3H/He mice having a low background incidence of the disease. Cell-free extracts were then serially passaged from C3H/He thymomas through the same strain of mice. After 15 successive passages, each one selecting for the most rapid thymoma-inducing filtrate, the final filtrate was able to induce thymomas in about 3 months in 95% of injected C3H/He mice. This highly leukemogenic extract was called Gross Passage A (GPA) (Gross, 1957).

The cell-free GPA extract was shown to contain a mixture of several MuLVs, namely ecotropic, xenotropic and MCF-type particles (Famulari, 1982). This mixture has been later passaged 24 times on NIH Swiss fibroblasts, and one type of virus has been biologically purified by end-point dilution (Buchhagen et al., 1980). This clone (Gross virus) gave a lower incidence of disease and had a longer latency period prior to onset of the disease. Nevertheless, the thymomas induced by this clone were identical to those induced by the original cell-free extract.

Recently, the ecotropic Gross virus has been molecularly cloned (Villemur et al., 1983). It appears that this virus is unable to induce thymomas by itself. However, the induction is associated with the formation of MCF-type recombinants, which would then be directly responsible for thymoma induction (Famulari et al., 1982). Recombinant DNA experiments

between the cloned Gross virus and the weakly leukemogenic Balb/c MuLV have shown that part of the *env* region, as well as the LTR of the Gross virus are responsible for its high leukemogenic potential and induction of thymomas (DesGroseillers et al., 1983). Therefore, the prolonged latency period for the biologically and molecularly cloned virus could be explained by the fact that more time was necessary after inoculation in order to generate the MCF-type recombinants, which would then directly induce thymus malignancies. Such MCFs may have been present in the original cell-free extracts obtained by Gross, which had shorter latencies than the cloned Gross virus.

## 2. The activation of cellular genes by insertional mutagenesis

### 2.1 Introduction

As seen in the previous section, retroviruses may be viewed as naturally occurring, self-propagating mutagens of humans and animals. They exert mutagenic effects by integration into host genome, a natural step in the viral replication cycle. Retroviral integration disrupts the host genome and may lead to activation or inactivation of cellular genes. Acute-transforming retroviruses will induce disease within 2 - 3 weeks post-infection. While slow-transforming retroviruses cause tumors with a latency of 3 - 9 months (Varmus, 1984; Bishop, 1987).

Studies of some diseases caused by slow-transforming retroviruses led to the observations that the tumors were clonal descendents of a single infected cell (Steffen and Weinberg, 1978; Cohen et al., 1979). Hayward et al., (1981) suspected that cellular oncogenes might be targets for proviral

integration. They screened ALV-induced bursal lymphomas in chickens for rearrangements of known oncogenes and found that most lymphomas contained provirus integrated in the 5' region of the c-*myc* oncogene.

The discovery that insertional mutagenesis plays an important role in oncogenes led to the use of provirus-tagging as a method to identify genes involved in this process. The strategy for identifying the relevant cellular genes is analogous to transposon-tagging, first applied by Drosophila geneticists (Bingham et al., 1981). Viral-host DNA junction fragments from one tumor are cloned and probes are derived from the cellular sequences flanking the proviruses. The presence of proviruses in a common region detected by the cellular probes in multiple, independent tumors, is taken as evidence for the presence of a cellular gene where activation has given the cell a growth advantage. The argument is based on the assumption that retrovirus integration sites in the cellular genome are too numerous to make detection of proviruses in the same genomic region in different tumors a frequent event, if no selective pressures are involved. Therefore, a common integration site indicates selection of one cell on its way to a tumorigenic state out of a large population of infected, but otherwise normal cells. This approach has led to the identification of new oncogenes (Table 1), revealed potential oncogene interactions during multistep process of oncogenesis, and provided clues of some aspect of gene regulation.

## 2.2 The mechanisms of activation of cellular genes

### 2.2.1 Promoter insertion

In this model, transcription of the cellular gene is under control of the LTR promoter.

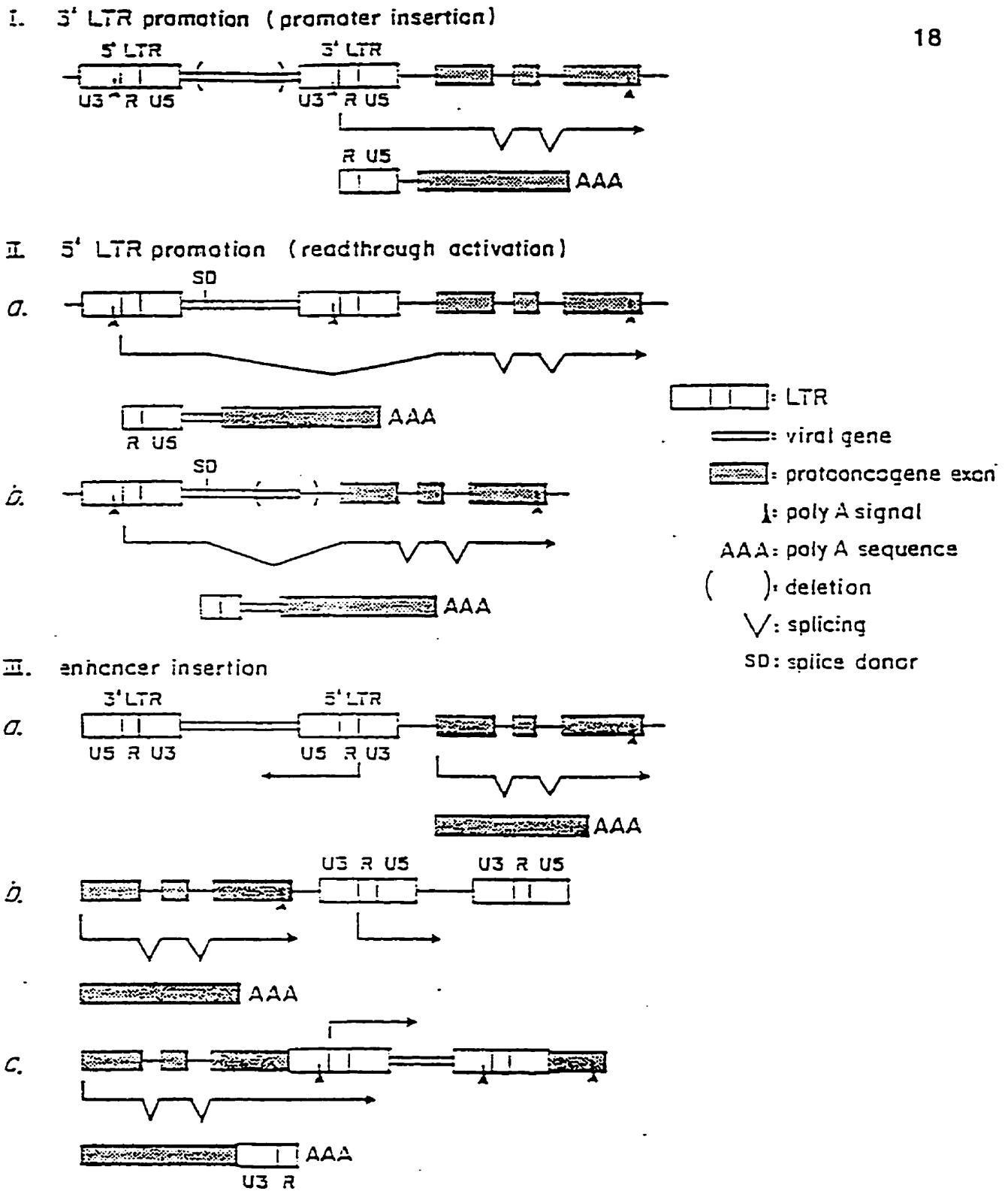



**Figure 4. The mechanisms of insertional activation of proto-oncogenes.** Arrows indicate direction of transcription, other symbols are defined above. Exon followed by AAA represents the mature transcripts of the the activated proto-oncogenes (figure adapted from Kung et al., 1991).

**Table 1: Common insertion sites** (adapted from van Lohuizen and Berns, 1990)

| Tumor | Insertion Site | Retrovirus | Frequency of Insertion | Mode of Activation |
|---|---|---|---|---|
| Erythroleukemias | *sfpi*-1 | SFFV | 95% | E |
| | *fli*-1 | F-MuLV | 75% | ? |
| | p53 | SFFV/F-MuLV | | E/I |
| Myeloid leukemias | c-*fms*/*fim*-2 | E-MuLV | 20% | E |
| | *evi*-1/*fim*-3/CB-1 | MuLV/F-MuLV | 100% | E |
| | *evi*-2 | MuLV | 10% | ? |
| | *fim*-1 | F-MuLV | 5% | ? |
| | GM-CSF | SFFV/R-MuLV | 35% | E |
| | multi-CSF(IL3) | SFFV/R-MuLV | 18% | E |
| | N-*myc* | MuLV | 3 cases | E |
| | *fis*-1 | F-MuLV | 5-10%* | ? |
| T cell lymphomas | c-*myc* | MuLV | 20-40% | E |
| | *mis*-1/*pvt*-1,*mlvi*-1 | MuLV | 20-30% | ? |
| | *mlvi*-2 | MuLV | <10% | ? |
| | N-*myc* | MuLV | 35% | E |
| | *pim*-1 | MuLV | 15-45% | E |
| | *lck* | MuLV | <10% | P |
| | c-Ha-*ras* | MuLV | 1 case | ? |
| | *gin*-1 | MuLV | 25% | ? |
| | *fis*-1 | F-MuLV | 5-10% | ? |
| B cell lymphomas | *pim*-1 | MuLV | 25% | E |
| | *evi*-1 | MuLV | 1 case | ? |
| | *ahi*-1 | A-MuLV | 16% | ? |
| | *bmi*-1 | MuLV | 25% | E/P |
| Mammary carcinomas | *wnt*-1(*int*-1) | MMTV | 30-80% | E/P |
| | *int*-2 | MMTV | 5-65% | E/P |
| | *int*-3 | MMTV | 8-40% | ? |
| | *wnt*-3(*int*-4) | MMTV | 10% | E |
| | *wnt*-4(*int*-5) | MMTV | ? | ? |

*, % difference (depends on host strain); E, enhancement; P, promoter insertion; I, inactivation due to insertion.

(1) 3' LTR promoter insertion (Fig. 4). Tumors have the provirus inserted at sites clustered at the 5' end of a proto-oncogene. The provirus is oriented in the same transcriptional direction as the gene. The transcript for the activated oncogene begins with R and U5 regions of the 3' LTR and contain no other viral sequences (Peters, 1990; Van Lohuizen, 1990; Kung et al., 1991).

(2) 5' LTR promotion (readthrough activation) (Fig. 4). This mechanism shares some characteristics with 3' LTR promotion, such as the clustering of proviruses at the 5' end of the gene. However, the proviruses involved in this activation remain intact and the oncogene transcripts usually contain leader sequences. Transcription extends through the viral genome as well as through cellular sequences downstream from the insertion site. Most of the primary transcripts are cleaved near the poly A signal of the 3' LTR to generate viral genomic RNA and spliced viral messenger RNAs. Approximately, 10 - 15% of primary transcripts, however, escape polyadenylation at the 3' LTR site and use a poly A signal of the downstream proto-oncogene. A readthrough transcript is generated including both the viral genes and the proto-oncogene. Splicing a donor site of the virus to an acceptor site of the proto-oncogene can result in a functional mRNA for an oncogenic fusion protein. DNA for viral 3' sequences (including the 3' LTR) is sometimes deleted which may increase the level of proto-oncogene message by eliminating interference due to polyadenylation at the 3' LTR site (Lazo ans Tsichlis, 1990; Peters, 1990; Van Lohuizen and Berns, 1990; Kung et al., 1991).

Activation by promoter insertion has been described for the following: c-*myc* (N), c-*erb* B, c-*myb* (N), c-Ha-*ras*, c-Ki-*ras*, c-*rel*, c-*lck*, *Mlvi*-4, *Evi*-1.

2.2.2 Enhancer insertion mechanism

Provirus insertion near a cellular gene may occur at a 3' position relative to the gene and in the same transcriptional orientation. Alternatively, it may occur 5' of the gene and in the opposite transcriptional orientation. The integrated provirus can either be full length or have internal deletions in various regions. The net effect of these proviral insertions is the transcriptional activation of a normally silent gene or transcriptional enhancement of a gene expressed normally at lower levels in the tissue of origin of the tumor. In either case, the gene is transcribed from its own promoter. If the transcription of the gene is normally under the control of more than one promoter, provirus integration may shift the balance of transcription toward one of them. This may be due to the interaction of the introduced viral enhancer with neighboring promoters. Insertion at sites up to 300 kbp away from a proto-oncogene has recently been reported (Lazo and Tsichlis, 1990; Tsichlis, 1990; Kung et al., 1991)

Activation of genes by enhancer insertion has been described for c-*myc*, N-*myc*, c-*mos*, *Pim-1*, *Spi-1*, *int-1/Wnt-1*, *int-2*, *int-4/Wnt-3*.

2.2.3 Truncation of gene: Synthesis of an abnormal gene product

Most of the proviral activation events observed in tumors do not perturb the structure of the gene product. However, in some instances, (i.e. c-*Erb* B, N-*myc* and c-*myb* ) provirus insertion occurs within the coding sequences or within an intron between two coding exons of an oncogene. The net result of these insertions is the synthesis of mRNA transcripts coding for proteins that are truncated either at their $NH_2$-terminal or COOH-terminal regions. ALV insertions in the c-*Erb* B oncogene lead to expression of an amino truncated EGF - receptor protein (Downward et al., 1984; Nilsen et al., 1985). The truncation removes most of the ligand-binding domain of the

receptor, thereby constitutively activating the signal-transducing kinase domain. Proviral insertions in c-*myb* also result in truncation of the myb protein. Analysis of acute-transforming viruses that transduce myb sequences indicate that v-*myb* is truncated at both 5' and 3' ends in these viruses. Similarly, the c-*myb* gene is truncated to the same extent at the 5' end in MLV-induced myeloid tumors, where viruses integrate upstream of the first v-*myb* homologous exon (Mushinski et al., 1983; Shen-ong et al., 1986). Carboxy-terminal truncations of c-*myb* have also been reported (Shen-ong et al., 1986) in the intron between c-*myb* exons 6 and 7 giving rise to mRNAs coding for a truncated c-*myb* protein product. All of the known proviral integrations do not interrupt coding domains, although viral insertions outside coding regions could potentially influence splicing patterns (Shen-ong, 1987; Rosson et al., 1987; Watson et al., 1987) and/or translational initiation (Marth et al., 1988).

2.2.4 Stability of RNA message

Although most cases of enhancer insertion do not perturb the coding regions of the gene, there are some examples in which the integration has occurred within the transcriptional unit and altered the structure of mRNA. Provirus insertion in the 3' untranslation region of a given oncogene, in some transcriptional orientation , results in transcription starting at the proto-oncogene promoter and polyadenylation occurring at the integrated viral 5' LTR polyadenylation site, thus removing 3' untranslated sequences from the proto-oncogene mRNA. In the case of *Pim-1*, activated in MuLV-induced lymphomas, this truncation results in the removal of AU-rich motifs, which can confer instability to mRNAs (Stelten et al., 1986; Domen et al., 1987). The net effect may be increased stability of the mRNA leading to an increase

in steady-state level of *Pim-1* transcripts in these tumors. Another gene that may be activated by a similar mechanism is N-*myc*. Provirus insetion occur within 100 bp of the translation stop codon, thus removing AU - rich motifs as well as the other evolutionary conserved domains (van Lohuizen et al., 1989). Another example has shown removal of 6 aa of the N-*myc* protein (Setoguchi et al., 1989).

2.2.5 Long distance gene activation

In the examples discussed in the preceeding sections, provirus insertions affect the expression or structure of genes in its immediate vicinity. In other cases, provirus insertion may affect the expression of genes over large distances, up to 300 kbp away (Peters, 1990; Tsichilis et al., 1990; Van Lohuizen and Berns, 1990; Kung et al., 1991). The best studied example of this phenomenon is the activation of c-*myc* in murine T cell lymphomas mediated through integrations in the *Mlvi-4* or *Mlvi-1/mis-1/pvt-1* loci, located 30 and 270 kbp 3' of the c-*myc* gene, respectively (Tsichlis et al., 1989, 1990; Lazo et al., 1990b). Cell hybrids were constructed between rat thymic lympoma cells bearing integration in either *Mlvi-1* or *Mlvi-4* and a murine T cell lymphoma line. The purpose of these cell fusions was to segregate the normal rat chromosome 7 (which contain c-*myc*, *Mlvi-1* , and *Mlvi-4* ) from the rearranged allele, and to see if the expression of c-*myc* was affected by one or the other proviral integration site (Lazo et al., 1990). A correlation was found among the expression of c-*myc*, *Mlvi-1* and the presence of proviral integrations in *Mlvi-4*. All cell hybrids retained both the normal mouse and rat c-*myc* alleles, but the rat c-*myc* was expressed only in hybrids having the rearranged *Mlvi-1* or *Mlvi-4* locus. The levels of c-*myc*

was undetectable in hybrids with the non-rearranged alleles. This led to the conclusion that elevated expression of rat c-*myc* is linked to proviral rearrangements of two distant loci.

Other examples of long distance activation of oncogenes by provirus integration includes activation of *Evi-1* by provirus integration in the *Fim-3/Cb-1* locus (Bartholomew et al., 1989) and the activation of the *Lyt-2* gene (CD8) by provirus insertion approximately 35 kbp 5' of the *Lyt-2* promoter (Tsichlis and Lazo, 1991).

These long range effects could represent cases in which the chromatin structure was altered, and thus allowing the "looping" of large DNA domains to bring enhancers close to promoter sequences.

2.2.6 Chromosomal rearrangements by homologous recombination between integrated proviruses

Provirus insertion may be identified by gross chromosomal rearrangements due to homologous recombination between integrated proviruses as in the case of a Mo-MuLV-induced rat T cell lymphoma (Lazo and Tsichlis, 1988). This tumor contained an integrated provirus 5' of the first exon of c-*myc* in a transcriptional orientation opposite to the gene and a second provirus 3' of the gene (Lazo and Tsichlis, 1991). The two proviruses integrated in opposite orientations. Homologous recombination between the 3' LTR of the c-*myc* provirus and the 5' LTR of the provirus distal to c-*myc* gave rise to an intrachromosomal inversion. One of the products of this recombination event was characterized and found to consist of an aberrant LTR structure flanked on one side by c-*myc* and the other side by cellular sequences derived from the site of integration of the second provirus (Lazo and Tsichlis, 1988).

Another gross chromosomal rearrangement due to recombination between integrated proviruses was detected in Mo-MuLV-induced murine T cell lymphoma. This tumor contained an integrated provirus in the intron between the 5' non-coding exon and the first coding exon of c-Ha-*ras* on mouse chromosome 7. Recombination between this and another provirus on the same chromosome resulted in the translocation of the c-Ha-*ras* coding sequences and enhanced expression of the gene. The translocated gene had the ability to transform fibroblasts in culture (Ihle et al., 1989). A similar type of rearrangement due to homologous recombination between integrated proviruses was also detected in an ALV-induced chicken lymphoma (Nottenburg et al., 1987; Lazo and Tsichlis, 1991).

## 2.3 The inactivation of cellular genes by retroviral insertional mutagenesis

Tumor development is associated with mutations in two types of genes, oncogenes and anti-oncogenes (recessive oncogenes) (Knudson, 1985). Mutations affecting oncogenes are usually dominant and they are associated with overexpression or enhancement of the biological activity of the gene product. Conversely, mutations affecting anti-oncogenes are usually recessive and associated with lack of expression of the anti-oncogene product. Alternatively, anti-oncogenes may suffer dominant negative mutations, where the mutant allele of the anti-oncogene produces a product that competes with the product of the normal allele (Lane and Benchimol,1990).

To date, only one anti-oncogene, *p53*, is known to be the target of insertional mutagenesis in retrovirus-induced tumors. Most tumors carrying

insertion mutations of *p53* lack a normal *p53* allele. Proviral integrations are frequently found in the *p53* gene in Abelson MuLV-induced pre-B cell lymphomas, (Wolf and Rotter et al., 1984; Rotter et al., 1984) and in Friends-MuLV-induced erythroleukemias (Mowat et al., 1985; Chow et al., 1987; Kovinski et al., 1987). While in some cases proviral integration completely abolishes *p53* expression (Hicks and Mowat, 1988; Ben-David et al., 1988), in most instances high levels of *p53* proteins are induced (Rovinski et al., 1987; Ruscetti and Scolnick, 1983). The overexpressed *p53* protein contains mutations, which can alter both the stability and/or the conformation of the protein (Hinds et al., 1987). Several reports indicated that the loss of function by mutation in *p53* is involved in erythroleukemia progression (Ben-David et al., 1988; Ben-David and Bernstein, 1991). Further studies show that the erythroleukemias induced by Friend virus result from the accumulation of a number of genetic changes, including activation of members of the *Ets* gene family (*Spi-1 / Pu-1* and *Fli-1*) and inactivation of the *p53* gene (Ben-David and Bernstein, 1991). Retroviral insertional activation of the *Fli-1* is the first detectable genetic event in F-MuLV induced primary erythroleukemias. Mutations in *p53* gene were observed in the Epo-dependent cell lines at late stages of tumor development (Howard et al., 1993). These data suggest that activation of the *Fli-1* gene may alter the self-renewal probabilities of erythroid progenitor cells and that *p53* mutations immortalize these cells, enabling them to grow *in vitro* in the presence of Epo.

## 2.4 Genes affected by provirus insertion

Provirus integration affects the expression of the structure of genes involved in the transduction of proliferation or differentiation signals from the cell membrane to the nucleus. These genes can be classified in at least 4 functional categories: (1) genes coding for growth factors; (2) growth factor receptors; (3) membrane associated or cytoplasmic proteins involved in signal transduction; (4) nuclear proteins, most of which are known to have transcription factor activity. A list of some of these genes are presented in Table 1.

# 3. Spermatogenesis

## 3.1 Introduction

Spermatogenesis is a unique and continual process in mammals whereby stem cells divide mitotically to become cells that either remain quiescent or give rise to those that undergo a series of mitotic and meiotic divisions, culminating in the onset of a remarkably complicated differentiating pathway. It is this differentiating process, called spermiogenesis, that results in the formation of immature spermatozoa. The frequency of stem cell division is tightly controlled in rodents. Only each 13 days do unknown environmental and/or genetic cues result in mitosis, and whereas one daughter cell begins its path to produce many genetically identical spermatogonia, the other cell becomes arrested before it divides and retains stem cell potential. Because of the incredibly intractable nature of the process, little is known about the molecular mechanisms that control spermatogenic stem cell renewal.

However, numerous studies have described in detail the dramatic morphological changes that the differentiating cells undergo and the unique structures that appear during the development and maturation of the male gamete. These structural data provide an excellent framework on which to study the changes in testicular gene expression that underlie the developmental events. Moreover, the testis provides a superior system to study certain eukaryotic control mechanisms. The ordered differentiation of germ cells allows analysis of the temporal regulation of gene expression for a series of defined proteins in a terminally differentiating system. Furthermore, because transcription terminates early during spermiogenesis, the translational regulation of stored mRNA is essential to synthesize many

of the proteins appearing during this haploid phase of spermatogenesis. A more detailed understanding of the molecular aspects and of the biochemistry of spermatogenesis will disclose many essential regulatory mechanisms unique to the development of the male gamete.

In this section, the literature on spermatogenesis and gene expression during spermatogenesis will be examined. Many excellent reviews of the biochemistry of spermatogenesis (Bellvé, 1979; Bellvé and O'Brien, 1983) and aspects of hormonal regulation (Steinberger, 1971; Fritz, 1978) are available. Therefore, most of this presentation will be restricted to an overview of spermatogenesis, with specific attention to spermiogenesis, as this process occurs without cell division and is one of the most phenomenal cell transformations in the mammalian body. As well, gene expression from both the diploid and haploid genomes during mammalian spermatogenesis will also be examined.

## 3.2 Scheme of spermatogenesis

The mammalian testis contains 2 discrete morphological compartments, interstitial tissue and the seminiferous tubules. Interstitial tissue forms the minor compartment and is composed primarily of vascular, lymphatic and connective-tissue elements, macrophages, fibroblasts and the androgen secreting interstitial cells of Leydig. The seminiferous epithelium forms the major compartment and contains the differentiating germ cells intercalated between the supporting Sertoli cells. Spermatogenesis is a continuum of germ cell differentiation which occurs in 3 principle stages: (1) stem cell differentiation and renewal; (2) meiosis; (3) spermiogenesis. The sequence of spermatogenesis in the mouse is presented in figure 5.

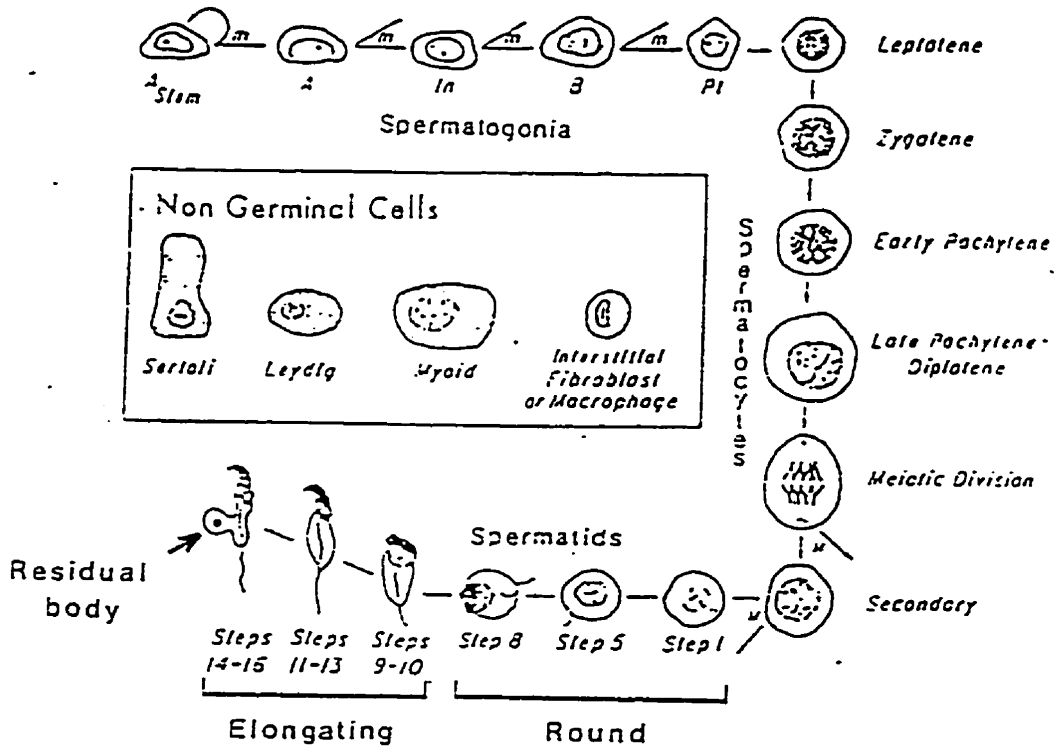



**Figure 5. Schematic diagram of mouse testicular germ cells and somatic cells.** Mitotic and meiotic divisions are indicated by m and M respectively. Other abbreviations: Stem cells, ($A_{stem}$); type A spermatogonia, (A); intermediate spermatogonia, (In); type B spermatogonia, (B); preleptotene spermatocytes, (Pl) (adapted from Meistrich, 1977).

3.2.1 Stem cell differentiation and renewal

During fetal development, primordial germ cells migrate to the genital ridge, where they continue to proliferate (Eddy, 1970; Jeon and Kennedy, 1973; Spiegelman and Bennett, 1973; Zamboni and Merchant, 1973). After a period of substantial cell growth and proliferation (Clark and Eddy, 1975), the germ cells, now called gonocytes or prespermatogonia, enter a nonproliferative growth phase and move to a position adjacent to the basal lamina of the seminiferous cords, where they differentiate to a transitional cell type, primitive spermatogonia (Baille, 1964; Franchi and Mandel, 1964). These cells, after several mitotic divisions, serve as progenitors of the definitive spermatogonia that produce primitive type-A spermatogonia and later, the presumptive type-A spermatogonia. It should be noted that the precise mechanism by which stem cells are continuously produced, giving rise to the differentiating type-A spermatogonia has not been resolved. One model by Clermont and Bustos-Obregon (1968) suggested that all type-A spermatogonia could be divided into 2 classes: The renewing stem cells (type $A_1$-$A_4$ spermatogonia) and the reserve stem cells ($A_0$ spermatogonia). Types $A_1$-$A_4$ would be responsible for forming more mature germ cells while $A_0$ spermatogonia would remain dormant, proliferating only upon destruction of the renewing stem cells. In contrast, Huckins (1971) proposed 3 categories of spermatogonia: (1) stem cells ($A_{isolated}$ or $A_{is}$); (2) proliferating cells ($A_{paired}$ or $A_{pr}$, $A_{aligned}$ or $A_{al}$); (3) differentiating cells ($A_1$-$A_4$, Aintermediate or $A_{in}$, B). The $A_{is}$ spermatogonia would divide occasionally and form either more $A_{is}$ or $A_{pr}$ cells. Once type $A_{pr}$ spermatogonia were generated, they would mature in the following sequence of spermatogonial cell types:

$A_{pr}$ --> $A_{al}$ --> $A_1$ --> $A_2$ -->$A_3$ --> $A_4$ --> $A_{in}$ --> B.

No matter what the preferred model, in mouse, all type-A spermatogonia appear in the seminiferous epithelium 7 days postnatally, and type-B spermatogonia are present in the testis by day 9 (Nebel, Amarose, and Hackett, 1961; Clermont, 1972; Bellvé et al., 1977). These early germ cell types are actively engaged in mitosis and constitute the mitotic phase of spermatogenesis. Little is known at the molecular level of the regulatory processes in spermatogonial differentiation, a time interval that is difficult to study.

3.2.2 Meiosis

During the second major phase in the approximately 34 day cycle producing spermatozoa in mice, type-B spermatogonia divide to form preleptotene primary spermatocytes, initiating the meiotic phase of spermatogenesis (Monesi, 1962). In mouse and rat species, each germ cell must undergo 6 mitotic divisions in the progression from spermatogonia to spermatocyte (Monesi, 1962; Clermont and Bustos-Obregon, 1968). The final round of semiconservative DNA replication occurs in preleptotene spermatocytes to increase the DNA content from 2N to 4N. Preleptotene spermatocytes differentiate into leptotene spermatocytes whose appearance signals the initiation of meiotic prophase. During this time, the chromatin condenses into chromosomes which remain unpaired. Pairing between homologous chromosomes occurs in the synaptonemal complexes of zygotene spermatocytes which succeed the leptotene cells. It is during the leptotene and zygotene stages of meiosis that the spermatocytes translocate from the basal compartment to the adluminal or central compartment of the seminiferous epithelium (Dym and Fawcett, 1970; Fawcett, Ross, 1977). The

duration of the first meiotic division is long, requiring approximately 12 days to complete (Kofman-Alfaro and Chandley, 1970). Zygotene spermatocytes then give rise to pachytene spermatocytes, cells in which sex vesicles and complete synaptonemal complexes can be observed and where chromosomes undergo homologous recombination. Finally, sufficient changes occur which trigger two more meiotic divisions, thus reducing the nuclear DNA content in the primary spermatocyte from a functional 4N level to N level in round spermatids. This haploid cell can never again divide but serves as the most immature cell in the spermiogenic differentiation pathway.

Several events that occur during the life of a spermatogonium are quite unusual and are undoubtedly programmed to ensure survival of several genetically identical germ cells. First, all of the offspring of a single cell develop as a clone or cohort (Huckins, 1972). The cells within each cohort remain attached by cytoplasmic bridges that allow the transfer of molecules, including proteins. Division within a cohort is synchronous and a certain number of such clones eventually die and degenerate at an identical stage within the cell cycle. The molecular principles that govern this unique form of programmed cell death are unknown (Russell et al., 1990). Second, the first meiotic cell becomes arrested in the cell cycle at metaphase II but continues to undergo remarkable morphological and biochemical changes. Chromatin condenses and the nucleus becomes quite large and distinct. Accompanying these morphological changes are an amazing array of transcriptional events. Nuclear RNA synthesis is extremely active in the mid to late pachytene spermatocyte stages (Monesi, 1964). Third, prior to completion of the first meiotic division, the cells pass from the basal to adluminal compartment (as mentioned earlier) within the seminiferous

tubule (Dym and Fawcett, 1970). The developing germ cells that can no longer divide mitotically thus become protected from the environment due to the "blood-testis barrier" that separates the 2 compartments. This barrier is formed by tight junctions between the adjacent Sertoli cells, within which the developing germ cells are imbedded. Finally, the entire process has evolved to function effectively at a temperature nearly 5°C lower than that of the body (Waites, 1970).

3.2.3 Spermiogenesis

The phase of spermiogenesis, an interval of about 14 days in the mouse (Nebel et al., 1961), is the period where the haploid round spermatid differentiates to the mature spermatozoon. Many unique morphological transformations occur, including remodeling and condensation of the nucleus into specie-specific shapes, assembly of the tail, reduction of number and localization of the mitochondria of the cell to the midpiece region of the developing sperm, formation of the acrosome in the nucleus, and topographical changes in the cell surface. Spermiogenesis can be subdivided into steps based on the morphological changes which occur in the acrosome forming adjacent to the spermatid nucleus. The number of steps of spermiogenesis varies amongst species so that in the mouse there are 16 steps whereas in the rat there are 19 steps (Leblond and Clermont, 1952; Oakberg, 1956). The duration of spermatogenesis from type A spermatogonia to spermatozoa also varies amongst species, requiring 34.5 days in the mouse and 48 days in the rat. (Oakberg, 1956)

(A) Nuclear shaping and condensation

Up to a certain point in spermiogenesis, the nucleus of the spermatid is roughly spherical. Thereafter, the sperm head of each species attains its

characteristic shape, i.e., the mouse head is sickle shaped. It has been argued that the nuclear shape may be the result of a pattern of chromatin condensation which is intrinsic to each species (Fawcett, Anderson and Phillips, 1971). Nuclear DNA packing is caused by changes in histones and other specific basic proteins that associate with DNA (Fawcett, Anderson and Phillips, 1971). The nuclear volume decreases substantially as the result of tighter packing of DNA and the probable elimination of fluid from the nucleus, creating a hydodynamically streamlined sperm head.

(B) Development of an acrosome

Although the general features of acrosomal development are similar in all mammals, each species differs from one another in fine details of acrosome formation and in the final shape of the acrosome over the sperm head. Acrosomal formation is a slow but continous process that is not complete until late spermiogenesis (Lalli and Clermont, 1981; Leblond and Clermont, 1952).

The most immature spermatids contain no acrosome, but show only a perinuclear Golgi apparatus. Shortly after the spermatids are formed, the Golgi apparatus is involved in producing small condensing or proacrosomal vacuoles, that contain dense material or proacrosomal granules. One to four very small membrane-enclosed proacrosomal granules eventually unite within one large membrane bounded vesicle containing a single granule known as the acrosomal vesicle. The acrosomal vesicle is rounded until it makes contact within the nucleus. Shortly thereafter, it indents the nucleus, becomes flattened on the side that makes contact with the surface of the nucleus and spreads out over the surface. The acrosomal granule remains dense, while the other material within the acrosome is less dense. The Golgi

apparatus is positioned nearby contributing more and more material to the developing acrosome. The membrane of the acrosomal vesicle in contact with the nucleus is called the inner acrosomal membrane and that not in contact with the nucleus is the outer acrosomal membrane. Acrosomal development is illustrated in figure 6.

Through an unknown mechanism, the spermatid nucleus moves to the cell surface (Russell et al., 1983). Here, the acrosomal region of the spermatid becomes tightly apposed to the cell surface (plasma membrane). This imparts polarity to the cell, signalling segregation of the head or nuclear (anterior) region of the cell from the tail or flagellar (caudal) region. Concomitantly, the cell begins to take on an elongate shape as the cytoplasm is stretched out along the flagellum. As time passes, the nucleus progressively elongates. The Golgi apparatus moves away from the acrosome and migrates to the caudal aspect of the cell, illustrated in figure 7. At this point, the acrosome appears not to grow further in mass, but undergoes a gradual process of increase in density. The acrosome is considered to be a sac of secreted enzymes necessary for penetration of the egg.

(C) Elimination of the cytoplasm

The spermatid is reduced in volume to approximately 25% of its original size before sperm release. Since the cell is smaller and streamlined, the motility apparatus is capable of propelling it through the fluid environment.

There are at least 3 phases in the process of making spermatids smaller and more streamlined. First, water may be eliminated from the nucleus and cytoplasm during the elongation of the spermatid. As a result,

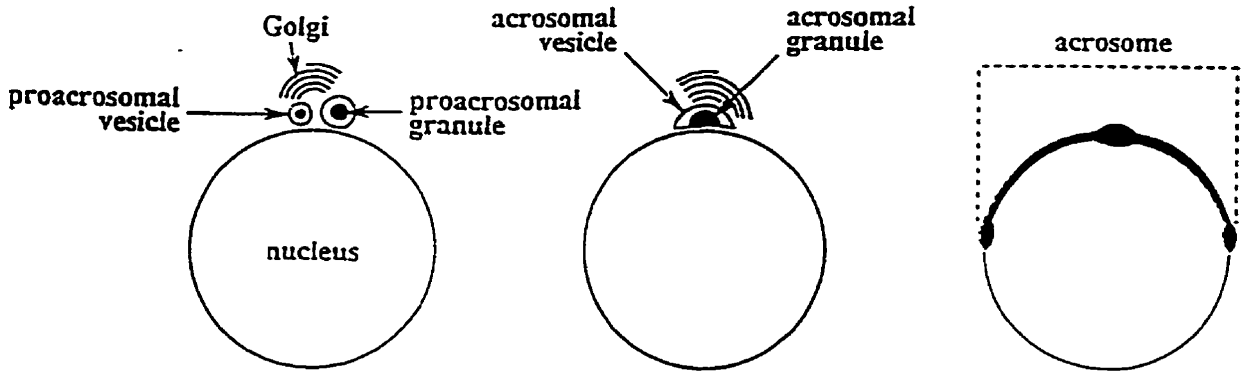



**Figure 6. Acrosomal development.** The proacrosomal vesicle(s) are the membrane bound elements, each of which contains a dense substance, the proacrosomal granule. The proacrosomal vesicle(s) may or may not touch the nucleus. The proacrosomal vesicle(s) coalesce to form a single acrosomal vesicle containing an acrosomal granule, the latter contacting the nucleus and flattening out over its surface. Together, the acrosomal vesicle and the acrosomal granule form the acrosomic system or acrosome (adapted from Russel et al., 1990).

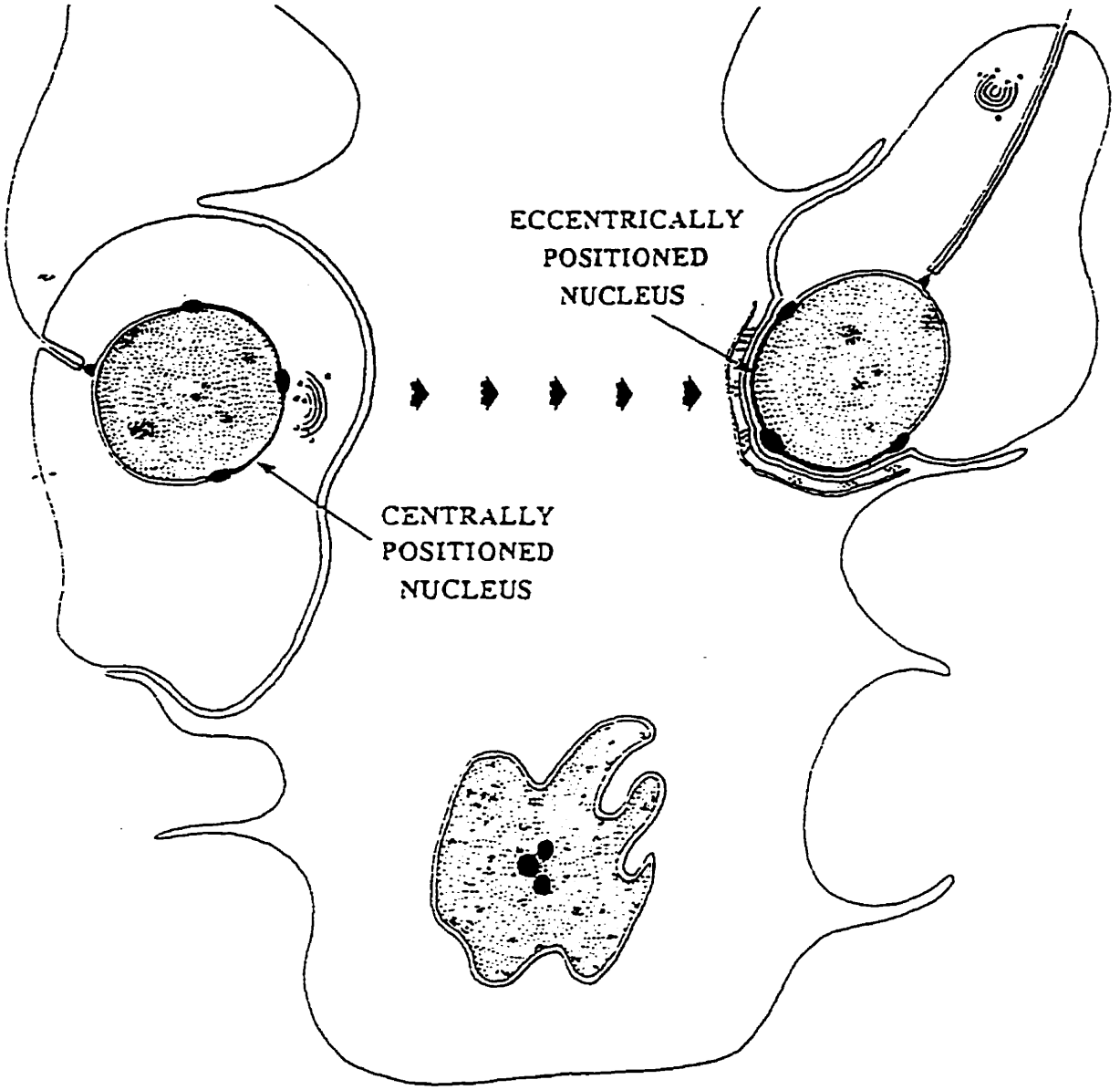



**Figure 7. Changes in the spermatid at mid-spermiogenesis.** The nucleus of the spermatid moves towards the cell surface. The acrosomal region of the nucleus abuts the spermatid plasma membrane and rotates within the cell to face the basal aspect of the Sertoli cell. The flagellum is seen at the opposite pole of the cell. These changes accomplished, the spermatid shows both polarity and a specific orientation within the seminiferous epithelium (adapted from Russell et al., 1990).

elongate spermatids may appear more dense than other cells. Second, some cytoplasm is eliminated just before sperm release by a minute structure called tubulobulbar complex (Russell and Malone, 1980). Third, the separation of a cytoplasmic package, the residual body, is responsible for about one-forth the volume reduction. Residual bodies are formed at sperm release as a lobe of cytoplasm is pinched off from the spermatid (Fawcett and Phillips, 1969; Russell, 1984). Residual bodies contain packed RNA and other packed organelles and inclusions which were used earlier by the spermatid but are no longer necessary for sperm survival after sperm have been released. These residual bodies are phagocytized by the Sertoli cell and transported to the base of the tubule where they are then digested by the Sertoli cell (Kerr and de Krester, 1974). After cytoplasmic elimination, a small amount of cytoplasm, the cytoplasmic droplet, remains around the neck of the spermatid.

(D) Development of the flagellum

The first evidence of a flagellum is seen in the very youngest spermatids. After the migration of the centriole pair to the cell surface, one of the pair forms an axoneme, a structure containing microtubules (9 + 2 arrangement) that causes the spermatid plasma membrane to protrude from the cell. The delicate forming flagellum rapidly extends into the tubular lumen. The centriole pair forming the flagellar axoneme moves from the surface of the cell to the nucleus and indents the nucleus. Although the flagellum may become implanted on any non-acrosomal area of the spermatid nucleus, it eventually becomes positioned directly opposite the acrosome, thus establishing acrosomal and flagellar poles of the nucleus.

Accessory components are added to the flagellum to form its middle, principle, and end pieces (Fawcett, 1975). Mitochondria are recruited from the cytoplasm to form a helical pattern around the middle piece (or mid-piece) of the flagellum. Outer dense fibers form in the mid-piece and principle pieces and a fibrous ring also forms in the principle piece. The various components are illustrated in figure 8. Flagellar development is a continuous process, lasting from the very start of spermiogenesis until sperm release. Although the flagellum imparts motility to the cell, sperm released in the testis are essentially immotile. The capability of motility is developed in the epididymis and not until sperm are deposited in the female reproductive tract are they vigorously motile.

3.2.4 Cycles, stages and waves in spermatogenesis

Leblond and Clermont (1952) have studied numerous cross-sections of seminiferous tubules and have shown that germ cell types are grouped into "cellular associations" As such, the least mature germ cell type of the cellular association are located towards the basement membrane of the tubule whereas the more mature cells are toward the lumen. Each cellular association can be refered to as a stage in spermatogenesis so that as one association replaces another, the region progresses into the next stage of development. Progression to the following stage involves the synchronous maturation of each cell type in the cellular association to its next respective phase in development. The stages are identified by the spermatids contained within and the step of spermiogenesis that these cells have reached. A cycle of seminiferous epithelium is therefore defined as a series of changes occurring in a given area of the seminiferous epithelium between two successive appearances of the same cellular association

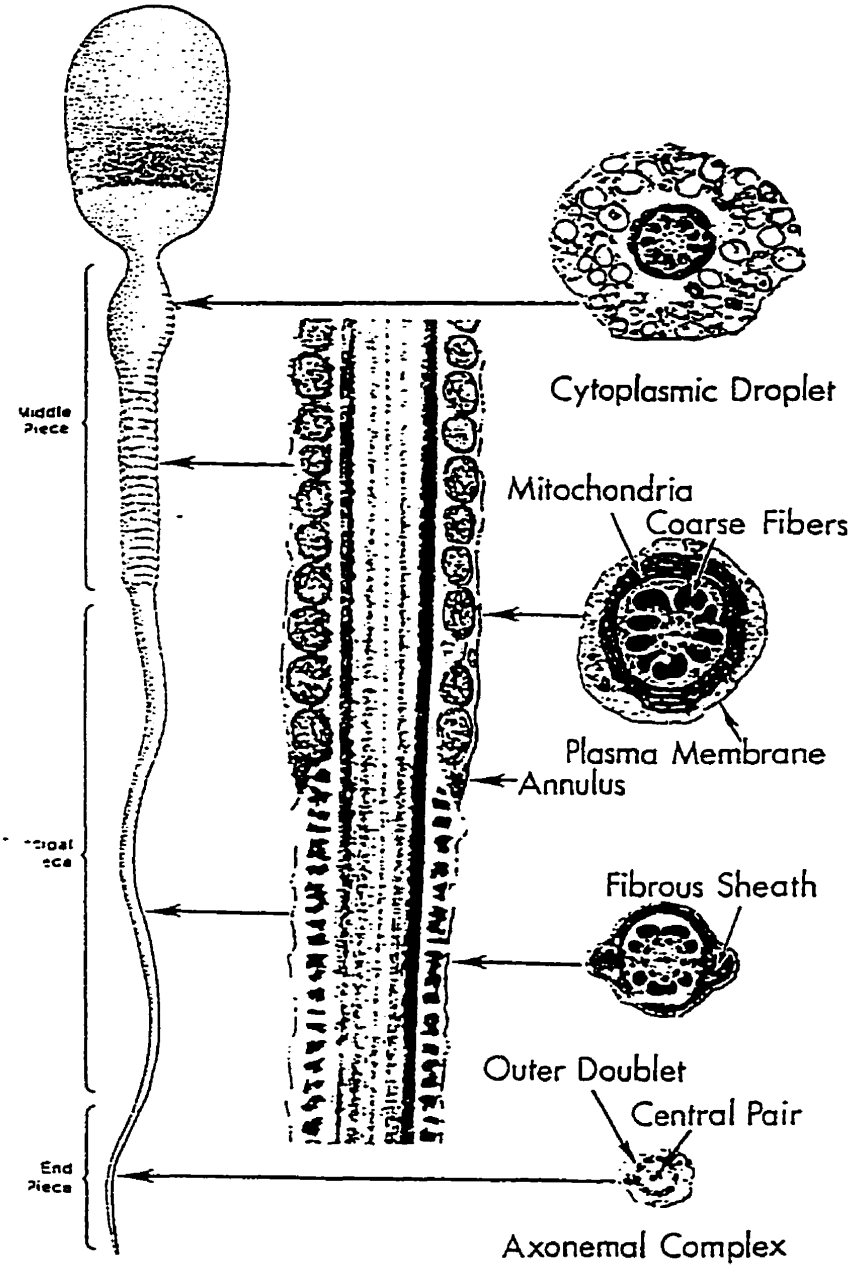



**Figure 8. Components of a flagellum.** The flagellum of a sperm is depicted and the major regional components labelled. A middle piece, principal piece and end piece are designated (adapted from Russell et al., 1990).

(Leblond and Clermont, 1952). In rats the succession of 14 stages or cellular associations make up one cycle of the seminiferous epithelium whose duration is 12.9 days in Sprague-Dawley rats, although this value varies amongst strains. In mouse however, one cycle consists of 12 stages and lasts 8.6 days (Fig. 9), indicating that as well as strain differences, there are also specie variations in the duration of the cycle.

## 3.3 Differential gene expression in testes

### 3.3.1 RNA synthesis

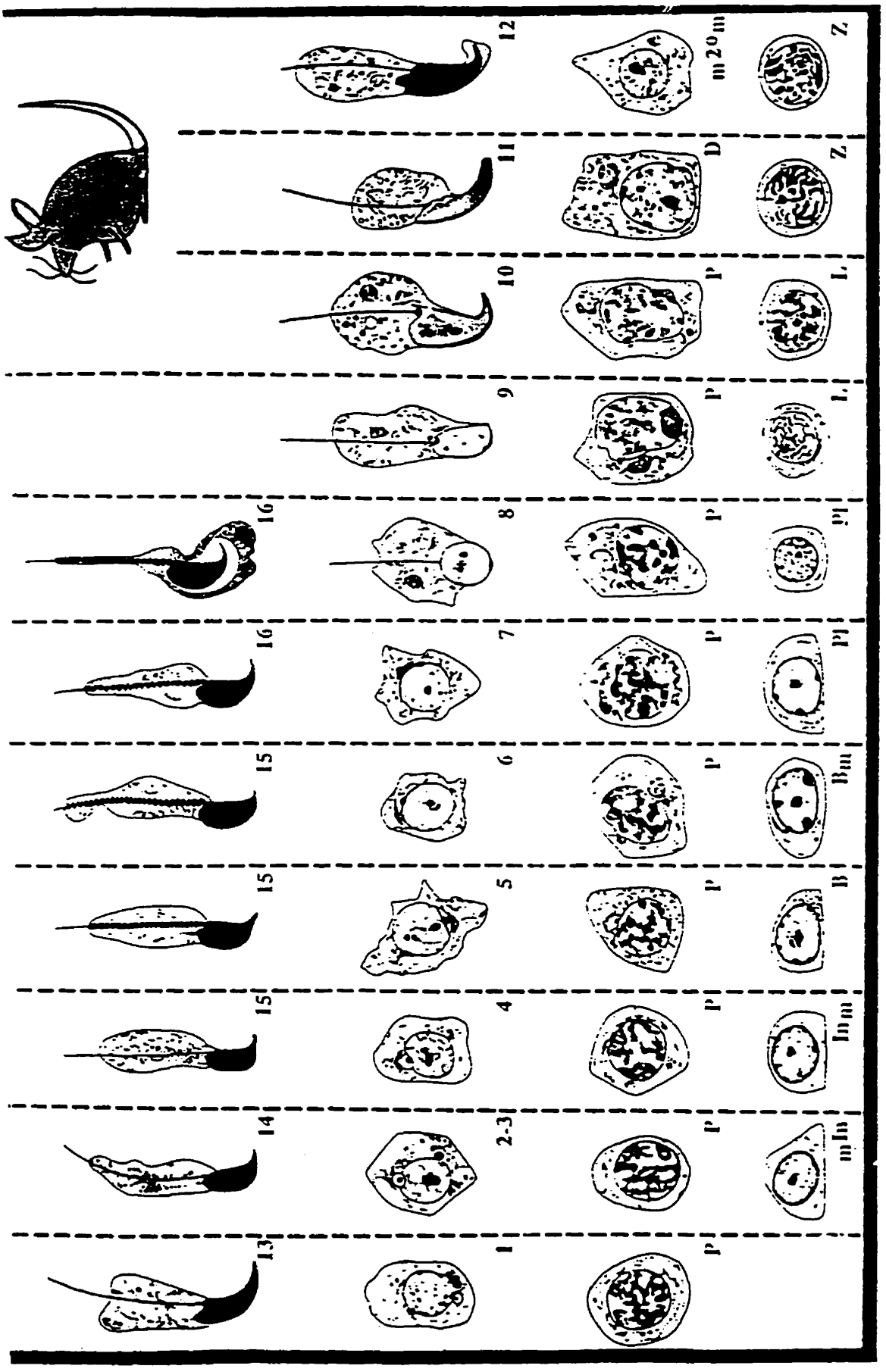
In the mouse, Monesi (1964a, 1965, 1971, 1978) observed that following incorporation of [$^3$H]-uridine, spermatogonia, midpachytene spermatocytes and spermatids up to step 8/9 were most active in RNA synthesis.

(A) RNA synthesis during meiosis

Some of the RNA synthesized in mouse primary spermatocytes was polyadenylated and appear on polysomes. This demonstrated that the meiotic transcripts are used to synthesize proteins (D'Agostino, Geremia, and Monesi, 1978). Furthermore, the presence of labeled poly (A)$^+$ mRNA in intermediate and late spermatids up to 14 days after intratesticular injection of [$^3$H]-uridine (Geremia et al., 1977) suggests that some of the mRNA synthesized in pachytene spermatocytes are long-lived. The synthesis of large numbers of polypeptides in elongating spermatids after the termination of transcription further argues for the need to storage and utilization of mRNAs from earlier cell types.

Some evidence for the storage and utilization of long-lived mRNAs in haploid cells comes from studies analyzing the regulation of protamine.

**Figure 9: Stages of the cycle.** Roman numerals indicate stages of the cycle; numbers indicate steps of the maturation of round spermatids to elongated spermatids to mature sperm. Letters indicate spermatogonia or spermatocytes (Pl=preleptotene; L=leptotene; Z=zygotene; P=pachytene; D=diplotene) (adapted from Russel et al., 1990).


I
II-III
IV
V
VI
VII
VIII
IX
X
XI
XII
P
mIm
Imm
B
Bm
P
P
P
P
P
P
P
D
P
1
2-3
4
5
6
7
8
9
10
11
12
13
14
15
15
15
16
16
16
Z
Z
L
L
Pl
Pl
m 2o m

Dixon and associates (Iatrou and Dixon, 1978) demonstrated that trout protamine mRNAs are synthesized in the pachytene spermatocytes, stored in the cytoplasm as ribonucleoprotein particles, and later activated and translated in condensing spermatids. Kleene, Distal and Hecht (1984) have demonstrated that in mice, protamine mRNA is first detectable in round spermatids, although the protamines are first synthesized much later in stages 12-15 of spermiogenesis.

(B) RNA synthesis during spermiogenesis

Proof that mRNA is synthesized during spermiogenesis was obtained by isolation of heterogenous poly $(A)^+$ from post-meiotic cells following a 1-3 hour labeling interval with [$^3$H]-uridine (Geremia, D'Agostino and Monesi, 1978; Erickson et al., 1980a). Furthermore, in a study by Kleene et al. (1983), the concentration of poly $(A)^+$ RNA was found to be twofold higher in round spermatids than in pachytene spermatocytes. Other studies (Erickson et al., 1980b) suggests that this increased poly $(A)^+$ RNA found in round spermatids codes for unique genes products (i.e. protamines) of the differentiating haploid cell.

(C) Translational regulation of mRNAs

Concurrent with the morphological changes seen as the spermatid differentiates to a spermatozoon, RNA transcription decreases and terminates (Monesi, 1964a). Thus, transcriptional regulation of stored mRNAs of either meiotic or early post-meiotic origin is thought to play a crucial role in the orderly synthesis of essential sperm components during spermiogenesis.

Cell fractionation experiments demonstrate that translational regulation occurs in meiotic and post-meiotic testicular cells. When the mRNAs from the polysomal and nonpolysomal fractions of a mouse testis extract are analyzed by a cell free translation assay, approximately two-thirds of the total mRNA or poly $(A)^+$ RNA is present in the nonpolysomal compartment (Gold and Hecht, 1981). This suggests, that in testes, some form of selective activation (or inactivation) or cytoplasmic sequestration regulates the loading of specific mRNAs onto polysomes to allow their temporal expression (Gold et al., 1983; Stern et al., 1983b). In contrast, over 80% of total liver mRNAs is found in the polysomal fraction. Although the majority of testicular mRNAs are in the nonpolysomal compartment, the distribution of individual mRNAs during spermatogenesis varies greatly. For instance, 82% of phosphoglycerate kinase 2 (PGK-2) mRNA is present in the polysomal fraction in total testis extracts; whereas 100% of PGK-2 mRNA is present in the nonpolysomal fraction in pachytene spermatocytes and more than half of the PGK-2 mRNA is found on polysomes in round spermatids. Another example is of the mouse protamines, which are the best studied proteins in post-meiotic cells. Kleene, Distal and Hecht (1984) found that approximately 85% of mouse protamine 1 (mP1) is found in the nonpolysomal fraction in round and elongating spermatids. Moreover, the nonpolysomal form of mP1 was approximately 580 nucleotides long, whereas the polysomal mP1 mRNA was about 450 nucleotides long. It was established that the size difference in mRNA length is due to a shortening of the poly (A) tail from about 160 nucleotides to approximately 30 nucleotides (Kleene, Distal and Hecht, 1984). These data indicate that mP1 mRNA is synthesized and stored in spermatids for up to 7 days before translation as an untranslated ribonucleoprotein of 580 nucleotides. Upon translation in

elongating spermatids, the polysomal mP1 mRNA becomes partially deadenylated. The reason for the loss of a sizable part of the 3' end of protamine mRNA concurrent with translation is unknown. Protamine 2 mRNA has also been shown to be similarly translationally regulated during spermiogenesis with a reduction of mRNA length between round and elongating spermatids.

3.3.2 Protein synthesis

Many studies by Monesi and associates (1964b, 1965, 1967, 1971, 1978) have shown that protein synthesis occurs in spermatogonia and pachytene spermatocytes and to a lesser extent in round spermatids.

Evidence for differential gene expression of specific proteins has been accumulated from analyses of a wide variety of proteins. The ability to analyze polypeptides from isolated populations of testicular cell types by two-dimensional polyacrylamide gel electrophoresis has provided evidence that large numbers of polypeptides are temporally regulated in the mammalian testis. Boitani, Polombi and Stefanini (1980) found differences in the polypeptides present in meiotic and postmeiotic cells by examining radiolabeled testicular polypeptides that has been synthesized when seminiferous tubules were incubated with [$^{3}$H]-leucine. By using cells obtained by centrifugal elutriation, Kramer and Erickson (1982) analyzed the *in vivo* $^{35}$S-methionine-labeled proteins from pachytene spermatocytes, round spermatids and elongated spermatids and found stage specific synthesis for approximately 15% of the soluble proteins. Using a combination of STAPUT sedimentation and Percoll density gradients to separate cell types, Stern et al. (1983a) analyzed the polypeptides synthesized in pachytene spermatocytes, round and elongated spermatids

and residual bodies following intratesticular injection of radiolabeled methionine and leucine. They concluded that for a constant number of cells, pachytene spermatocytes incorporated 5.4 times more isotope than round spermatids, which incorporated 2.4 times more isotope than elongated spermatids - results in close agreement with the 10:2:1 incorporation ratio reported by Kramer and Erickson (1982). These experiments demonstrated that different cell types, including haploid cells, did indeed synthesize cell-type specific proteins.

## 3.4 Novel genes and proteins of the testis

One interesting aspect of gene expression in male germ cells is in the existence of a large number of novel proteins and unique isozymes variants (i.e. phosphoglycerate kinase, lactate dehydrogenase and cytochrome c) in the testis (Table 2). In addition, the testis contains many other novel enzymes and structural proteins (Table 2). Some such as acrosin and the protamines are unique to testes. Others such as the meiotic histones are highly enriched in the testis. Most of these proteins are required for specific functions and structures of the testis and their expression is temporally controlled. For example, during spermatogenesis, the tubulins and actins are believed to play important roles in the mitotic and meiotic divisions, in changing cell structure and shape, in the formation of the specie-specific shapes of sperm heads and in the synthesis of the axoneme of the sperm tails. Both tubulins and actins are encoded by multigene families and may have distinct functional isoforms of these proteins (Cleveland et al., 1980; Minty et al., 1982).

**Table 2: Novel genes and proteins of the testis** (adapted from Wolgemuth and Watrin, 1991)

| Gene | Class &/or possible function | Spermatogenic expression | Somatic &/or Embryonic expression | Translation regulation |
|---|---|---|---|---|
| Hox 1.4 | Homeobox gene | Spermatocytes (P) to spermatids (R & E) | embryonic expression | ? |
| Zfp-35 | zinc finger | spermatocytes to spermatids | yes | ? |
| Zfy-1, Zfy-2 | zinc finger | spermatocytes to spermatids | yes | ? |
| c-*abl* | proto-oncogene | spermatids (R & E) | yes | yes |
| c-*mos* | proto-oncogene | spermatocytes (late) to spermatids (R) | yes, low levels | ? |
| c-*pim*-1 | proto-oncogene | spermatids | yes | ? |
| c-*raf*-1 | proto-oncogene | spermatogonia (A & B) to spermatids | yes | ? |
| c-*kit* | proto-oncogene | all stages, highest in spermatogonia (A & B) to spermatids | yes | ? |
| Ha-*ras* | proto-oncogene | spermatocytes to spermatids | yes | ? |
| Ki-*ras* | proto-oncogene | spermatocytes | yes | ? |
| N-*ras* | proto-oncogene | spermatocytes to spermatids | yes | ? |
| c-*fos* | proto-oncogene | spermatogonia B | yes | ? |
| c-*myc* | proto-oncogene | most abundant in spermatogonia B | yes | ? |
| c-*jun* | proto-oncogene | most abundant in spermatogonia B | yes | ? |
| *wnt*-1 | proto-oncogene | spermatids (R & E) | yes | ? |
| NGF | growth factor | spermatocytes to spermatids | yes | no |
| hsp-70.2 | heat shock gene | spermatocytes | ? | ? |
| hsp-70.1 | heat shock gene | spermatids | yes | yes |
| hsp-86 | heat shock gene | spermatogonia to spermatids | yes | ? |
| pro-opiomelano-cortin | neuropeptide | spermatocytes to spermatids (R) | yes | yes |
| pro-enkephalin | neuropeptide | spermatocytes to spermatids (R & E) | yes | ? |
| LDH-A | lactate dehydrogenase subunit | spermatocytes (P) to spermatids (E) | yes | yes |
| LDH-B | lactate dehydrogenase subunit | spermatogonia | yes | ? |
| LDH-C (ldh-x) | lactate dehydrogease subunit | spermatocytes (L) to spermatids (E) | no | yes |
| PGK-2 | phosphoglycerat e kinase | spermatocytes to spermatids | no | yes |

| | | | | |
|---|---|---|---|---|
| B1,4-galtase | B1,4-galactosyl transferase enzyme | spermatogonia then spermatids | yes | ? |
| Cs | cytochrome c | spermatogonia to spermatids | yes | yes |
| Ct | cytochrome c | spermatogonia to spermatocytes to spermatids | no | yes |
| M-α-3,-7 | tubulin | ? | no | ? |
| actin | smooth muscle γ actin | spermatids (E) | yes | ? |
| H2B | histone | spermatids (R) | no | yes |
| MP1, MP2 | protamines | spermatids (R & E) | no | yes |
| TP1 | transition nuclear protein | spermatids (R & E) | no | yes |
| TP2 | transition nuclear protein | spermatids (R & E) | no | ? |
| Mea | male enhanced Ag | spermatogonia to spermatids | yes | ? |

E, elongated spermatids; L, leptotene; P, pachytene; R, round spermatids; ?, not determined.

In addition, a large number of proto-oncogenes are expressed in the testis (reviewed in Propst et al., 1988). These genes will be discussed in the following section.

More genes and their products have yet to be identified. A more complete understanding of the unique components and the genes that regulates their functions is likely to provide an explanation for sperm defects of animals and man.

## 3.5 Proto-oncogene expression during premeiotic, meiotic and postmeiotic steps of spermatogenic cell development

During the 1980s, it was recognized that proto-oncogenes are expressed in spermatogenic cells during their proliferation and differentiation. A summary of the various proto-oncogenes expressed in the testis is presented in Table 2. Although the specific significance of *c-kit* in testicular development and initiation of spermatogenesis (*c-kit* is linked to the generation of primordial germinal cell) is well defined (Manova et al., 1990), very little is known about the function of other proto-oncogenes expressed in the testis.

Testicular proto-oncogenes are known to express at least 3 distinct classes of proteins. For example, proto-oncogenes in mouse spermatogenic cells express products belonging to the protein kinase family (*c-kit*, *c-abl*, *c-raf*, *c-mos*, *pim-1*), GTP-binding proteins with GTPase activity (*c-rasH*, *K* and *N*) and nuclear transcription factors (*c-fos*, *c-jun*). A fourth class of proto-oncogenes includes the Wnt proto-oncogene family represented in the testis by 3 members: *Wnt-1*, *Wnt-5B* and *Wnt-6* (Gavin et al., 1990). *Wnt-1* (or *Int-1*) is involved in mammary gland tumorigenesis and is normally expressed during mouse early neural development (Gavin et al., 1990). In addition,

*Wnt-1* is a homolog of *Wingless*, a gene with a role in *Drosophila* embryonic segmentation. Related *Wnt-1* DNA isolated from *Xenopus*, *Drosophila* and *C. elegans* indicate multiple roles of the proto-oncogene family, in particular during mesoderm formation. In the mouse, *Wnt-1* expression is limited to the round spermatids (Shackelford and Varmus, 1987). Although the function of *Wnt*-encoded proteins is not well defined, it is known that the *Wnt-1* product is secreted (Papkoff et al., 1987) and binds to the extracellular matrix and/or cell surfaces to elicit developmental changes, probably as a local growth factor or as a cell adhesion molecule. Different *Wnt* genes are expressed during early development of the central nervous system, but the testis is regarded as a unique site where *Wnt* expression continues in the adult. Both the selective expression of *Wnt-1* during mouse early spermiogenesis and the presence of high *Wnt-5B* and *Wnt-6* transcript levels in testis suggests that these proto-oncogenes may be involved in regulatory pathways during sperm formation.

The *c-mos* proto-oncogene is expressed during mouse and rat spermatogenesis and its product has serine-threonine protein kinase activity. The *c-mos* transcripts of different sizes were detected in mouse ovary and mouse and rat testis: transcripts in mouse oocytes are smaller in size (1.4 kb, Propst and Vande-Woude, 1987) than in mouse spermatids (1.7 kb). Rat spermatogenic cells express 3 *c-mos* transcripts: 5.0, 3.6 and the more abundant 1.7 kb (Van der Hoorn et al., 1991) directed by seminiferous tubule-extracted transcription factors binding to the *c-mos* promoter region. In the mouse testis, *c-mos* encodes a $p39^{mos}$ as well as a protein of slightly higher molecular mass [$p43^{mos}$ (Herzog et al., 1988)]. The function of *c-mos*-encoded products during spermatogenesis is presently unknown. In the

ovary, the *c-mos* product ($p39^{mos}$) is involved in meiotic maturation of fully grown oocytes (Goldman et al., 1987).

The *c-abl* proto-oncogene encodes a protein with specific tyrosine kinase activity and is expressed throughout mouse prenatal development and during mouse spermatogenesis (Müller et al., 1982) but not during oogenesis (Ponzetto and Wolgemuth, 1985). Primary spermatocytes contain larger size *c-abl* transcripts (8.0 and 6.2 kb) than early spermatids (4.7 kb) (Ponzetto and Wolgemuth, 1985). The existence of a single copy of the *c-abl* proto-oncogene indicates that its expression is developmentally regulated during spermatogenesis.

Three closely related and evolutionary conserved *c-ras* proto-oncogenes encoding GTP-binding protein with GTPase activity are expressed during mouse spermatogenesis (Sorrentino et al., 1988; Wolfes et al., 1989). While *c-ras H* transcripts 1.2 - 1.4 kb are expressed in spermatocytes and spermatids, *c-ras K* (2.0 - 2.2 kb) is detected in pachytene spermatocytes and *c-ras N* (1.4 - 1.6 kb) predominate in spermatids (Sorrentino et al., 1988). Nuclear transcription factors (*c-fos*, 2.2 kb and *c-jun*, 2.7 kb) are expressed during premeiotic and meiotic steps of spermatogenesis (Müller et al., 1982). Hopefully, in the near future, investigators will study the expression of other cellular oncogenes during spermatogenesis. The testis could provide an excellent system to clarify the function(s) of oncogene products in normal cell growth and differentiation.

## 3.6 Transgenic animal in male reproduction research

Transgenic animal technology is becoming one of the most valuable tools in biomedical research. This approach allows the study of gene

function, regulation and dysfunction *in vivo*. Moreover, the study of expression of a transgene is sometimes the only way to investigate the role and regulation of non-coding sequences of genomic DNA, such as promoters, enhancers and *cis*-acting elements, and to discover tissue-specific transcription factors. Many genes, especially those expressed during spermatogenesis, are now being studied *in vivo* by transgenic animal technology. This may provide further insight to determine their role in spermatogenesis.

Transgenic mice are produced by introducing sequences of foreign DNA, containing the gene of interest, into the mouse genome. At present, the technique of choice is pronuclear injection, but it is increasingly being replaced by the introduction of transformed embryonic stem cells into blastocyst-stage recipient embryos (otherwise known as gene targeting by homologous recombination or gene knock-out).

Very few studies have been designed specifically to investigate the function of genes or regulatory sequences on spermatogenesis. Most of the data with transgenic mice relevant for the male reproductive function had been obtained by chance, as a result of insertion mutagenesis or unexpected expression in the testis of genes not known to be involved in spermatogenesis. However, an increasing number of researchers are using a transgenic strategy to address specific problems related to male (in)fertility.

An example of these studies is the elimination of the α-inhibin gene that was obtained by homologous recombination (Matzuk et al., 1992). Heterozygous males were normal and fertile, while homozygous inhibin-deficient animals were normal but developed mixed or incompletely differentiated gonadal stromal tumors, uni- or bilaterally. This model showed that inhibin is a very important regulator of the proliferation of gonadal

stromal cells, and that it is a potent local tumor suppressor, without any direct, notable effect on germ cell maturation. Other exapmles are summarized in a review by Simoni (1994).

The limitations of the transgenic approach can be multifold. Most of the work till now has been carried out with pronuclear injection. Expressing or overexpressing transgenes in the testis may result in complex perturbations of testicular function, sometimes difficult to interpret. Thus, knock-out experiments will have more relevance. However, it has to be expected that many genes involved in spermatogenesis might also be involved in other developmental events. In this case, the interpretation of experiments of targeted elimination of genes in transgenic animals could be problematic and developmental problems might become prominent. An alternative strategic approach would be targeted delivery of antisense oligonucleotides to living adult animals, allowing a local block of selected functions (Baertschi, 1994).

**Objectives of the research**

The *Pom-1* gene was initially identified (from the *Gin-1* region) as a common insertion provirus integration site in 11 of 44 GPA-MuLV induced thymomas (Villemur et al., 1987). By using single copy probes spanning a 60 kbp region of cloned sequences around the integration site, a testis specific gene has been identified and a part of this gene was isolated (Monczak, 1990). The remaining sequences needed to be cloned in order to characterize this gene in tumorigenesis as well as in spermatogenesis. This body of work will discuss the cloning of the remaining sequences and the characterization of the *Pom-1* gene.

**References**

Albritton , L.M.,Tseng, L., Scadden, D., Cunningham, J.M. (1989) A putative murine ecotropic retrovirus receptor gene encodes a multiple-membrane-spanning protein and confers susceptibility to virus infection. Cell 57, 659-666.

Baertschi, A.L. (1994) Antisense oligonucleotide strategies in physiology. Mol. Cell Endocrinol. 101: R15-R24

Baillie, A.H., (1964) The histochemistry and ultrastructure of the gonocyte. J. Anat. 98, 641-645.

Bartholomew, C., Morishita, K., Askew, D., Buchberg, A., Jenkins, N.A., Copeland, N.G., and Ihle, J.N. (1989) Retroviral insertions in the CB-1/Fim-3 common site of integration activate expression of the Evi-1 gene. Oncogene 4, 529-534.

Bellvé, A.R. (1979) The molecular biology of mammalian spermatogenesis. *In* Oxfords Reviews of Reproductive Biology, ed. C.A. Finn, pp.159-261. Oxford University Press.

Bellvé, A.R., Cavicchia, J.C., Millette, C.F., O'Brien, D.A. Bhatnagar, Y.M., and Dym, M., (1977) Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. J. Cell Biol. 74, 68-85.

Bellvé, A.R., and O'Brien, D.A. (1983) The mammalian spermatozoon: structural and temporal assembly. *In* Mechanisms and Control of Fertiilization, ed. J.F. Hartman, pp.55-137. New York: Academic Press.

Ben-David, Y., Prideaux, V.R., Chow, V., Benchimol, S., and Bernstein, A. (1988). Inactivation of the p53 oncogene by internal deletion or retroviral integration in erythroleukemic cell lines induced by Friend leukemia viruses. Oncogene 3, 179-185.

Ben-David, Y., Giddens, E.B., and Bernstein, A. (1990). Identification and mapping of a common proviral integration site Fli-1 in erythroleukemia cells induced by Friend murine leukemia virus. Proc. Natl. Acad. Sci. USA 87, 1332-1336.

Ben-David, Y., and Bernstein, A. (1991). Friend virus-induced erythroleukemia and multistage nature of cancer. Cell 66, 831-834.

Bingham, P.M., Levis, R., and Rubin, G.M. (1981) Cloning of DNA sequences from the white locus of *D. melanogaster* by a novel and general method. Cell 25, 693-704.

Bishop, J.M. (1985) Viral Oncogenes. Cell 42, 23-38.

Bishop, J.M. (1987) The molecular genetics of cancer. Science 235, 305-311.

Boitani, C., Geremia, R., Rossi, R., and Monesi, V. (1980) Electrophoretic pattern of polypeptide synthesis in spermatocytes and spermatids of the mouse. Cell. Differ. 9, 41-49.

Chow, V., Ben-David, Y., Bernstein, A., Benchimol, S., and Mowat, M. (1987). Multistage Friend erythroleukemia: independent origin of tumor clones with normal or rearranged p53 cellular oncogenes. J. Virol. 61, 2777-2781.

Clark, J.M., and Eddy, E.M. (1975) Fine sructural observations on the origin and associations of primordial germ cells of the mouse. Dev. Biol. 47, 136-155.

Clermont, Y., (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelial cycle and spermatogonial renewal. Physiol. Rev. 52,198-236.

Clermont, Y., and Bustos-Obregon, E. (1968) Re-examination of spermatogonial renewal in the rat by means of seminiferous tubule mounted "in toto". Am. J. Anat. 122, 237-248.

Cleveland, D.W., Lopata, M.A., McDonald, R.J., Cowan, N.J., Rutter, W.J., and Kirschner, M.W. (1980) Number and evolutionary conservation of $\alpha$ and $\beta$ tubulin and cytoplasmic $\beta$ and $\gamma$ actin genes using specific cloned cDNA probes. Cell 20, 95-105.

Cohen, J.C., Shank, P.R., Morris, V.L., Cardiff, R., and Varmus, H.E. (1979) Integration of the DNA mouse mammary tumor virus-infected normal and neoplastic tissue of the mouse. Cell, 16, 333-345.

Coffin, J.M. (1990) Retroviridae and their replication. in Fields, B.N. and Knipe, D.M. (eds): Virology. New York, Raven Press, pp.1437-1499.

Dahlberg, J.E. (1988) An overview of retrovirus replication and classification, in Perk, E. (ed): Immunodeficiency disorders and retroviruses. New York, Academic Press, pp. 1-35.

D'Agostino, A., Germia, R., and Monesi, V. (1978) Post-meiotic gene activity in spermatogenesis of the mouse. Cell. Differ. 7, 175-183.

DesGroseillers, L., and Jolicoeur, P. (1983) Physical mapping of the Fv-1 tropism host range determinant of Balb/c murine leukemia viruses. J. of Virol. 48, 685-696.

DesGroseillers, L., Rassart, E., and Jolicoeur, P. (1983) Thymotropism of murine leukemia virus is conferred by its long terminal repeat. Proc. Natl. Acad. Sci. USA 80, 4203-4207.

Domen, J., von Lindern, M., Hermans, A., Breuer, M., Grosveld, G., and Berns, A. (1987) Comparison of the human and mouse *pim-1* cDNAs: nucleotide sequence and immunological identification of the *in vitro* synthesized *pim-1* protein. Oncogene Research 1, 103-112.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P.,. Ullrich, A., Schlessinger, J., and Waterfield, M.D. (1984) Close similarity of

epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307, 521-527.

Dulbecco, R., and Ginsberg, H. (1988) Virology. pp.355-375. Second Edition. J.B. Lippincott Company. Philadelphia.

Dym, M., and Fawcett, D.W. (1970) The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol. Reprod. 3, 308-326.

Eddy, E.M. (1970) Cytochemical observations on the chromatoid body of the male germ cells. Biol. Reprod. 2,114-128.

Erickson, R.P. Erickson, J.P., Betlach, C.J. and Meistrich, M.L. (1980a) Further evidence for haploid gene expression during spermatogenesis: heterogeneous, poly[A]-containing RNA is synthesized postmeiotically. J. Exp. Zool. 215, 13-19

Erickson, R.P. Kramer, J.M., Rittenhouse, J., and Salkeld, A. (1980b) Quantitation of mRNAs during mouse spermatogenesis: protamine-like histone and phosphoglycerate kinase 2 mRNAs increase after meiosis. Proc. Natl. Acad. Sci. U.S.A. 77, 6086-6090.

Evans, L.H., and Cloyd, M.W. (1984) Generation of mink cell-focus forming viruses by Friend murine leukemia virus: recombination with specific endogenous proviral sequences. J. of Virol. 49, 772-781.

Fawcett, D.W. (1975) Ultrastructure and function of the Sertoli cell. *In*: Handbook of Physiology, eds. Hamilton, D.W. and R.O. Greep, Vol. 5, Male Reproductive System, pp.21-55, American Physiological Society, Washington, D.C.

Fawcett, D.W., and Phillips, D.M. (1969) Observation on the release of spermatozoa and on changes in the head during passage through the epididymis. J. Reprod. Fert. (Suppl. 6), 405-418.

Fawcett, D.W., Anderson, W.A., and Phillips, D.M. (1971) Morphogenetic factors influencing the shape of the sperm head. Dev. Biol. 26, 220-251.

Franchi, L.L., and Mandl, A.M. (1964) The ultrastructure of germ cells in foetal and neonatal male rats. J. Embryol. Exp. Morphol. 2, 289-308.

Fritz, I.B. (1978) Sites of action of androgens and follicle stimulating hormone on cells of the seminiferous tubule. *In* Biochemical Actions of Hormones, ed. G. Litwack, Vol. 5, p.249. New York: Academic Press.

Gavin, B.J., McMahon, J.A., McMahon, A.P. (1990) Expression of multiple novel *Wnt-1/int-1*-related genes during fetal and adult mouse development. Gene Dev. 4, 2319-2332.

Germia, R., Boitani, C., Conti, M., and Monesi, V. (1977) RNA synthesis in spermatogenesis and spermatids and presevation of meiotic RNA during spermiogenesis in the mouse. Cell Differ. 5, 343-355.

Germia, R., D'Agostino, A., and Monesi, V. (1978) Biochemical evidence of haploid gene activity in spermatogenesis of the mouse. Exp. Cell Res. 111, 23-30.

Goff, S.P. (1992) Genetics of retroviral integration. Ann. Rev. Gen. 26, 527-544.

Gold, B. and Hecht, N.B. (1981) Differential compartmentalization of messenger ribonucleic acid in murine testis. Biochemistry 20, 4871-4877.

Gold, B., Stern, L., and Hecht, N.B. (1983) Gene expression during mammalian spermatogenesis. II. Evidence for stage-specific differences in mRNA populations. J. Exp. Zool. 225, 123-134.

Goldman, D.S., Kiessling, A.A., Millette, C.F., Cooper, G.M. (1987) Expression of *c-mos* RNA in germ cells of male and female mice. Proc. Natl. Acad. Sci. U.S.A. 84, 4509-4513.

Gross, L. (1951) Spontaneous leukemia developing in C3H mice following inoculation, in infancy, with AK-leukemic extracts, or AK-embryos. Proc. Soc. Exp. Med. 76, 27-32.

Gross, L. (1957) Development and serial cell-free passage of a highly potent strain of mouse leukemia virus. Proc. Soc. Exp. Med. 94, 767-771.

Hayward, W.S., Neel, B.G., and Astrin, S.M. (1981) Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature 290, 475-480.

Herzog, N.K., Singh, B., Elder, J., Lipkin, I., Trauger, R.J., Millette, C.F., Goldman, D.S. Wolfes, H., Cooper, G.M. Arlinhaus, R.B. (1988) Identification of the protein product of the *c-mos* proto-oncogene in mouse testes. Oncogene 3, 225-229.

Hicks, G.G. and Mowat, M. (1988). Integration of Friend murine leukemia virus into both alleles of the p53 oncogene in an erythroleukemic cell line. J. Viol. 62, 4752-4755.

Hinds, P.W., Finlay, C.A., Frey, A.B., and Levine, A.J. (1987). Immunological evidence for the association of p53 with a heat shock protein, hsc 70, in p53-plus-ras-transformed cell lines. Mol. Cell. Biol. 7, 2863-2869.

Howard, J.C., Yousefi, S., Cheong, G., Bernstein, A., and Ben-David, Y. (1993). Temporal order and functional analysis of mutations within the Fli-1 and p53 genes during the erythroleukemias induced by F-MuLV. Oncogene 8, 2721-2729.

Huckins, C. (1971) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation, and maturation. Anat. Rec. 169, 533-558.

Huckins, C. (1972)

Iatrou, K., and Dixon, G.H. (1978) Protamine messanger RNA: its life history during spermatogenesis in rainbow trout. Fed. Proc. 37, 2626-2633.

Ihle. J.N., Smith-White, B., Sisson, B., Parker, D., Blair, D.G., Schultz, A., Kozak, C., Lunsford, R.D., Askew, D., Weinstein, Y., and et al., (1989) Activation of the c-H-ras proto-oncogene by retrovirus insertion and chromosomal rearrangement in a Moloney leukemia virus-induced T-cell leukemia. J. Virol. 63, 2959-2966.

Jeon, K.W., and Kennedy, J.R. (1973) The primordial germ cells in early mouse embryos: light and electron microscope studies. Dev. Biol. 31, 275-284

Jolicoeur, P. (1979) The Fv-1 gene of the mouse and its control of murine leukemia virus replication. Curr. Top. Microbiol. Immunol. 86, 67-122.

Kelly, M., Holland, C.A., Lung, M.L., Chattopadhyay, S.K., Lowry, D.R., and Hopkins, N.H., (1983) Nucleotide sequence of the 3' end of MCF 247 murine leukemia virus. J. of Virol. 45, 291-298.

Knudson, A.G., Jr. (1985) Hereditary cancer, oncogenes, and anti-oncogenes. Cancer Research 45, 1437-1443.

Kramer, J.M., and Erickson, R.P. (1982) Analysis of stage-specific protein synthesis during spermatogenesis of the mouse by two-dimensional gel electrophoresis. J. Reprod. Fertil. 64, 139-144.

Kerr, J.B. and de Krester, D.M. (1974) The role of the Srtoli cell in phagocytosis of the residual bodies of spermatids. J. Reprod. Fertil. 36, 439-440

Kleene, K.C., Distel, R.J., and Hecht, N.B. (1983) cDNA clones encoding cytoplasmic poly(A)+ RNAs which first appear at detectable levels in haploid phases of spermatogenesis in the mouse. Dev. Biol. 98, 455-464.

Kleene, K.C., Distel, R.J., and Hecht, N.B. (1984) Translational regulation and coordinate deadenylation of a haploid mRNA during spermiogenesis in the mouse. Dev. Biol. 105, 71-79.

Kofman-Alfaro, S., and Chandley, A.C. (1970) Meiosis in the male mouse. An autoradiographic investigation. Chromosoma 31, 404-420.

Kung, H.J., Boerkoel, C., and Carter, T.H. (1991) Retroviral mutagenesis of cellular oncogenes: a review with insights into the mechanisms of insertional activation. Current Topics in Microbiology and Immunology 171, 1-25.

Lalli, M.F., and Clermont, Y. (1981) Structural changes of the head components of the rat spermatid during spermatogenesis. Am. J. Anat. 160; 419-434.

Lane, D.P. and Benchimol, S. (1990) p53: oncogene or anti-oncogene? Genes and Dev. 4, 1-8.

Lazo, P.A. and Tsichlis, P.N. (1988) Recombination between two integrated proviruses, one of which was inserted near *c-myc* in a retrovirus-induced rat thymoma: implications for tumor progression. J. Virol. 62, 788-794.

Lazo, P.A. and Tsichlis, P.N. (1990) Biology and pathogenesis of retroviruses. Seminars in oncology 17, 269-294.

Lazo, P.A., Lee, J.S., and Tsichlis, P.N. (1990) Long distance activation of the myc proto-oncogene by provirus insertion in Mlvi-1 or Mlvi-4 in rat T-cell lymphomas. Proc. Natl. Acad. Sci. USA 87, 170-173.

Leblond, C.P., and Clermont, Y. (1952) Spermiogenesis of rat, mouse, hamster, and guinea pig as revealed by the periodic acid-fuchsin technique. Am. J. Anat. 90, 167-215.

Majors, J. (1990) The structure and function of retroviral long terminal repeats. Curr. Top. Microbiol. Immunol. 157, 49-92.

Manova, K., Nocka, K., Besmer, P., and Bachvarova, R.F. (1990) Gonadal expression of *c-kit* encoded at the *W* locus of the mouse. Development 110, 1057-1069.

Marth, J.D., Overall, R.W., Meier, K.E., Krebs, E.G., and Perlmutter, R.M. (1988) Translational activation of the lck proto-oncogene. Nature 332, 171-173.

Matzuk, M.M., Finegold, M.J., Su, J-GJ., Hseueh, A.J.W., and Bradley, A., (1992) Inhibin is a tumor suppressor gene with gonadal specificity in mice. Nature 360: 313-319.

Minty, A.J., Alonso, S., Caravatti, M., and Buckingham, M.E. (1982) A fetal skeletal muscle actin mRNA in the mouse and its identity with cardiac actin mRNA. Cell 30, 185-192.

Monesi, V., (1962) Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of the mouse testis using teitiated thymidine. J. Cell Biol. 14, 1-18

Monesi, V., (1964a) Ribonucleic acid synthesis during mitosis and meiosis in the mouse testis. J. Cell Biol. 22, 521-532.

Monesi, V., (1964b) Autoradiographic evidence of a nuclear histone synthesis during mouse spermiogenesis in the absence of detectable quantities of nuclear ribonucleic acid. Expl. Cell. Res. 36,683-688.

Monesi, V., (1965) Synthetic activities during spermatogenesis in the mouse: RNA and protein. Expl. Cell Res. 39, 197-224.

Monesi, V., (1967) Ribonucleic acid and protein synthesis during differentiation of male germ cells in the mouse. Arch. Anat. Microsc. Morphol. Exp. 56, 61-74.

Monesi, V., (1971) Chromosome activities during meiosis and spermiogenesis. J. Reprod. Fertil. Suppl. 13, 1-14.

Monesi, V., (1978) Biochemistry of male germ cell differentiation in mammals: RNA synthesis in meiotic and post-meiotic cells. Curr. Top. Dev. Biol. 12, 11-36.

Mowat, M., Cheng, A., Kimura, N., Bernstein, A., and Benchimol, S. (1985) Rearrangements of the cellular p53 gene in erythroleukemic cells transformed by Friend virus. Nature 314, 633-636.

Müller,R., Slamon, D.J., Tremblay, J.M., Cline, M.J., and Verma, I.M. (1982) Differential expression of cellular oncogenes during pre- and post-natal development of the mouse. Nature 299, 640-644.

Mushinski, J.F., Potter, M., Bauer, S.R., and Reddy, E.P. (1983) DNA rearrangement and altered expression of the *c-myb* oncogene in mouse plasmacytoid lymphosarcomas. Science 220, 795-798.

Nebel, B.R., Amarose, A.P., and Hackett, E.M. (1961) Calendar of gametogenic development in the prepubertal male mouse. Science 134, 832-833.

Nilsen, T.W., Maroney, P.A., Goodwin, R.G., Rottman, F.M., Crittenden, L.B., Raines, M.A., and Kung, H.J. (1985) c-erb-B activation in ALV-induced erythroblastosis: novel RNA processing and promoter insertion result in expression of an amino-truncated EGF receptor. Cell 41, 719-726.

Nottenburg, C., Stubblefield, E., and Varmus, H.E. (1987) An aberrant avian leukosis virus provirus inserted downstream from the chicken c-myc coding sequence in a bursal lymphoma results from intrachromosomal recombination between two proviruses and deletion of cellular DNA. J. of Virol. 61, 1823-1833.

Oakberg, E.F. (1956) Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am. J. Anat. 99, 507-516.

Papkoff,J., Brown, A.M.C., and Varmus, H.E. (1987) The *int-1* proto-oncogene products are glycoproteins that appear to enter the secretory pathway. Mol. Cell Biol. 7, 3978-3984.

Peters, G. (1990) Oncogenes at viral integration sites. Cell Growth and Differentiation 1, 503-510.

Ponzetto, C., and Wolgemuth, D.J. (1985) Haploid expression of a unique c-abl transcript in the mouse male germ line. Mol. Cell Biol. 5, 1791-1794.

Propst, F.,and Vande Woude, G. (1985) Expression of *c-mos* proto-oncogene transcripts in mouse tissues. Nature 315, 516-518.

Propst, F., Rosenberg, M.P., Vande Woude, G. (1988) Proto-oncogene expression in germ cell development. Trends in Genetics 4, 183-187.

Ross, M.H. (1977) Sertoli-Sertoli junctions and Sertoli-spermatid junctions after ductule ligation and lanthanum treatment. Am. J. Anat. 148, 49-56.

Rosson, D., Dugan, D., and Reddy, E.P. (1987) Aberrant splicing events that are induced by proviral integration: implications for myb oncogene activation. Proc. Natl. Acad. Sci. USA 84, 3171-3175.

Rotter, V., Wolf, D., Pravtcheva, D., and Ruddle, F.H. (1984). Chromosomal assignment of the murine gene encoding the transformation-related protein p53. Mol. Cell. Biol. 4, 383-385.

Rovinski, B., Munroe, D., Peacock, J., Mowat, M., Bernstein, A., Benchimol, S., (1987). Deletion of the 5'-coding sequences of the cellular p53 gene in mouse erythroleukemia: a novel mechanism of oncogene regulation. Mol. Cell. Biol. 7, 847-853.

Ruscetti, S.K., and Scolnick, E.M. (1983). Expression of a transformation-related protein (p53) in the malignant stage of Friend virus-induced diseases. J. Virol. 46, 1022-1026.

Russell, L.D. (1979) Spermatid-Sertoli tubulobulbar complexes as devices for elimination of cytoplasm from the head region of the late spermatids of the rat. Anat. Rec. 194, 233-246.

Russell, L.D. (1984) Spermiation - the sperm release process: Ultrastructural observations and unresolved problems. *In*: Electron Microscopy in Biology

and Medicine, Ultrastructure of Reproduction, eds. J. Van Blerkom and P.M. Motta, Chapter 5, pp.46-65, Plenum Press New York.

Russell, L.D., Hikim, A.P.S., Ettlin, R.A., and Clegg, E.D. (1990) Mammalian spermatogenesis. *In* Histological and Histopathological evaluation of the testis, Chapter 1, pp.1-161, Cache River Press, USA.

Russell, L.D., and Malone, J.P. (1980) A study of Sertoli-spermatid tubulobulbar complexes in selected mammals. Tissue and Cell. 12, 263-285.

Russell, L.D., Lee, I.P., Ettlin, R., and Peterson, R.N. (1983) Development of the acrosome and alignment, elongation and entrenchment of spermatids in procarbazine-treated rats. Tissue and Cell. 15, 615-626.

Setoguchi, M., Higuchi, Y., Yoshida, S., Nasu, N., Miyazaki, Y., Akizuki, S., and Yamamoto, S. (1989) Insertional activation of *N-myc* by endogenous Moloney-like murine retrovirus sequences in macrophage cell lines derived from myeloma cell line-macrophage hybrids. Mol. Cell Biol. 9, 4515-4522.

Shackleford, G.M., and Varmus, H.E. (1987) Expression of the proto-oncogene *int-1* is restricted to post-meiotic germ cells and the neural tubule of mid-gestational embryos. Cell 50, 89-95.

Sheng-Ong, G.L., Morse, H.C., Potter, M., and Mushinski, J.F. (1986) Two modes of *c-myb* activation in virus-induced mouse myeloid tumors

[published erratum appears in Mol. Cell Biol. 1986 6, 2756] Mol. Cell Biol. 6, 380-392.

Sheng-Ong, G.L. (1987) Alternative internal splicing in *c-myb* RNAs occurs commonly in normal and tumor cells. Embo J. 6, 4035-4039.

Shih, C.C., Stoye, J.P., and Coffin, J.M. (1988) Highly preferred targets for retroviral integration. Cell 53, 531-537.

Simoni, M. (1994) Transgenic animals in male reproduction research. Exp. Clin. Endocrinol. 102: 419-433.

Sorrentino, V., McKinney, M.D., Giorgi, M., Geremia, R., Fleissner, E. (1988) Expression of cellular proto-oncogenes in the mouse male germ line: a distinctive 2.4 kilobase pim-1 transcript is expressed in haploid postmeiotic cells. Proc. Natl. Acad. Sci. U.S.A. 85, 2191-2195.

Spiegelman, M., and Bennett, D. (1973) A light- and electron-microscopic study of primordial germ cells in the early mouse embryo. J. Embryol. Exp. Morphol. 30, 97-118.

Steffen, D., and Weinberg, R.A. (1978) The integrated genome of murine leukemia virus. Cell 15, 1003-1010.

Steinberger, E. (1971) Hormonal control of mammalian spermatogenesis. Physiol. Rev. 51,1

Stern, L., Gold, B., and Hecht, N.B. (1983a) Gene expression during mammalian spermatogenesis . I. Evidence for stage-specific synthesis of polypeptides "in vivo". Biol. Reprod. 28, 25-41.

Stern, L., Kleene, K.C., Gold, B., and Hecht, N.B. (1983b) Gene expression during mammalian spermatogenesis . III. Changes in populations of mRNA during spermiogenesis. Exp. Cell Res. 143, 247-255.

Tsichlis, P.N., Shephard, B.M., Bear, S.E. (1989). Activation of the Mlvi-1/mis-1/pvt-1 locus in Moloney murine leukemia virus-induced T-cell lymphomas. Proc. Natl. Acad. Sci. USA 86, 5487-5491.

Tsichlis, P.N., Lee, J.S., Bear, S.E., Lazo, P.A., Patriotis, C., Gustafson, E., Shinton, S., Jenkins, N.A., Copeland, N.G., Huebner, K., Croce, E., Levan, G., Hanson, E. (1990) Activation of multiple genes by provirus integration in the Mlvi-4 locus in T-cell lymphomas induced by Moloney murine leukemia virus. J. Virol. 64, 2236-2244.

Tsichlis, P.N. and Lazo, P.A. (1991). Virus-host interactions and the pathogenesis of murine and human oncogenic viruses. Curr. Topics. in Microbiol. and Immunol. 171, 95-171.

Van der Hoorn, F.A., Spiegel, J.E., Maylie-Pfenninger, M.-F., Nordeen, S.K. (1991) A 43 kD *c-mos* protein is only expressed before meiosis during rat spermiogenesis. Oncogene 6, 929-932.

Van Lohuizen, M., and Berns, A. (1990) Tumorigenesis by slow-transforming retroviruses -- an update. Biochem Biophys Acta 1032, 213-235.

Varmus, H.E. (1984) The molecular genetics of cellular oncogenes. Annual Review of Genetics 18, 553-612.

Varmus, H. and Brown, P. (1989): Retroviruses. in Berg, D.E. and Howe, M.M. (eds): Mobile DNA. Washington, American Society for Microbiology, pp.53-108

Wang, H., Kavanaugh, M.P., North, R.A., Kabat, D., Cell-surface for ecotropic murine retroviruses is a basic amino-acid transporter. Nature 352, 729-731.

Watson, R.J., Dyson, P.J., and McMahon, J. (1987) Multiple *c-myb* transcript cap sites are variously utilized in cells of mouse haematopoietic origin. Embo J. 6, 1643-1651.

Weiss, R., Teich, N., Varmus, H., and Coffin, J. (1984) RNA tumor viruses. Molecular biology of tumor viruses. Second edition. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.

Weiss, R., Teich, N., Varmus, H., and Coffin, J. (1985) RNA tumor viruses. Molecular biology of tumor viruses. Second edition. Supplement and appendices. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.

Wolf, D., and Rotter, V. (1984). Inactivation of p53 gene expression of Moloney murine leukemia virus-like DNA sequences. Mol. Cell. Biol. 4, 1402-1410.

Wolfes, H., Kogawa, K., Millette, C.F., Cooper, G.M. (1989) Specific expression of nuclear proto-oncogenes before entry of meiotic prophase of spermatogenesis. Science 245, 740-743.

Zamboni, L., and Merchant, H. (1973) The fine morphology of mouse primordial germ cells in extragonadal locations. Am. J. Anat. 137, 299-336.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Phage Library Screenings

### 2.1.1 Titration of recombinant bacteriophage

Serial dilutions of a recombinant phage suspension was prepared in 100 µl of phage buffer, to which 100 µl of Y1090 *E. coli* (grown in LB and 0.2% maltose and 200 µl of 10 mM $MgCl_2/CaCl_2$) were added. These dilutions were incubated for 15 mins at 37°C to allow the phage particles to bind to their receptors on the surface of the bacteria. 4.5 ml of liquified top agar was added to the infected bacteria, and the suspension was poured onto an OBA (ordinary bottom agar) petri plate. After solidification, plates were incubated overnight at 37°C. Phage plaques were counted and the titer was determined (i.e. 100 plaques on a $10^{-6}$ petri plate would give a titer of $1 \times 10^8$ recombinant phages per ml.

### 2.1.2 Plating of phage library

Large square (22 x22 cm) agarose petri plates were pre-heated at 37°C. $1 \times 10^6$ phages were added to 1 ml of Y1090 bacteria, 1 ml of phage buffer and 2 ml of 10 mM $MgCl_2/CaCl_2$ and incubated for 15 mins at 37°C. 35 ml of liquified top agarose was added to the phage-bacteria suspension and evenly poured over the petri plates. The plates were incubated overnight at 37°C.

### 2.1.3 Phage plaque transfers to nylon membranes

Hybond (Amersham) nylon membranes were placed onto the plated phage library plate in order to absorb the free phage cDNA from each lysis plaque, for a duration of 10 mins. Reference points were marked with a needle, piercing both the membrane and agarose. The filters were removed

and soaked in 0.5M NaOH - 1.5M NaCl for 10 min, after which they were neutralized in a solution of 0.5 M Tris-HCl pH 7.0 - 3M NaCl for a further 10 min. Meanwhile, a replicate filter was prepared in the same manner with the same reference points marked on the duplicate filter as the original. All filters were air-dried and fixed under UV light (DNA cross-linking). Filters were rinsed in 0.1X SSC and hybridized with an appropriate probe.

2.1.4 Phage purification

Phage plaques that hybridized to a specific probe were picked off with the wide end of a pasteur pipette, and the agarose plug was incubated for 4 hours in 0.5 ml of phage buffer. Serial dilutions were made, and these were plated out as previously descibed. The phage plaques were transferred onto nylon membranes and hybridized with the original probe. Isolated positive plaques were then picked with the small end of the pasteur pipette, eluted in phage buffer and plated again in order to verify the purity. Once all plaque hybridize to the probe, it is considered 100% pure.

2.1.5 Small scale phage cDNA preparation

The method for rapid, small scale preparation of phage DNA was described by Grossberger (1987). Briefly, one plaque was picked with a pasteur pipette and added to a 14 ml tube (Falcon #2059) containing 0.3 ml 10 mM $MgCl_2/CaCl_2$ and 0.2 ml Y1090 bacteria grown in LB - 0.4% maltose. This was incubated for 10 min at 37°C without shaking. 10 ml of LB containing 10 mM $MgCl_2$ and 0.1 % glucose was added to the tube and was incubated overnight at 37°C in a shaking incubator.

The tube was centrifuged for 10 min at 2000 rpm in a bench-top centrifuge to pellet the bacterial debris. The supenatant was then centrifuged

in an SW41 rotor (Beckman) for 30 mins at 30,000 rpm, 16°C. The pellet was resuspended in 200 µl of plaque buffer and transferred to a 1.5 ml eppendorf tube. 200 µl of proteinase K (1 mg/ml) was added and incubated for 2 hours at 37°C. The preparation was extracted once with phenol, once with phenol/chloroform, and once with chloroform. 100 µl of 7.5 M ammonium acetate and 1 ml of EtOH was added to precipitate the DNA. The tube was centrifgued for 15 min, the pellet was washed once with 75% EtOH and resuspended in 100 µl of $H_2O$. 10 µl aliquots were used for restriction enzyme analysis.

2.1.6 Large scale phage cDNA preparation

Four large petris, plated to confluence (1 x $10^6$) were eluted in 35 ml of phage buffer for 4 hours, shaking at room temperature. The buffer was collected and centrifuged at 6 000 g for 10 min to pellet bacterial debris. 50 µg/ml of RNase (1mg/ml) and 1µg/ml DNase I were added to the supernatant and incubated for 30 min at 37°C. 14 ml of this preparation was layered onto a discontinuous glycerol gradient made up as follows: 7 ml 5% glycerol (in phage buffer) over 7 ml 40% glycerol (in phage buffer). The gradient was centrifuged for 210 min at 24 000 rpm in N SW27 rotor (Beckman) at 4°C.

The supernatant was quickly decanted, and the tubes were kept inverted for 10 min to allow adequate draining. The pellet was resuspended in 0.5 ml phage buffer. The suspension was adjusted to 0.5% SDS and 50 µg/ml proteinase K, and incubated for 30 min at 37°C. The phage cDNA was extracted twice with phenol, once with phenol/chloroform, and once with chloroform, and precipitated with EtOH.

2.1.7 Bacteria and phage media

- LB medium: 0.2% $MgCl_2$, 1% N-Z amine (Sheffield), 0.5% yeast extract (DIFCO), 0.5% NaCl, 0.5% casamino acid (DIFCO), pH 7.3.
- Plaque buffer: 100 mM NaCl, 10 mM $MgSO_4$, 20 mM Tris-HCl, pH 7.5, 0.01% gelatin.
- OBA agar: 0.2% $MgCl_2$, 1.5% bacto agar (DIFCO), 1% N-Z amine (Sheffield), 0.5% yeast extract (DIFCO), 0.5% NaCl, 0.1% casamino acid (DIFCO).
- Top agarose: 0.2% $MgCl_2$, 1% agarose (Sigma), 1.3% N-Z amine (Sheffield), 0.6% NaCl, 0.1% casamino acid (DIFCO).

## 2.2 Endonuclease Digestions, Agarose Gel Electrphoresis, Hybridization Procedure and Sequencing

### 2.2.1 Restriction enzyme digestions

Genomic or cloned phage/cosmid DNA or cDNA was digested with various restriction endonucleases (New England Biolabs, Inc., Beverly, Mass.; Boehringer Mannheim Biochemicals, Montreal; and P-L Pharmacia, Montreal) under conditions recommended by the manufacturers, as previously described (Maniatis et al., 1982). Briefly, DNA is digested with 1 unit of enzyme per μg of DNA at 37°C for 1 h in the presence of an appropriate buffer (New England Biolabs, Inc., Beverly, Mass.; Boehringer Mannheim Biochemicals, Montreal; and P-L Pharmacia, Montreal).

### 2.2.2 Agarose gel electrophoresis

DNA or cDNA fragments were separated by electrophoresis in agarose gels. Various concentrations of agarose were used, ranging from 0.7% - 2.0%, in order to separate high molecular weight DNA to very small

molecular wight DNAs, respectively. Overnight gels were electrophoresed in a Tris-acetate buffer (40 mM Tris-acetate pH 7.7, 1 mM EDTA) under constant voltage of approximately 40 - 50 volts (35 mA), whereas minigels were run at a much higher voltage of 100 - 150 in a Tris-borate buffer (89 mM Tris-borate, 2 mM EDTA). The DNA samples were loaded in 1X loading buffer (10 X buffer: 80% sucrose, 0.2% w/v SDS, 0.5% bromophenol blue).

2.2.3 Ethidium bromide staining

DNA was visualized in an electrophoresis agarose gel by staining with ethidium bromide (EtBr). The agarose gel was soaked in 0.3 ml of 0.5 µg/ml EtBr for 20 min, then destained in water for 10 min, after which it was photographed under UV illumination. Alternatively, 20 µl of 10 mg/ml EtBr was added to the liqiud agarose before pouring to make the gel mold.

2.2.4 Southern blotting

This technique has been described elsewhere (Southern, 1975). Briefly, after electrophoresis, the agarose gel is treated with NaOH (0.5 M NaOH, 1.5 M NaCl) for 30 min to denature the DNA. The gel is then neutralized in Tris/NaCl (O.5 M Tris-HCl pH 7, 3 M NaCl) for 30 min. Six layers of Whatman 3M paper were soaked in 20X SSC (1X SSC: 150 mM NaCl, 15 mM sodium citrate) and stacked one on top of each other. The agarose gel is laid on top of the paper, and covered with nylon membrane (Hybond, Amersham). Two layers of Whatman 1M paper, soaked in 2X SSC, were placed on top of the membrane, followed by a thick layer of absorbant paper. The transfer was allowed to proceed for 4 - 16 hours, after which the nylon membrane was air-dried and UV fixed.

When high molecular weight DNA was to be transferred, the gels were first soaked in 0.25 M HCl for 10 min. This causes a partial depurination of the DNA; subsequent treatment of the DNA with NaOH causes a breakage in the DNA molecules at the site of depurination. This allows for more efficient transfer of large molecular weight DNA from the gel to the membrane.

2.2.5 Electroelution of DNA or cDNA fragments

Specific DNA or cDNA fragments were isolated from agarose gels by eluting the DNA onto NA45 membrane (DEAE cellulose, Schleicher and Schuell). Briefly, a thin slit was made in the gel below the fragment of DNA to be isolated, into which a strip of NA45 membrane was inserted. Electrophoresis was continued until the DNA was completely transferred onto the membrane. As well, a second strip of NA45 membrane was inserted into the gel just above the DNA of interest to prevent contaminating DNA to co-transfer onto the membrane. The membrane containing the DNA of interest was then washed in low salt NET buffer (20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.1 mM EDTA), and the DNA was eluted in 400 µl high salt NET buffer (20 mM Tris-HCl pH 8.0, 1 M NaCl, 0.1 mM EDTA) at 65°C for 45 min in an eppendorf tube. The DNA was extracted with phenol: phenol/chloroform: chloroform and precipitated with EtOH.

2.2.6 Hybridizations

$^{32}$P-labelled probes ($^{32}$P: 0.8 mCi/ml, Amersham Corp.) were labelled by the random priming method with the Klenow fragment of DNA polymerase I in the presence of hexamers (Feinberg and Vogelstein, 1983). Nylon membranes were hybridized in SET 4X (1X is 0.15M NaCl, 30mM

Tris, 2mM EDTA, pH 7.4) with 0.1% sodium pyrophosphate, 0.2% sodium docecyl sulfate (SDS), 10% dextran sulfate and 0.5 mg of heparin/ml at 65°C for 16 h. After hybridization, all membranes were washed in 2X SSC for 15 min at room temperature, 0.1X SSC and 0.5% SDS for 1 h at 65°C and three times 2 min in 0.1X SSC at room temperature. Membranes were air-dried and exposed on RP-Royal X-Omat film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex Lightening-Plus intesifying screen (DuPont Co., Wilmington, Del.) at -70°C.

### 2.2.7 Sequencing

Each fragment of interest was purified and subcloned into an appropiate vector. Sequencing was performed by the $^{35}$S-chain termination method (Sanger et al, 1977), ($^{35}$S-methionine: >1,000 Ci/mmol, Amersham Corp.) utilizing synthetic primer and subclones of fragments derived with suitable restriction endonucleases. For each clone, both strands were fully sequenced using SP6 and T7 or T3 and T7 promoters (P.L. Pharmacia, Montreal), depending on the vector, according to the manufacturer's protocol. Sequencing comparison was made using the program BESTFIT, fom the Wisconsin GCG package (Devereux et al., 1984), on a VAX computer.

## 2.3 DNA/cDNA Cloning

### 2.3.1 DNA/cDNA ligation reactions

Ligation reactions were carried out as follows: linearized plasmid, dephosphorylated by calf intestinal phosphatase (CIP), was added to a two-fold excess of insert. 2 µl of 10X ligation buffer (0.5 M Tris-HCl pH 7.5, 0.1 M

$MgCl_2$, 0.2 M DTT, 10 mM ATP) were added to the eppendorf, and the volume was completed to 19 µl with water. 1 µl of ligase (5 Weiss units) was added to the solution, and the tube was incubated overnight at 14°C.

2.3.2 Bacterial transformations

Transformation competent bacteria (i.e. *E. coli* 1061) were thawed on ice, and 100 µl of the bacteria was added to a pre-chilled Falcon tube (#2059) containing 5 µl of the ligation reaction diluted in $H_2O$ to 20 µl. This was incubated on ice for 15 min. The bacteria and DNA were 'heat shocked' by incubating the reaction at 42°C for 90 secs, followed by an incubation on ice for 2 min, after which 1 ml of LB medium was added to the tubes. The tubes were incubated at 37°C for 1 h in a shaking incubator.

50, 100, 150, and 200 µl of the reaction was spread on a nylon membrane in small OBA petri plates having the appropriate amount of antibiotics. The plates were incubated overnight at 37°C.

2.3.3 Replicas of transformed bacterial spreads

A nylon membrane with bacterial colonies on its surface (from the transformation) was removed from the petri plate and a replica (mirror image) was made by applying a second nylon membrane to its surface. Reference points were made using a needle and piercing the membranes. The replicate was removed from the original and incubated for 6 h at 37°C, while the original was stored at 4°C.

The replicate was denatured in NaOH/NaCl and neutralized in Tris/NaCl as described earlier. The membranes were soaked for 1 min in EtOH, rinsed in 0.1X SSC and fixed under UV light for 3 min.

2.3.4 Rapid small scale plasmid preparations

Isolated bacterial colonies were picked off the agar plates with a sterile toothpick and incubated overnight in a shaking incubator at 37°C in 5 ml LB containing antibiotics.

1 ml of bacterial culture was transferred to an eppendorf tube and briefly centrifuged at 4°C to pellet the bacteria. The supernatant was decanted and the pellet was resuspended in 100 µl of TEG (25 mM Tris-HCl pH 8, 10 mM EDTA, and 50 mM glucose), and 200 µl NaOH/SDS (0.2 N NaOH, 1% SDS) was added. The solution was gently mixed by inversion several times. 150 µl of 3M:5M KOAc (3M K, 5M acetate, pH 4.8) was added, and mixed by inversion. The tube was centrifuged for 15 mins at 4°C in an eppendorf centrifuge, and the supernatant was transferred to another tube and extracted once with phenol:chloroform, and once with chloroform. The plasmid DNA was then precipitated with two volumes of EtOH, and resuspended in 100 µl of $H_2O$.

2.3.5 Large scale plasmid preparation

This technique is similar to that of the small scale preparation as described above, but makes use of additional purification steps to yield pure, noncontaminated DNA in large amounts. Concentrations of solutions were the same as above unless otherwise stated.

One liter of an overnight bacterial culture was centrifuged at 3.5 K for 15 mins. The pellet was resuspended in 35 ml TEG, 5 ml of lysozyme (40 mg/ml in TEG) was added and incubated at room temperature for 10 mins. 120 ml of NaOH/SDS was added, the preparation gently swirled and incubated on ice for 10 mins. 60 ml KOAc was added, again gently mixed and incubated on ice for an additional 20 mins. The solution was centrifuged

at 3.5 K for 15 min at 4°C, and its supernatant was filtered through a cheese-cloth. 0.6 volumes (120 ml) of isopropanol was added to the filtrate, mixed, and incubated at room temperature for 60 min. The solution was then centrifuged at 3.5 K for 15 min and the pellet was resuspended in 7.5 ml of TE. 2.5 ml $NH_4OAc$ (10M) was added to the suspension and kept on ine for 20 min,.and centrifuged at 7.5 K for 15 min. The supernatant was transferred to a 30 ml Corex tube, and precipitated on ice with 2 volumes (20 ml) of EtOH for 20 min, followed by a 15 min centrifugation at 7.5 K. The pellet was resuspended in 7 ml TE, and 7 $\mu$l of RNase A (1 mg/ml) was added, and incubated at 37°C for 15 min. 3 ml NaCl (5 M) and 2.5 ml PEG 6000 (30% PEG 6000 in 1.5 M NaCl) was added amd kept on ice for 30 min. This was centrifuged at 9 K for 15 min. The supernatant was decanted and the tubes inverted on paper tissue to air-dry. The pellet was resuspended in 200 $\mu$l of $H_2O$ and transferred to an eppendorf tube. 200 $\mu$l of 2X PK buffer (2X: 0.2 Tris-HCl pH 7.5, 25 mM EDTA, 0.3 M NaCl, 2% w/v SDS) and 40 $\mu$l proteinase K (5 mg/ml) was added to the suspension, and incubated at 37°C for 30 min. The plasmid DNA was extracted once with phenol, once with phenol:chloroform, once with chloroform and precipitated with EtOH. The pellet was washed twice in 75% EtOH, and resuspended in 1 ml of $H_2O$. The final yield of plasmid DNA was between 300 and 1000 $\mu$g.

## 2.4 RNA Isolation and Northern Blot Analysis

### 2.4.1 Organ dissection and freezing

Mice were sacrificed with $CO_2$, and ventrally dissected to expose the internal cavity. Organs of interest were removed and immediately frozen in

liquid nitrogen. Tissues were stored at -70°C in sterile vials for RNA extractions.

2.4.2 Total RNA extractions

Total cellular RNA was extracted by the method of Chomczynski and Sacchi (1987). Two hundred mg of frozen tissue was homogenized (Tissumizer, Teckmar) for 20 to 30 s in 4 ml of solution D (4M guanidium thiocynate, 25 mM sodium citrate pH 7, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol). The tubes were placed on ice and the following reagents were sequentially added: 0.4 ml of 2 M sodium acetate pH 4, 4 ml phenol (water saturated) and 2.4 ml chloroform-isoamyl alcohol (24:1). The tubes were shaken vigorously for 30 s and allowed to stand on ice for 15 mins. The samples were centrifuged at 10 000g for 20 min at 4°C (Sorvall).

The aqueous phase was transferred to a fresh tube and 4 ml of isopropanol was added, mixed by inversion, and incubated for 60 min at -20°C to precipitate the RNA out of the solution.

The samples were centrifuged at 10 000g for 20 min at 4°C (Sorvall). The supernatant was quickly decanted, the pellet was resuspended in 0.3 ml of solution D and transferred to an eppendorf tube. The RNA was precipitated a second time with 1 volume of isopropanol at -20°C for 60 min.

The samples were centrifuged in a microfuge for 15 min, and the pellet were rinsed twice with 75% EtOH. The pellet was resuspended in 100 µl of $H_2O$, and its concentration adjusted to 1 µg/µl by optical densitometry. Two µg of the RNA sample was electrophoresed on an agarose gel and visualized by etidium bromide staining under UV light in order to verify the quality of the extraction. The remainder of the RNA extract was stored in 2 volumes of EtOH at -70°C.

2.4.3 mRNA (Poly $A^+$ RNA) extraction

Poly $A^+$ RNA was prified from total RNA extracts as described by Maniatis et al (1982). Briefly, total RNA extracts are slowly passed through an affinity column containing small cellulose beads to which short chains of deoxythymidines (oligo $dT_{12}$) have been fixed. At a specific salt concentration, RNA molecules having poly $A^+$ tails will bind to the oligo dT-column. The bound poly $A^+$ mRNA is then eluted at a different salt concentration. Average final yields of poly A mRNA are approximately 5% of initial RNA quantity.

One to four hundred μg of oligo-dT cellulose (P.L. Pharmacia, Montreal) was resuspended in 10 ml of $H_2O$ and loaded into an appropriate column. The column was washed with 50 ml of $H_2O$, 50 ml of 0.1 M NaOH - 5 mM EDTA, 50 ml $H_2O$, and finally with 50 ml of Solution 1 (10mM Tris-HCl pH 7.5, 0.5 M NaCl, 1mM EDTA, 0.1% SDS).

The total RNA extract was adjusted to a concentration of 400 μg/ml with $H_2O$, heated at 65°C for 10 mins and immediately quenched on ice for 5 min. One volume of 2X solution 1 was added to the RNA extract, and this was passed through the affinity column 4 times. The column was then washed with 50 ml of solution 1.

The poly $A^+$ mRNA was eluted from the column with 5 ml solution 2 (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.05% SDS). The eluate was collected in 1 ml fractions in eppendorf tubes. One μl of EtBr was added to 10 μl of each faction and was visualized under UV light. The fractions containing RNA were pooled and precipitated with 0.2 M Na acetate pH 5.5 and 2 volumes EtOH. The pelleted RNA was resuspended in 25 μl $H_2O$ and its concentration was adjusted to 1μg/μl busing optical densitometry.

2.4.4 RNA electrophoresis

RNA and mRNA samples were electrophoresed in a denaturing agarose-formamide gel as follows: 3 g of RNA-grade agarose was dissolved by boiling in 200 ml $H_2O$. Six ml 50X MOPS buffer (1.0 M MOPS, 250 mM Na acetate, 10 mM EDTA) and 15.3 ml formaldehyde was added to the agarose solution and the volume was adjusted to 300 ml. The agarose solution was poured into a mold and allowed to solidify.

Twenty $\mu$g of total RNA or 2-10 $\mu$g of poly $A^+$ RNA were dried under vacuum in eppendorf tubes and resuspended in 30 $\mu$l of RNA loading buffer (0.75 ml de-ionized formamide, 0.03 ml 50X MOPS, 0.24 ml formaldehyde, 0.1 ml $H_2O$, 0.1 ml glycerol, 0.02 ml bromophenol blue). The samples were heated to 65°C for 15 min, and 1 $\mu$l of EtBr (10 mg/ml) wad added to each sample before loading on gel. Elecrophoresis was carried out for 16 -18 h at 50 volts in 1X MOPS buffer. The gel was photographed under UV light and transferred to a nylon membrane as described before (Maniatis et al., 1982). Washings were performed for 15 min at room temperature in 2X SSC, for 1 h at 65°C in 0.1X SSC - 0.5% SDS, and in 0.1X SSC three times for 2 min each at room temperature.

2.4.5 RNase H Treatment

Selective digestion of poly ($A^+$) tails was performed essentially as described by Kleene et al (1984). Briefly, aliquots consisting of 20 mg of RNA and 1 $\mu$g of oligo (dT) (1 mg/ul) were dissolved in DEPC-treated distilled $H_2O$ to a final volume of 70 ml. Each sample was incubated for 2 min at 65 °C followed by the addition of 20 ml of digestion buffer (20mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 100mM KCl; and 5% sucrose). Samples were

then allowed to cool on ice for 15 min. Digestion of poly ($A^+$) tails was initiated by the addition of 2 U of RNase A (Boehringer Mannheim, 1U/ml) to each sample followed by a 37 °C incubation for 30 min., extracted with phenol:chloroform, and precipitated with ethanol. Reaction volumes were as follows: 20 mg testis RNA, 1 mg oligo (dT), 2 U RNase H, 100 ml reaction.

2.4.6 Isolation of Polysomal RNA

Polysomal RNA from 40 day old mouse C57BL/6 testes was isolated by methods utilizing sucrose gradients as described by Kleene et al (1984). Briefly, testes were homogenized in 2.0 ml of ice cold buffer consisting of 5mM $MgCl_2$, 50mM KCl, 0.5% TritonX-100, 50 U/ml RNase inhibitor (RNasin; Promega), and 50mM Tris-HCl, pH 7.5. In control experiments, $MgCl_2$ was replaced by 10 mM EDTA. After the removal of nuclei by centrifugation at 12,000 x g for 15 min, the samples were loaded over 10-40 % (w/v) linear sucrose gradients with a 60% (w/v) sucrose cushion and centrifuged at 40,000 rpm for 110 min in a Beckman SW-40 rotor. The pelleted material and supernatant (referred to as polysomal and nonpolysomal fractions, respectively) were collected and RNA loaded on 1% agarose-formamide gels as above.

## 2.5 Cell culture, transfection and virus infections

All Rat-1 derived cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine calf serum. The BOSC 23 cells were cultured in DMEM supplemented with 10% fetal calf serum.

Transfections of Rat-1 cells were performed as previously described (Zarbl et al., 1987). Transfections in BOSC 23 cells were performed as described (Pear et al., 1993). Retroviruses were collected 48 h post transfection and filtered through a 0.22 μM Millipore filter. Retroviral infections were done 24 h after seeding 1 x $10^5$ cells on 60 mm petri dishes. Cells were infected with 0.2 ml of virus suspension containing 8 μg/ml of polybrene at 37 °C. Cultures were agitated periodically, fed after 1 h with normal medium, incubated for 2 days before addition of puromycin at 4 μg/ml . Clones were selected 2-3 weeks postinfection.

## 2.6 Sperm Cell Fractionation: Purification of Pachytene Spermatocytes and Spermatids

Male CD-1 mice, at least 40 days old, were obtained from Charles River Breeding Laboraotries. The protocol for purification of spermatocytes and spermatids is essentially the same as Romerell et al (1976), Meistrich et al (1981), Stern et al (1983) and Kleene et al (1983). Briefly , 10 mice testes were excised into enriched Krebs Ringer bicarbonate (EKRB) and incubated with 1mg/ml collagenase at 34°C for 12 mins. The testes were washed twice and incubated with 2.5 mg/ml trypsin and 1mg/ml DNase 1at 34°C for 12 min. To release the sperm cells further from the seminiferous tubules, the suspension was greatly agitated with a pasteur pipette. The Leydig and Sertoli cells were filtered using a Nitex mesh (70 micron) and the flow-through (the spermatogenic cells) was directly added to the STAPUT chamber. This apparatus allows the spermatogenic cell suspension to sediment at unit gravity on a 2-4 % bovine serum albumin gradient (Bellvé et al., 1977). Fractionation of sedimented cells was achieved using a fraction collector set at 250 drops / tube (12 ml / tube).The appropriate cells are

pooled and the purity of pachytene spermatocytes and spermatids was determined by light microscopy and found to be 80-85% and 85-90 %, respectively.

## 2.7 *In situ* Hybridization

### 2.7.1 Synthesis of RNA probes

The probe synthesis reactions are carried out with linearized plasmid as template as previously described (Melton et al., 1984). Briefly, the plasmid subclone is linearized by restriction enzyme digestion at a single site upstream or downstream of the insert. This site should be oriented such that the synthesized probe will be truncated by polymerase runoff. To synthesize a labelled probe that hybridizes to mRNA, the antisense strand of the cloned insert must be used as the template in the polymerase reaction. The control reaction is the sense strand of the cloned insert which also gets labelled.

In an eppendorf tube, the reaction is as follows:

1 µl linearized plasmid template (1 µg)

2 µl 10X T7/T3/SP6 RNA polymerase buffer

2 µl 10X stock of ATP, GTP, CTP

80µCi $^{35}$S-UTP

1µl placental ribonuclease inhibitor (60 units)

1µl T7/T3/SP6 RNA polymerase (20-70 units)

2 µl DTT (0.1 M)

Xµl DEPC-treated $H_2O$ (adjust volume to 20 µl)

The reaction is incubated at 40°C for 60 min. Following this incubation, the following is added: 20 µl 10X T7/T3/SP6 RNA poymerase buffer, 140 µl DEPC-treated $H_2O$, 15 µl RNase-free Yeast total RNA (100 µg), and 2 µl

placental ribonuclease inhibitor (120 units), and 1 µl DNase I (23 units) and incubated at 37°C for 15 min. To stop the reaction, 10 µl 0.5M EDTA and 2 µl 1M DTT are added and kept on ice while checking the % incorporation. The reaction is then extracted with phenol, phenol:chloroform, and chloroform, and precipitated with two volumes of ethanol and resuspended in 50 µl of DEPC $H_2O$, 10 mM DTT.

2.7.2 Hydrolysis of RNA probes

Alkaline hydrolysis of probes is carried out in a carbonate buffer system essentially as described by Cox et al. (1984). Briefly, 50 µl $Na_2CO_3$ hydrolysis buffer is added to the samples, incubated at 60°C for approximately X min (for 600 nucleotides of original transcript, X=60-90 min). and quenched on ice. Samples are neutralized with 100 µl of ice cold acetate neutralization buffer. Twenty µl of 3 M NaOAc pH 5.5 is added, followed by precipitation with 2 volumes ethanol. Pellets are resuspended in 50 µl of DEPC $H_2O$, 10 mM DTT at a final cpm which is the required number of cpm/section in 2.5 µl. The probe length before and after hydrolysis is checked by sequencing gel electrophoresis ($10^6$ cpm); the gel is dried and exposed overnight at -70°C with a screen.

2.7.3 Preparation of paraffin-embedded sections

Paraffin-embedded sectioned tissues were done as previously described (Hogan et al., 1986). Briefly, mice were anesthesized and perfused with 4% phosphate-buffered paraformaldehyde solution through the left heart. The organs of choice were removed and placed in ice cold 1X PBS solution containing 10 µg/ml BSA. When all tissue have been removed, they were transferred to ice cold 1X PBS followed by 10 ml ice cold 4%

paraformaldehyde. Tissues were fixed overnight at 4°C with regular slow rotation. The overnight solution was then replaced with 10 ml 1X PBS and incubated for 30 min at 4°C. The 1X PBS is then replaced with 10 ml 0.85% saline and incubated for 30 min at 4°C. The saline solution was successively replaced with 10 ml ethanol:saline (1:1) and then 2X 10 ml 70% ethanol, incubating each time for 30 min at 4°C. Dehydration of the tissues is completed by equilibration in increasing percentage ethanol at room temperature. The second 70% ethanol solution is replaced with 10 ml 85% ethanol, 95% ethanol, and 2X 100% ethanol, incubating each time for 30 min. The ethanol is then removed from the tissues by first incubating in 100% ethanol:toluene (1:1) followed by two equilibrations in toluene, incubating each time for 30 min. The final step is to replace the toluene in the sample with paraffin (i.e. impregnation). This is accomplished with four changes of paraffin, each for 30 min at 60°C. The tissues are then transferred to molds containing paraffin at 60°C, oriented as desired and allowed to set.

2.7.4 Pretreatment of slides

Slides are soaked overnight in a 4% solution of Extran 300 (gentle detergent). Slides are then rinsed in running tap water for 45 min., and rinsed in milli-pure water. Slides are baked in a Pasteur oven overnight. Incubate cooled slides at room temperature for 30 s in 2% 3-Aminopropyl-triethoxysilane (Rentrop et al., 1986) made in absolute methanol. Slides are washed twice, 1 min each, in methanol, followed by a 1 min wash in milli-pure water. Slides are baked to dry overnight at 37°C.

2.7.5 Sectioning of tissues

The paraffin blocks containing the tissues are cut with a razor blade to a trapezoid and the tissue is ribboned into 3 um sections on a hand cranked microtome. To spread the sections and remove any creases each group of four sections is lowered onto a waterbath at 45°C. Sections are then lifted out on a slide.The slides are incubated to 37°C for 20 min, put into a box containing dessicant and kept on a slide warmer overnight. The slides are now ready to be used.

2.7.6 Pretreatment of slides prior to hybridization

Paraffin is removed from the sections with three 10 min xylene washes. Following paraffin removal, the sections are cleared of xylene by incubating the slides for 2 min. in 95% ethanol. The sections are rehydrated by putting the slides through a decreasing ethanol series at 2 min. for each step:

a. 95% ethanol
b. 95% ethanol
c. 80% ethanol
d. 60% ethanol
e. 30% ethanol

Basic proteins are removed from the sections by incubating in 0.2 M HCl for 20 mins. Slides are rinsed in DEPC $H_2O$ for 1 min with gentle agitation, and incubated in a preheated 2X SSC solution for 30 min at 70°C. Slides are rinsed in $dH_2O$ for 2 min and incubated for 10 min in 0.625 mg/ml pronase in 1X P buffer (20X P buffer=1 M Tris (pH 7.5, 0.1 M EDTA), followed by incubation for 30 sec of 0.2% glycine in 1X PBS (to block pronase reaction). Slides are rinsed twice in 1X PBS for 30 sec each before re-fixing in 4% paraformaldehyde by incubating slides for 20 min. The carriage containing

slides is quickly dipped in 1X PBS twice for 3 min each, and attached to a 1 liter beaker with a magnetic stirrer containing acetic anhydride diluted 1:400 in 0.1 M triethanolamine pH 8. Once the anhydride is completely dispersed, the stirrer is turned off, and slides are allowed to sit for 10 min. The sections rinsed in 1X PBS for 1 min and are dehydrated by putting the slides through an increasing ethanol wash, 2 min each step:

a. 30% ethanol

b. 60% ethanol

c. 80% ethanol

d. 95% ethanol

Slides are air-dried and are ready for prehybridization.

2.7.7 Hybridization/hybridization of slides

Heat the 5X probe to 80°C for 1 min. Microfuge for 20 seconds and mix with hybridization buffer, estimating 25 µl per 22 x 22 mm coverslip. Take slides after air-drying and pipet 25 µl onto the coverslip and gently overlap the slide onto the coverslip (sections are facing down). Incubate overnight at 50°C in a sealed sandwich box containing paper towels saturated with 1X Salts, 50% formamide.

stock solutions

1. 10X Salts: 3 M NaCl, 0.1 M Tris-HCl, 0.1 M $NaPO_4$ (pH 6.8), 50 mM EDTA, Ficoll 400 (0.2% w/v), polyvinylpyrolidone (0.2% w/v). After the above reagents have dissolved, add BSA Fraction V (0.2% w/v) and dissolve by gentle stirring. Make in DEPC $H_2O$ and store in 50 ml aliquots at -20°C.

2. hybridization buffer:

| | ml |
|---|---|
| 10X Salts | 1.25 |

| | |
|---|---|
| formamide | 5.0 |
| 50% dextran sulfate | 2.5 |
| total RNA (50mg/ml) | 0.25 |
| 1 M DTT | 0.1 |
| DEPC $H_2O$ | 0.90 |
| total volume | 10.0 |

Final volume including probe is 12.5 ml.

2.7.8 Posthybridization washings

Digestion with RNase A destroys any single-stranded unhybridized probe while leaving double-stranded hybrids intact. The washing steps are very important to remove backgroud, and variations in time and temperature may be necessary for optimal results.

Slides are incubated in 50% formamide, 1X salts, 10 mM DTT at 50°C for 15 min. Coverslips are gently removed, solution changed, and washing is continued for 2 1/2 h. Slides are incubated in 0.5 M NaCl in TE at 37°C for 15 min, followed by an incubation in 0.5M NaCl in TE containing 20 μg/ml RNase A at 37°C for 30 min, and an incubation in 0.5 M NaCl in TE at 37°C for 5 min. Slides are washed in 4 liters of 2X SSC (30 min at room temperature) with gentle stirring followed by 4 liters of 0.1X SSC (30 min at room temperature). Slides are dehydrated through: 30% EtOH in 300 mM $NH_4Ac$ for 2 min; 60% EtOH in 300 mM $NH_4Ac$ for 2 min; 95% EtOH in 300 mM $NH_4Ac$ for 2 min. Slides are air-dried at 37°C in incubator.

2.7.9 Autoradiography

All steps should be carried out in a darkroom.

Use Ilford K5D emulsion. Melt 1:1 dilution at 42°C in dipping apparatus. Dip clean slides into emulsion and hold up to light to check for the presence of bubbles or lumps of uncompletely melted or mixed emulsion. Continue dipping clean slides until slides are uniformly coated with emulsion. Dip experimental slides and leave to dry on rack for atleast 10 min. Store in light tight box which contains a small packet of freshly prepared dessicant, at 4°C for 3-30 days.

2.7.10 Autoradiography-Developing

All solutions should be exactly at the same temperature (18°C) because variations between solution temperatures cause swelling and contracting of emulsion, leading to cracking and loss of emulsion layer. Steps 1-5 inclusively are with gentle agitation every 30 s for 5 s:

1. D19 developer, 4 min at 18°C
2. $dH_2O$, 2 min at 18°C
3. 30% sodium thiosulfate, 4 min at 18°C
4. $dH_2O$, 2 min at 18°C
5. $dH_2O$, 2 min at 42°C. This clears unreacted emulsion from the slide.
6. return to $dH_2O$ at 18°C.

2.7.11 Staining of slides

Stain for 30 s to 20 min at room temperature in 1:10 dilution in $dH_2O$ of stock hematoxylin. Staining time varies according to tissue type, section thickness, and age of hematoxylin. Rinse 2 x 2 min in $dH_2O$. Immerse 5 s only in 1:20 dilution in $dH_2O$ of stock $LiCO_3$. Rinse 2 x 2 min in $dH_2O$. Incubate slides in 30 % EtOH, 1 min 20 s; 60% EtOH, 1 min; 80% EtOH, 1 min. Eosin stain for 20 s. with continuous agitation. Dilute stock in 1:3 in

EtOH and add 0.5 ml glacial acetic acid/100 ml just prior to use. Incubate in 95% EtOH 2 x 1 min, and xylene 2 x 2 min. Mount with a minimum amount of Permount.

## 2.8 Protein Synthesis and Extraction

### 2.8.1 Protein synthesis *in vitro*

The cloned *Pom-1* cDNA was digested with *Eco RI* and *Xba I* (stop codon removed) and ligated to the GEM 4 vector which was also digested with the same restriction enzymes. The *Pom-1* plasmid was digested with *Pst I* and ligated to a *PstI* fragment containing a second orf (start codon removed). The new subclone was digested with *Sac* I and linearization of the DNA was checked on a 1 % agarose gel. Linearized DNA (5 mg) was used for transcription *in vitro* with SP6 RNA polymerase, as previosly described (Melton et al., 1984) in 50 x reaction buffer (300mM NaCl, 10 mM Tris-HCl, 5 mM EDTA) containing ATP, CTP, UTP, GTP, each at 500 $\mu$M; 10 mM DTT; 80-100 U of RNase inhibitor (RNasin; Promega); and 25 U SP6 RNA polymerase. RNA species were checked by formaldehyde gel and used for rabbit reticulocyte translations as recommended by the manufacturer, with 50 $\mu$Ci of $^{35}$S-methionine. Translation reaction was performed for 60 min at 37 °C with 35 ml of nuclease treated rabbit reticulocyte lysate (Promega), 1 ml of 1 mM methionine-free amino acid mixture (Promega), 1 mg of synthesized RNA in 2 ml of $H_2O$, 5 ml of $^{35}$S-methionine (1,200 Ci/nmol) at 10 mCi/ml, 1.25 ml of RNasin (Promega) RNase inhibitor at 40 U/ml, 5.75 ml of $H_2O$ in a total volume of 50 ml. The

resulting products were inspected by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE).

2.8.2 Small scale protein purification

Bacteria carrying either the parental pGex2T (ß-galactosidase) or the recombinant GST-*Pom-1* were grown overnight (with appropriate antibiotics, where necessary), diluted 1/5, and after 1 h of additional growth, IPTG was added to a final concentration of 250 µg/ml and grown for 4 h. Cells (1 ml) were then harvested (10 min at 4000 rpm, 4°C), resuspended in 40 µl lysis buffer (10% sucrose; 100 mM Tris pH 8; 1.5 mM EDTA), 2 µl lysozyme (0.5 mg/ml) was added, and incubated on ice for 15 min. 325 µl of extraction buffer ( 10 mM Tris pH 8; 0.16 mM EDTA; 0.43 mM O-phenantroline; 1% sucrose; 0.5 mM DTT; 50 mM NaCl) was added, followed by the addition of 7.5 µl of 10% N-Laurylsarcosine, and sonicated 3 times for 10 s each time. Debris were spun down at 10,000 x g for 15 min at 4°C. The supernatant was mixed with 30 µl of 25% Triton-X-100, 0.3 µl 1 M $MgCl_2$, and 0.5 µl 1 M $CaCl_2$, and incubated on ice for 5 mins. The preparation was spun down at 10,000 rpm for 10 min. The supernatant was mixed with 400 µl of PBS and 20 µl glutathione agarose resin (Pharmacia) that was previously equlibrated with PBS and left on a rotary shaker for 60 min at 4°C. The resin was collected by centrifugation (2000 rpm, 4°C) and washed three times with 0.5 ml PBS. Pellet was resuspended in 30 µl of PAGE-SDS buffer and was analyzed for the purities and quantities of the bound GST proteins on SDS-PAGE that were Coomassie blue stained.

2.8.3 Large scale protein purification

Fifty ml TB medium (900 ml=12 g tryptone; 24 g yeast extract;

8 ml 50% glycerol) was inoculated with either the parental pGex2T (ß-galactosidase) or the recombinant GST-*Pom-1* and grown overnight (with appropriate antibiotics, where necessary), at 37°C. The overnight culture was transferred to a flask containing 400 ml TB medium, 44 ml 10 X $KPO_4$ buffer and antibiotic. The culture is grown for 1-1 1/2 h and IPTG (25uM final) induced for 6 h. Bacteria is spun for 15 min at 4°C. Pellet is resuspended in 4 ml lysis buffer and 80 µl lysozyme (5 mg/ml) is added and incubated for 30 min. Extraction buffer (32.5 ml), and 750 µl 10% N-Laurylsarcosine is added, and sonicated 2-3 times at 30 s each time. Debris is spun down at 16,250 rpm for 15 min at 4°C. Three ml of 25% Triton X-100 is added to the supernatant and incubated on ice for 15 min. $MgCl_2$ and $CaCl_2$ are added to a final concentration of 1.25 mM and 1.06 mM, respectively, and incubated on ice for 20 min. Preparation is spun for 30-60 min at 10,000 rpm. One volume PBS is added to the supernatant, filtered on a 0.45 µm filter, and added to a Glutathione-agarose column. An aliquot of this preparation and its flowthrough is kept as contols. The column is washed 2X with 5 volumes of PBS, and the protein is eluted with 30 ml of DE (10 mM glutathione, 50 mM Tris pH 8) in 1.5 ml aliquots (in an eppendorf tube). Thirty µl of each aliquot and controls are checked on a PAGE-SDS gel. Positive protein aliquots are pooled and dialyzed 3X 2 liters against 50 mM Tris pH 7.5. An aliquot is rechecked on a PAGE-SDS gel and quantified with a BIO-RAD micro assay.

2.8.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were resuspended in SDS-PAGE buffer and solubilized for 5 min at 100°C and run on a linear gradient (5-15%) polyacrylamide gel. Up to 50 µg of protein per lane was used. Apparent molecular weights were

determined from the mobility of the low molecular weight standards (Pharmacia). Gels were stained with Coomassie Brilliant Blue stain.

2.8.5 Western blotting

Electrophoretic transfer of proteins from gels to nitrocellulose paper was done in 25 mM Tris-HCl, 192 mM glycine, and 20% methanol. Electrophoretic transfer was carried out at 0.5-0.6 A for 1-4 h, depending on the gel type used. After removal of the transfer apparatus, gels were stained with Coomassie Brilliant Blue and the nitrocellulose blots were temporarily stained with 0.2% rouge Ponceau in 3% trichloracetic acid (TCA) to determine the effectiveness of the transfer.

2.8.6 Immunoblotting

Washed and preselected nitrocellulose filters were wet with methanol and saturated in a blocking solution (0.5% skim milk) in TBS for 30-60 min at room temperature or overnight at 4°C. Filters were washed in TBS and incubated with the 1° antibody diluted in TBS at room temperature for 1-2 hours. Filters were washed again before adding the 2° antibody (anti-rabbit HRP) in TBS + Tween, and incubated for 60 min at room temperature. The filters were washed 3X in TBS + Tween and 1X in TBS.

2.8.7 ABC Immunolabelling

Slides are deparaffinized by three 10 min washes in 100% xylene and hydrated by 3 min washes in:

1. 95% EtOH
2. 95% EtOH
3. 80% EtOH

4. 60% EtOH

5. 30% EtOH

Slides were blocked in 10% goat serum in TBS (40 $\mu$l) for 10 min and washed in TBS + 1% Tween. Slides were incubated for 90 min at 37°C with affinity-purified Ab, washed 4X in TBS + 1% Tween, 5 min each wash, and blocked again with 10% goat seum for 5 min. Slides were incubated with biotinylated goat anti-rabbit 2° Ab for 30 min at 37°C, and avidin-biotin conjugated to peroxidase in TBS for 30 min at 37°C. Slides were washed 3X in TBS + Tween, 5 min each wash, and incubated in TBS containing 0.03% $H_2O_2$, 0.1 M imidizole, and 0.05% diaminobenzidine tetrahydrochloride (DAB) pH 7.4 (DAB is polymerized in the presence of peroxidase and hydrogen peroxide to form an insoluble brown polymer which is deposited at the site of the Ag-Ab complex). Slides are washed in $dH_2O$ and counterstained with 0.1% methylene blue for 10-12 min. Slides are dehydrated and mounted with Permount.

## 2.9 Generation of Antibodies

### 2.9.1 Generation of antibodies

Antibodies were raised against bacterially expressed fusion proteins. The expression vector used was pGex2T (ß-galactosidase). The anti-*Pom-1* antibody was raised against a GST (Glutathione *S*-Transferase)-*Pom-1* fusion protein by immunizing rabbits against the fusion protein. Three animals were injected with 300 µg of the antigen in 0.9% NaCl:complete Freund's adjuvant (1:1). Four booster inoculations (300 µg each) were given, every four weeks, in 0.9% NaCl:incomplete Freund's adjuvant (1:1),

and each time the animals were bled 7 to 10 days post-inoculation. The activity of the third and fourth bleed were tested for its capacity to immunoprecipitate *in-vitro* translated proteins. This was verified against pre-immune serum as a control.

2.9.2 Affinity Purification of antibodies

To obtain antibodies specific to *Pom-1*, GST-*Pom-1* fusion protein (150 μg) was adsorbed onto portions of nitocellulose filters and allowed to air dry. The filters were washed with TBS for 5 min and incubated for 30 min in blocking solution (5% skim milk in TBS). Filters were washed 3X in TBS for 2 min each time. The protein bound nitrocellulose filter was incubated with the appropriate antibody ( GST-*Pom-1* antibody) (1:20) in TBS for 60 min. Filters were washed 3X in TBS + 1% Tween for 5 min each wash. Filters were then saturated with free GST protein (10X amount of GST Ab) for 30 min, and washed 4X in TBS + 1% Tween for 5 min each wash. Affinity purified Ab was eluted using 20 mM glycine pH 2.5 for 2 min, and quickly neutralized with 5 ml 2X TBS pH 12. The eluate was concentrated with an Amicon Centricon microcentrator (Amicon Corp., Danvers, MA). This is the affinity purified antibodies to be used for Westerns, immunoprecipitations, and immunocytochemisrty.

The nitrocellulose strips can be maintained for future use by washing with TBS 2X for 5 min each, and freezing.

## References

Bellvé, A.R., Cavicchia, J.C., Millette, C.F., O'Brien, D.A. Bhatnagar, Y.M., and Dym, M., (1977) Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. J. Cell Biol. 74, 68-85.

Chomczynski, P. and and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Analytical Biochem. 162,156-159

Cox, K.H., DeLeon, D.V., Angerer, L.M., and Angerer, R.C. (1984) Dev. Biol. 101, 485.

Devereux, J., Haeberli, P., and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Research 12, 387-395.

Feinberg, A.P., and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments tohigh specific activity. Anal. Biochem. 132, 6-13

Hogan B., Costantini, F., and Lacy, E. (1986) Manipulating the mouse embryo: A laboratory manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY., pp.228-243.

Kleene, K.C., Distel, R.J., and Hecht, N.B. (1983) cDNA clones encoding cytoplasmic poly(A)+ RNAs which first appear at detectable levels in haploid phases of spermatogenesis in the mouse. Dev. Biol. 98, 455-464.

Kleene, K.C., Distel, R.J., and Hecht, N.B. (1984) Translational regulation and coordinate deadenylation of a haploid mRNA during spermiogenesis in the mouse. Dev. Biol. 105, 71-79.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, USA.

Meistrich, M.L., Longtin, J., Brock, W.A., Grimes, S.J., Mace, M.L. (1981) Purification of rat spermatogenic cells and preliminary biochemical analysis of these cell. Biol. Reprod. 25, 1065-1077.

Melton D.A., Kreig, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Research 12, 7035-7056.

Pear W.S., Nolan, G.P., Scott, M.L., and Baltimore, D. (1993) Proc. Natl. Acad. Sci. USA 90, 8392-8396.

Rentrop (1986). Histochem J. 18, 271-276.

Romerell, L.J., Bellve, A.R., and Fawcett, D.W. (1976) Separation of mouse spermatogenic cells by sedimentation velocity. A morphological characterization. Dev. Biol. 49,119-131.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74, 5463-5467.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Stern, L., Gold, B., and Hecht, N.B. (1983a) Gene expression during mammalian spermatogenesis . I. Evidence for stage-specific synthesis of polypeptides "in vivo". Biol. Reprod. 28, 25-41.

Stern, L., Kleene, K.C., Gold, B., and Hecht, N.B. (1983b) Gene expression during mammalian spermatogenesis . III. Changes in populations of mRNA during spermiogenesis. Exp. Cell Res. 143, 247-255.

Zarbl, H., Latreille, J., and Jolicoeur, P. (1987) Revertants of v-fos transformed fibroblasts have mutations in cellular genes essential for transformation by other oncogenes. Cell 51, 357-369.

# CHAPTER 3

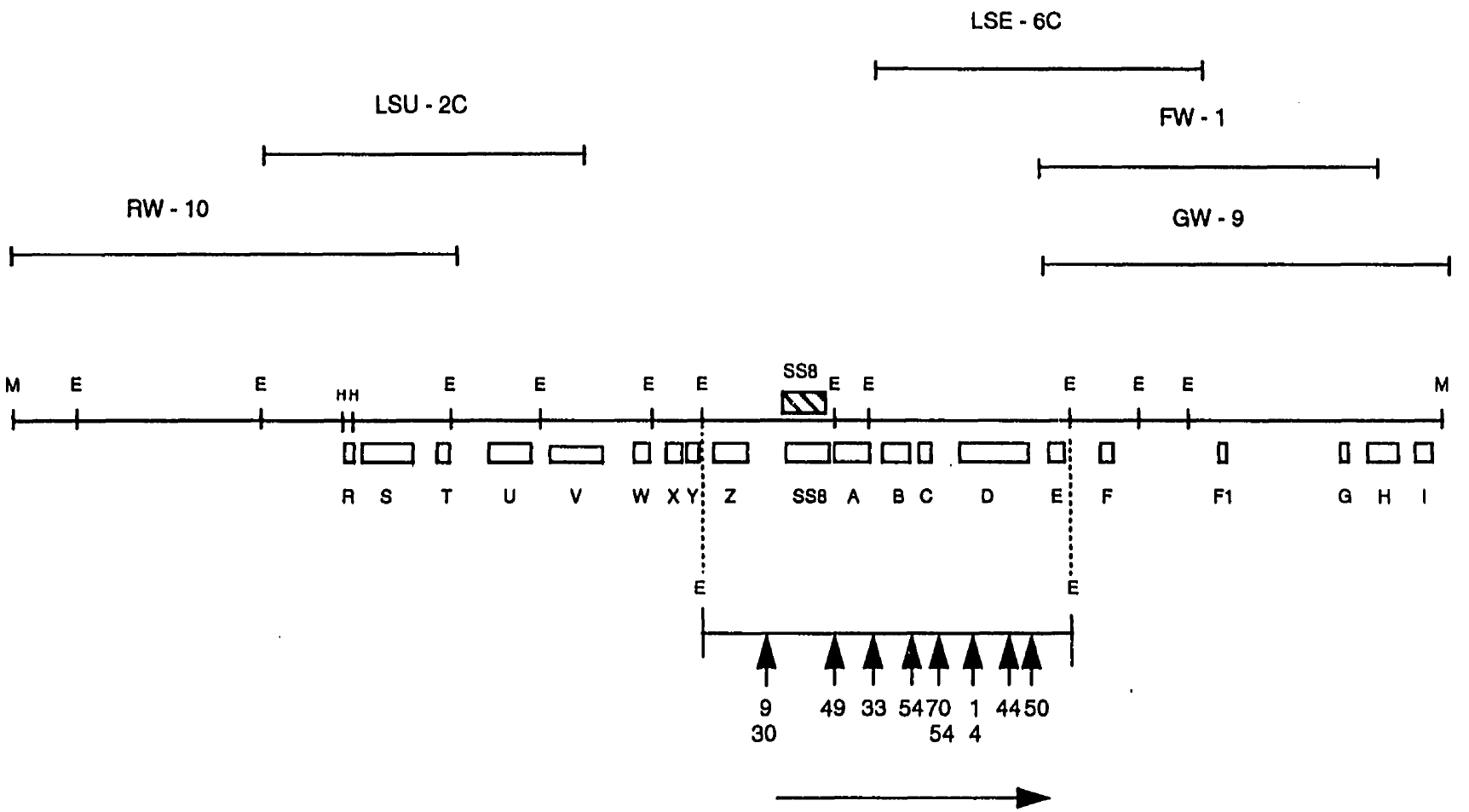
# RESULTS

The origin of the *Gin-1* region has been described in detail elsewhere (Villemur et al., 1987). Briefly, a common integration site, *Gin-1*, was identified in mice thymomas induced by Gross Passage A Murine Leukemia virus (GPA MuLV). However, using various *Gin-1* genomic DNA probes, no RNA transcript was detected from this 28 kbp genomic region. Thus, genomic walking in either direction of the *Gin-1* region was employed in order to identify its transcript.

As summarized in Monczak (1990), three different genomic DNA libraries, a Sim.S partial *Eco RI* genomic DNA library (λ-EMBL4), a partial *Hind* III genomic DNA library (λ-2001), a C57BL/6 partial *Mbo* I genomic DNA library (λ-EMBL3), were screened in the 3' and 5' direction with various genomic DNA probes to extend the *Gin-1* region. In summary, genomic walking in the 3' direction from the *Gin-1* integration site generated a total of 15.5 kbp of novel DNA and 5 new single copy probes, and genomic walking in the 5' direction from the *Gin-1* proviral integration site enabled the subcloning of 18 kbp of novel genomic DNA and 3 new single copy probes were derived from this region. The total length of genomic DNA cloned around the *Gin-1* integration site is 61.5 kbp (figure 1). The 8 new single copy probes were used to identify a transcript.

As seen in Monczak (1990), to identify a transcript encoded by this region, total RNA was isolated from adult C57BL/6 tissues including: testis, epididymis, ovary, spleen, thymus, heart, lung, liver, kidney, brain, intestine, stomach, and whole embryo (E8 and E19). Several Northern blots were used and were hybridized with the novel 8 probes mentioned in Figure 1 (data not shown). Only the $^{32}$P- labeled R probe had a signal in testis and its transcript size was a doublet of 1.0 and 1.2 kbp (data not shown). No signal

Figure 1: Partial restriction endonuclease map of the *Gin-1* locus and location of the Gross Passage A MuLV insertion sites. Recombinant phage LSE-6C, FW-1, GW-9, LSU-2C and RW-10 were isolated by hybridizing various mouse genomic libraries with $^{32}$P-labeled probes, E, F, G, U, and R respectively. DNA from these clones were digested with restriction enzymes to derive a restriction map of the region. The digested DNA was electrophoresed on a 1% agarose gel and visualised with ethidium bromide. Fragment sizes were estimated using *Hind III* digested lambda markers. Non-reiterated fragments (identified as open boxes in this figure) were subcloned into pBR322 or pGEM 3, to be used as hybridizing probes. Arrows indicate the site and direction of insertion of MuLV proviruses. Numbers indicate the thymoma number. Abbreviations: E,*Eco RI*; M,*Mbo I*

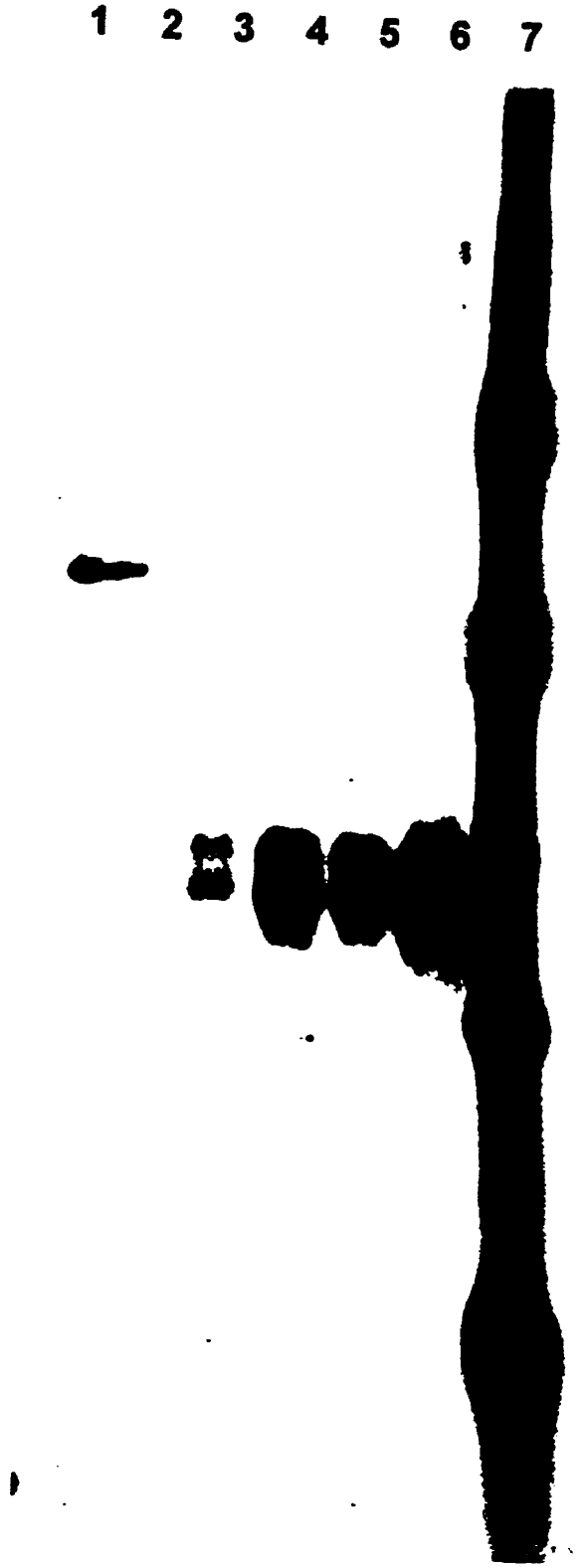

LSE - 6C
LSU - 2C
FW - 1
RW - 10
GW - 9
M
E
HH
SS8
R
S
T
U
V
W
X
Y
Z
A
B
C
D
F
F1
G
H
I
9
30
49
33
54
70
54
1
4
44
50
2 kbp

was detected in any other tissues screened, even after longer exposures (data not shown).

## 3.1 Molecular Cloning and Sequencing of Mouse *Gin-1* cDNA

To determine the identity of *Gin-1* and to map its location on the genome, we first attempted to clone its cDNA. A C57BL/6 mouse testis cDNA library was screened with the genomic R probe. Approximately, 1 million cDNA clones were screened, and 4 positive clones were isolated (Monczak,1990). These clones were analyzed by restriction endonuclease mapping and partial sequencing (data not shown). This analysis showed that all clones contained a cDNA insert of 648 bp. When used as a probe for RNA detection by Northern analysis, this 648 bp cDNA fragment revealed the same pattern of hybridization previously observed with the genomic R probe (Fig. 2), indicating that part of the *Gin-1* gene had been identified (the band in lane 1 is an artifact from hybridization as it is not specific to lane 1). Because the cDNA clones isolated from the mouse cDNA library were not full length, we used this 648 bp cDNA insert as a probe to screen other mouse testis cDNA libraries. Six mouse testes cDNA libraries were screened (4 Clonetech libraries (C57Bl/6 and Balb/C), 2 libraries generated in our laboratory). More than 6 x $10^6$ cDNA plaques of each library were screened using the 648 bp cDNA probe. No positive signals were identified. As well, 5' RACE PCR was tried without success. We obtained another cDNA testis library generously donated by Dr. D. Wolgemuth (Columbia University, New York). Eight positive clones were obtained after screening 4 x $10^5$ cDNA clones. The cDNA was isolated and used as a probe on Northern blots to verify the pattern of hybridization (data not shown). The longest cDNA insert, 800 bp, was sequenced (double stranded sequencing)

Figure 2: Identification of *Gin-1* transcripts. Mouse total RNA and poly $A^+$ mRNA extracts from mouse tissues were electrophoresed on a denaturing 1% agarose gel, transferred to nylon membrane and hybridized with the $^{32}P$-labeled R probe. Lane 1: 5 μg total RNA of 8 day old embryo; lane 2: 5 μg of poly $A^+$ mRNA from adult thymus; lane 3: 5 μg total RNA from adult testis; lane 4: 1μg poly $A^+$ mRNA from adult testis (extract #1); lane 5: 1μg poly $A^+$ mRNA from adult testis (extract #2); lane 6: 1μg poly $A^+$ mRNA from adult testis (extract #3); lane 7: lambda *Hind III* marker (Monczak,1990).


1 2 3 4 5 6 7

(Figure 3A) and the two longest potential open reading frames (orf) were noted. One orf has the capacity to code for a protein of 58 amino acids (aa) while the other could encode a 48 aa-long protein. A search through the GeneBank database did not reveal any DNA sequences with homology to *Gin-1*. A search through the SwissProt database for any known related proteins revealed no similarity between the two putative orfs and the proteins in the database. Thus, *Gin-1* is likely to be a new gene which is not significantly related to any of the known genes represented in the databases searched.

To date, we have been unable to clone the longer (1.2 kbp) *Gin-1* cDNA. The screening of 6 different testis cDNA libraries never resulted in the cloning of the additional sequences. The presence of secondary structures within these complementary stretches of cDNA are present, causing improper reverse transcription may explain this problem. Other alternative techniques are presently being employed to isolate these remaining sequences.

## 3.2 Characterization of *Gin-1* cDNA

### 3.2.1 Exon Mapping

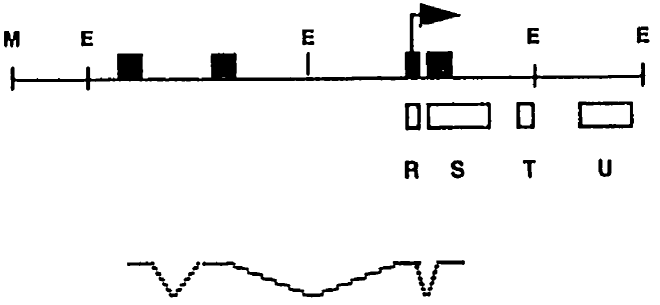
To localize the exact positions of the exons of this gene, probes were derived from the mouse cDNA and were used for hybridization of the cloned genomic fragment by Southern blot hybridization (data not shown). A schematic representation of the exon-intron boundries is shown in Figure 3B.

Figure 3: (A) Nucleotide sequence of the *Pom-1* cDNA. Initiation codon is underlined in bold for the 58 aa orf. and the initiation codon for the 42 aa orf is underlined. The poly-adenylation signal is also underlined. (B) Location of *Pom-1* exons. The 5' region of the genomic map as seen in Fig.1 shows open boxes to indicate non-reiterated probes used in the identification of the transcript. Hatched boxes indicate the exons of *Pom-1*. Abbreviations are the same as in Fig. 1.

(A)

```
1    GATCGAGGACATTAGGCGAGTGACGAGGGAGCCCTCAAGGCGATGGCAGGAAGAGGAATT
61   GCCAAGGTGAATGGAGTCTGAGAACACTAGTTATTGTAAGGGACAGAGTATGTAAAAACA
121  CGAGGAAAAGCCCTTCAGCGGACAGTCACCATTTTGGGACTTCCCACCCCGGTGAGATCT
181  GGGACTGGACAGCTCTGTTGTAGGGGGCTGTCCTGTGCAGCTTCACTGAGATCGGGAAAT
241  AACGGTGAGCCAGATCCATACTGTCCTGGTCTCGTGGATCAAAGATGAGCCCTGTTGAAG
301  CCATCTTGCTTAAATAGTCCTTGGCCTCTTCCGCTGGAGTTGTGGATTTAGAGTGAGAGG
361  ATACGGAGCAGCTGACATGGAGGCCTCATCCAATCCAGTCTAAGCCTTACTCAGTAATTG
421  CGGCCCCTGCCTGGACACTGGAGCTTTCAGCTGCTAACCCACCCCACACTAGGTAGGTGG
481  GCTTCGAAGGCGGACGTCCCCGGCGCCTCAAGGTTTTGTTAACACTGAGAATTGAAAGAC
541  CCGGCGCACCATAGGAAACATGCAGGATGCTTCAAGATAGGAAACATGCAGGATGCTTCC
601  AGACACAAGCTTGACAACGGTGCACAGGAGACTTCCTGCCGGTCCCCAACTGAAAAGAAT
661  GGGAATTGGCGACAGTTTACAGCTGACTTATTTCTTAGACTTCATTTCTAGAGTTACATT
721  TAGTCAACTTTTAACAATGATGAAAAAAACAATACCACCTGTGTAGCCCTCAAAGAATAA
781  ACCCAGAAGGCTTCCGTACAAAAC
```

(B)



### 3.2.2 Size Determination of Poly (A) Tail: The Poly (A) Tail Is Not Responsible for the Observed Differences Between the Two Testis-Specific *Gin-1* Transcripts

To determine the size of the poly ($A^+$) tail and to assess if the *Gin-1* 800 bp cDNA is full length, total RNA from adult testis and lung C57BL/6 mice were subjected to RNase H digestion in the presence of oligo (dT) to remove poly ($A^+$) tails. The sizes of the resulting RNAs were determined by Northern blot analysis following hybridization with $^{32}P$-labelled *Gin-1* cDNA. Analysis of untreated adult RNA reveals the two species (1.0 and 1.2 kbp) of the testis-specific *Gin-1* transcripts. Removal of the poly ($A^+$) tail by RNase H digestion resulted in a reduction in size of the transcripts of 200 bp for each transcript, generating transcripts of 0.8 and 1.0 kbp in length (Figure 4).

Since removal of the poly ($A^+$) tract gave rise to a transcript of 0.8 kbp and since our sequencing of the *Gin-1* cDNA reveals that it was 0.8 kbp in length in absence of the poly ($A^+$) tail, these results suggest that the cloned *Gin-1* cDNA is, in fact, full length. Furthermore, since removal of the poly ($A^+$) tail did not result in a single transcript, we can conclude that the poly ($A^+$) tract is not resposible for the size differences between the two *Gin-1* testis-specific transcripts.

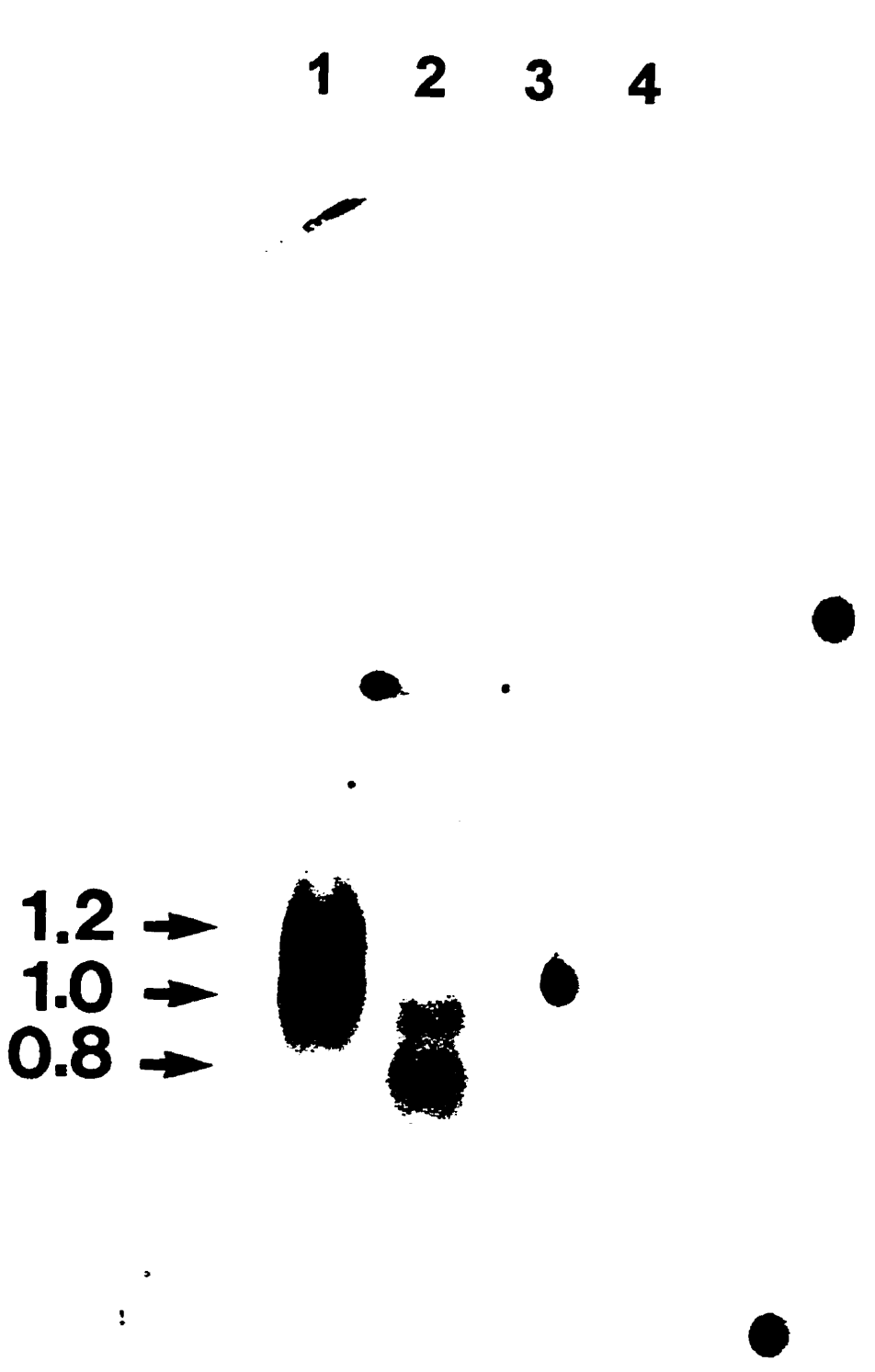
## 3.3 Role of *Gin-1* in Tumorigenesis

### 3.3.1 *Gin-1* Retroviral Construction and Infection

#### 3.3.1.1 Production of Recombinant Retroviruses Expressing *Gin-1*

To investigate the phenotypic effects of an expressed *Gin-1* gene in Rat-1 cells, we constructed a recombinant retrovirus containing the *Gin-1*

Figure 4: Measurement of the length of the poly $A^+$ tract of *Pom-1* transcripts. Oligo d(T) was annealed to total RNA and RNAse H was used to digest the RNA-DNA hybrid. The RNA was electrophoresed on a denaturing 1% agarose gel, transferred to nylon membrane, hybridized with $^{32}P$-labelled *Pom-1* cDNA, and exposed to film for 3 days. Lane 1 total testis (C57/BL6) RNA; lane 2: total testis (C57/BL6) RNA annealed with oligo d(T) and digested with RNAse H; lane 3: total lung (C57/BL6) RNA; lane 4: total lung(C57/BL6) RNA annealed with oligo d(T) and digested with RNAse H.


1 2 3 4
1.2
1.0
0.8

gene along with a puromycin gene, which confers resistance to the antibiotic puromycin and can serve as a selectable marker. The 0.8 kb *Eco R1* cDNA fragment was cloned into the *Eco R1* site of pBabe-Puro. Control contsructs were also made, where the 0.8 kb *Gin-1* cDNA fragment was subcloned in the anti-sense direction. Restriction enzyme analysis (data not shown) revealed the sense and anti-sense orientations. Other controls were recombinant retrovirus containing the *fos* oncogene, a *fos* mutant, and the vector alone.

Helper free virus stocks were derived from these constructed plasmids using the BOSC 23 cell line. Viruses produced were used to infect Rat-1 cells, and ecotropic virus stocks were ultimately obtained from clonal lines derived from single colonies of infected Rat-1 cells. Virus stocks gave titers of approximately 2 x $10^5$ colony forming units per milliliter when assayed for tranduction of puromycin resistance.

3.3.1.2 Infection with Recombinant Retroviral *Gin-1* Viruses Does Not Cause Morphological Changes in Rat-1 Cells

Virus stock of recombinant retroviral *Gin-1*, sense and anti-sense gene, *fos* gene, *fos* mutant gene and pBabe-Puro vector alone were used to infect Rat-1 cells. After two weeks post-selection, no morphological changes (data not shown) in the cells infected with the *Gin-1* gene in either orientation were evident. When individual puromycin resistant colonies were picked and grown separately, no morphological changes were noted. However, the virus stocks of *fos* retroviral induced cells, showed morphological changes, as in transformation of Rat-1 cells.

3.3.1.3 Virus Infected Cells Contain Recombinant Retroviral *Gin-1* proviruses and Express Proviral Transcripts of the Expected Size

To confirm that the infected Rat-1 cells contain *Gin-1* proviruses, Southern (data not shown) and Northern blotting revealed that the DNA had inserted in the genome and that RNA transcripts were expressed from the proviruses in the infected cells (Fig. 5). All the infected cultures (except one, lane 3) contained a 4.0 kbp RNA specie that hybridizes with the *Gin-1* probe. One infected culture had a smaller transcript, 2.5 kbp (lane 2) and was not investigated further. Little RNA was loaded in lanes 5 and 9 as seen by ethidium bromide staining.

3.3.1.4 Transfection with Recombinant Retroviral *myc* and *ras* Genes Do Not Cause Morphological Changes in Recombinant Retroviral *Gin-1* Cell Lines

As mentioned above, virus stocks of recombinant retroviral *Gin-1* gene (sense and anti-sense) were used to infect Rat-1 cells. Clonal lines were derived from single colonies of infected Rat-1 cells and maintained with puromycin selection. Each cell line was transfected with recombinant retroviral *myc* plasmid and a recombinant retroviral *ras* plasmid and selected with G418. No transformation of cells were noted (data not shown) with the *myc* gene. Hence, *Gin-1* does not act in cooperation with *myc*. To investigate the possibility that *Gin-1* may act as an antioncogene, the recombinant retroviral *ras* gene was used for transfections. No block in transformation from *Gin-1* was noted with this *ras* plasmid.

3.3.2 *in situ* hybridization of mice E15 - birth with *Gin-1* cDNA probe

To determine if *Gin-1* is expressed in a subpopulation of cells not seen in adult thymus, a series of *in situ* hybridization experiments was

Figure 5: Virus infected cells express recombinant retroviral *Gin-1* proviruses of the expected size. Total RNA was extracted from infected cultures and migrated on a 1% denaturing agarose gel. The gel was transferred to nylon membrane for 16 h, UV-fixed, and hybridized with a *Gin-1* probe. Lane 1: Rat-1 cells as a negative control; lane 2: Rat-1 cells infected with pBabe/puro; lanes 3-10: Clones of rat-1 cells infected with pBabe/puro-*Gin-1* showing the expected 4 kbp transcript.


1 2 3 4 5 6 7 8 9 10

performed on sections from mouse C57/BL6 thymus (E15-birth), adult thymus, liver (E15-birth), adult liver, and testis. Facs analysis was performed to determine the exact population of cells of fetal thymus and to verify the age of the animal. Sense and anti-sense RNA probes derived from the *Gin-1* cDNA clone were used for experimental and control hybridizations. No signal above background was seen in any of the thymus sections, nor liver sections. However, a signal was observed in all tubules from adult testis. This signal is exclusive to post-meiotic spermatids. No signal above background was seen in any other area of the testis section.

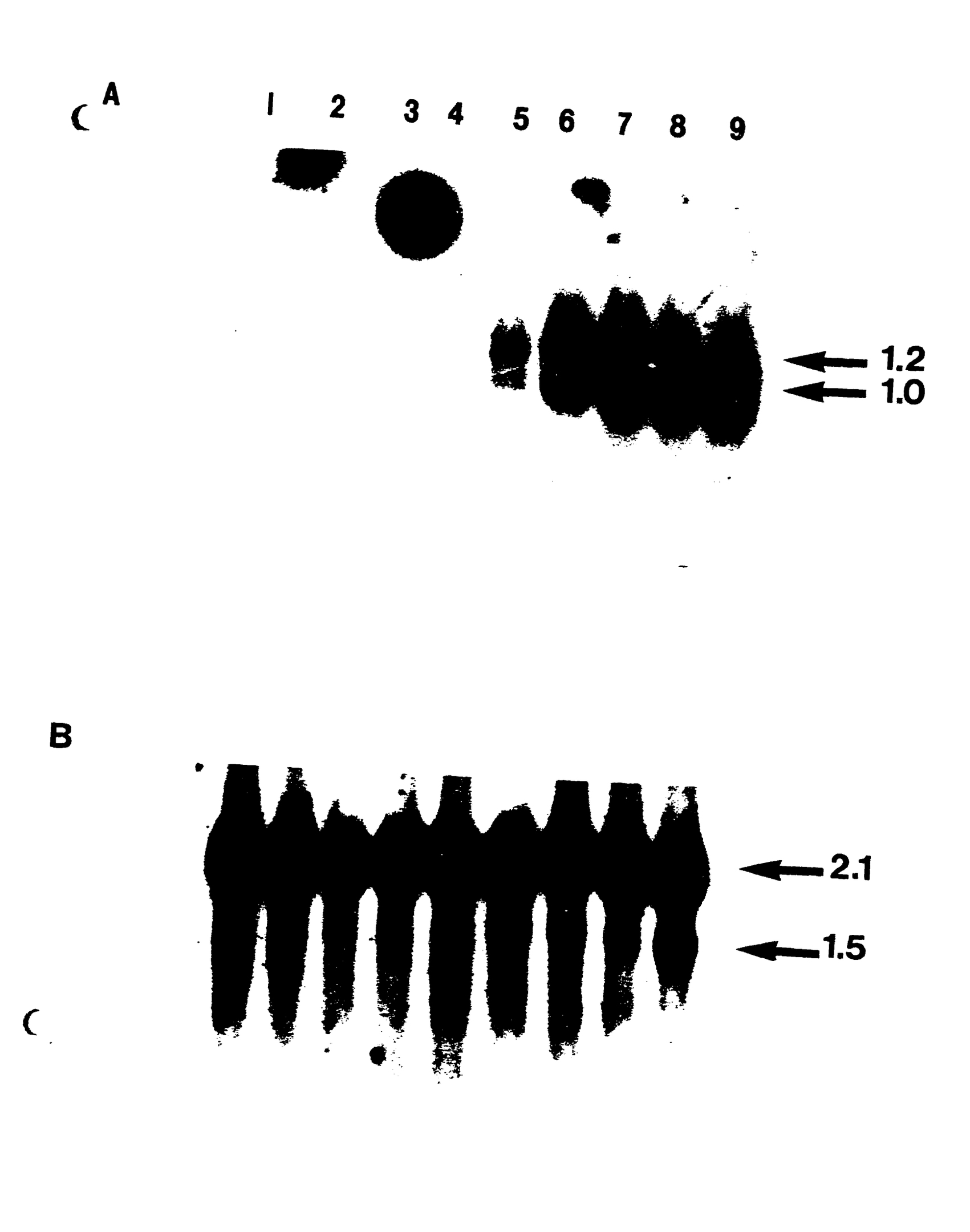
### 3.3.3 *Gin-1* Is Not Expressed In GPA-MuLV Induced Thymomas

To determine if *Gin-1* is expressed in GPA-induced thymomas, RNA was isolated from *Gin-1* rearranged and non-rearranged thymomas. No RNA was detected with the *Gin-1* cDNA $^{32}$P-labelled probe in tumors exhibiting *Gin-1* provirus insertion (data not shown). The control RNA testis lane reveals the doublet transcript, as expected. The filter was stripped and re-hybridized with a $^{32}$P-labelled actin probe to control for RNA presence and loading. This indicated that this novel transcript detected with probe R was unlikely to be the *Gin-1* gene affected by provirus insertion. Therefore, the gene encoding the testis-specific transcripts was re-named *Pom-1* (post-meiotic gene).

## 3.4 Temporal Expression of *Pom-1*

Northern analysis of C57BL/6 testis RNA samples from birth to adult, showed that the gene is expressed only in postpuberal mouse testis (data not shown), where the entire complement of germ cells is present. Fig. 6A is

Figure 6: (A) Time course of *Pom-1* expression on C57/BL6 testis RNA. Northern analysis of total RNA from testis day 20 to 28 after birth. 20 μg was electrophoresed on a denaturing 1% agarose gel, transferred to nylon membrane and hybridized with $^{32}$P-labelled *Pom-1* cDNA. Lane 1 - 9 is RNA from day 20 - 28 consecutively in each lane,; transcript sizes are 1.0 and 1.2 kbp. (B) This panel shows the same filter as in (A) stripped of the probe and re-hybridized with $^{32}$P-labelled actin as a control for RNA loading, showing the 2.1kbp and the testis specific 1.5 kbp transcripts.


A
1
2
3
4
5
6
7
8
9
1.2
1.0
B
2.1
1.5

a Northern blot of C57BL/6 testis RNA samples from day 20 to 28 after birth. *Pom-1* expression begins weakly on day 20 but increases thereafter to day 26 where its expression is stably maintained. The decrease of *Pom-1* expression seen in day 22 can be the result of individual expression for the gene. The filter was stripped and rehybridized with radiolabelled actin to control for RNA loading (Fig. 6B).

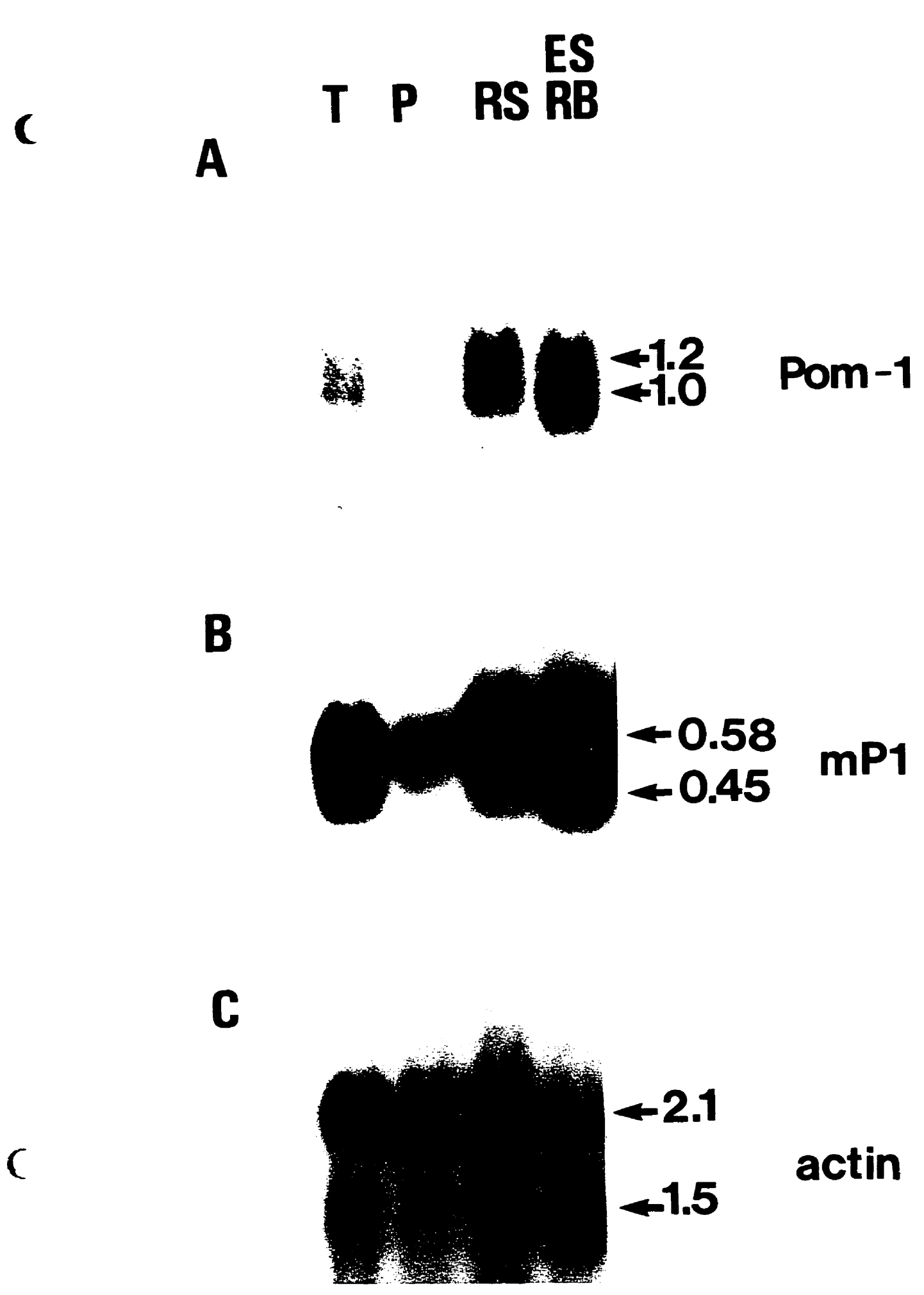
These results suggest that *Pom-1* is tightly regulated during differentiation of the testis and is expressed beginning on day 20, where the majority of the germ cells are in the haploid stage of spermatogenesis.

## 3.5 Spatial Expression of *Pom-1*

### 3.5.1 *Pom-1* Expression in Germ Cells of Testis

To determine which spermatogenic cells express the *Pom-1* transcript, enriched populations of meiotic prophase spermatocytes (predominantly in the pachytene stage of meiosis), postmeiotic early spermatids, and a mixture of elongated spermatids and residual bodies were obtained by the STAPUT technique (see Materials and Methods). Total RNA was isolated from the various cell types and analyzed by Northern blot hybridization with $^{32}$P-labelled *Pom-1* cDNA (Fig. 7A). The transcripts were very abundant in the postmeiotic fractions: round spermatids and the mix of elongated spermatids with residual bodies. The presence of *Pom-1* transcripts in the latter fraction suggests that this RNA is stable, since transcriptional activity ceases at stage 9 of spermatogenesis (Kierszenbaum & Tres, 1975). *Pom-1* expression was also seen in the pachytene spermatocyte fraction, although the signal was weak.

Figure 7: Size and distribution of *Pom-1* transcripts in purified populations of spermatogenic cells. 20 μg of RNA from total testis (T), pachytene cell (P), round spermatids (RS), elongated spermatids and residual bodies (ES & RB) were electrophoresed on a denaturing 1% agarose gel and blotted to nylon membrane. Autoradiograms were obtained after hybridization with probes encoding (A) *Pom-1* cDNA (B) protamine and (C) actin. The same filter was stripped between hybridization with each probe.


T
P
RS
ES
RB
A
1.2
1.0
Pom-1
B
0.58
0.45
mP1
C
2.1
1.5
actin

To confirm the reliability of fractionation of testicular germ cells with STAPUT, expression of protamine was examined (Figure 7B) as it was previously reported (Kleene et al., 1984) not to have any expression in the pachytene population. However, we detected some expression of this gene in the pachytene fraction, indicating that our preparation of pachytene cells were slightly contaminated with round spermatid cells.

To test for RNA loading, the filter was stripped and rehybridized with $^{32}$P-labelled actin probe (Fig. 7C).

3.5.2 *In situ* Hybridization Analysis

To further define the cellular expression of *Pom-1*, a series of *in situ* hybridization experiments were performed on tissue sections from C57BL/6 mice. Antisense and sense $^{32}$P-labelled RNA probes derived from the *Pom-1* cDNA clone were used for experimental and control hybridizations. A signal was observed in all tubules from adult testis, independent of the stage of their seminiferous epithelial cycle (as described by Oakberg, 1956). Examination of the cellular distribution revealed that this signal was not exclusive to postmeiotic spermatids (Fig. 8). No signal above backgound was noted in the spermatogonial cells in the periphery of the tubules, nor in the late steps of elongated spermatids which are located adjacent to the lumen. No signal above background was detected in the interstitial regions of the adult testis. In short, detectable signals were found in all stages of the cycle, including late pachytene spermatocytes (stages IX-XII), in all steps of round spermatids and in elongated spermatids (steps 10-early 15). No detectable signal was found in steps late 15 and 16 of elongated spermatids, nor in the early meiotic spermatocytes of any stage. This data is in agreement with the STAPUT data of Figure 7.

Figure 8: *In situ* hybridization analysis of testis sections from normal adult animals: (A) dark field of a tubule in stage I showing pachytene spermatocytes and round spermatids negative for *Pom*-1 expression, and positive in elongated spermatids; (B) dark field of a tubule in stageV showing pachytene spermatocytes and elongated spermatids negative, and round spermatids positive for *Pom*-1 expression; (C) dark field of several tubules (magnification 25 X) in stages XI (top left hand corner: diplotene and step 11 elongated spermatids both positive), stage VII (top center: pachytene spermatocytes have weak expression, round spermatids are positive while elongated spermatids are negative), and stage IX (bottom left hand corner: pachytene spermatocytes and round spermatids are both positive); (D) light field of (B); (E) light field of (C); (F) control sens slide showing background labeling.

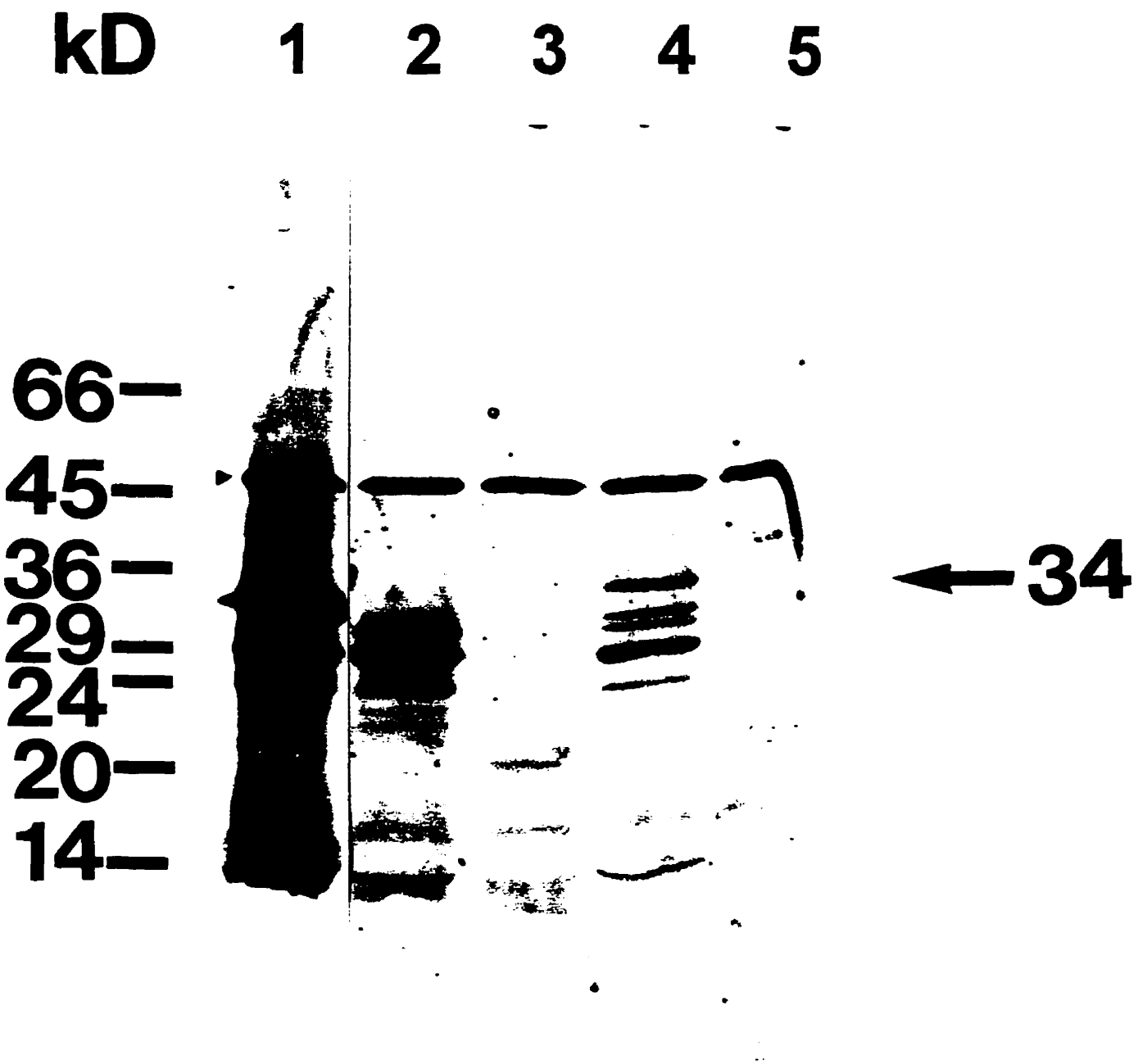
## 3.6 *Pom-1* is Actively Translated in Mouse Testes

### 3.6.1 *in vitro* Translation of *Pom-1*

#### Synthesis of *Pom-1* Fusion Protein by Reticulocyte Extracts

In order to determine which of the two potential open reading frames codes for a protein, *in vitro* translation was performed. To circumvent the technical problems of working with a peptide of 6 kD, *Pom-1* was fused, in frame, to a second open reading frame (orf) in pGEM 4, generating a new protein size of 34 kD (the stop codon of *Pom-1* was removed and subcloned in frame with a second orf whose start codon was removed). Each subclone was sequenced to verify the integrity of the coding frame (data not shown). The resultant plasmids were linearized with *Sac* I downstream of the inserted cDNA. *In vitro* transcription with SP6 polymerase yielded RNA species which were translated in the rabbit reticulocyte lysate system *in vitro*. The 58 aa *Pom-1* orf fused to the second orf (lane 5) gave the expected 34 kD protein as well as other species which may be degradation forms of the original full length protein (Fig. 9). The second *Pom-1* orf of 42 aa fused to the second orf did not give the expected major protein (lane 3). This can be a result of complete degradation of the full length protein or lack of a strong start codon. As negative controls, *Pom-1* cDNA in antisense orientation was fused to the above mentioned second orf. These constructs did not yield any high molecular weight protein at all (lanes 4 and 6). These results indicate that the 58 aa orf has a start codon that can be used for the translation of a *Pom-1* product.

Figure 9: Autoradiogram of $^{35}$S-methionine-labeled proteins translated from *Pom-1* orf fused to a second orf RNA in a reticulocyte lysate system. Lane 1: second orf alone; lane 2: low molecular weight markers; lane 3: 42 aa orf fused to second orf; lane 4: 42 aa orf antisense orientation fused to second orf; lane 5: 58 aa orf fused to second orf; lane 6: 58 aa orf antisense orientation fused to second orf.


kD
1
2
3
4
5
66
45
36
29
24
20
14
34

3.6.2 Polysomal RNA Expression of *Pom-1*

The status of translational activity for *Pom-1* was determined by Northern analysis of RNA extracted from polysomal and nonpolysomal fractions in the presence of $Mg^{++}$ (lanes 2 and 3) or EDTA (lanes 4 and 5) from adult mouse testes. The results of this experiment show that in adult mice the testis-specific $^{32}P$-labelled *Pom-1* cDNA probe hybridized to the $Mg^{++}$ polysomal fraction (lane 2) and very faintly in the nonpolysomal fraction (lane 3) (Fig. 10). Furthermore, upon disruption of the polysome with EDTA, the majority of the signal appeared to be in the nonpolysomal fraction (lane 5). Thus, the testis-specific *Pom-1* appears to be translated in the adult mouse testis, and may be a functional gene.

## 3.7 Immunolabelling of testis specific cells with *Pom-1* antibodies

Antibodies to the GST-*Pom-1* polypeptide were raised in rabbits and used in immunolabelling experiments of mouse testis cells. Under the light microscope, a strong positive reaction was observed over the Leydig cells (figure 11a and b). Control experiments using pre-immune serum did not show any sign of reactivity over any part of the rat or mouse testis (figure 11c).

Since GST was later found to react in mouse testis (Veri et al., 1993), specifically in Leydig cells, protein to GST and *Pom-1* was used to saturate the antibody before immunolabelling. The reaction was similar in both rat and mouse testis. The GST-*Pom-1* antibody saturated with 10 μg of

Figure 10: Measurement of the size and disribution in polysomal and non-polysomal fractions in mouse C57/BL6 testis. Polysomal and nonpolysomal RNA were isolated using sucrose gradients (see materials and methods). Testes were homogenized in cold buffer containing 5mM $MgCl_2$ (to preserve the polysomes) or 10mM EDTA (to dissociate the polysomes). After centrifugation (40,000 rpm for 110 min) on a sucrose gradient, the pelleted material and supernatant (referred to as polysomal and nonpolysomal fractions, respectively) were collected and electrophoresed on a denaturing 1% agarose gel, blotted to nylon membrane and hybridized with $^{32}P$-labelled *Pom-1* cDNA. The pellet (lane 2) and supernatant (lane 3) were prepared in the presence of $Mg^{++}$. Lanes 4 (pellet) and 5 (supernatant) were prepared in the presence of EDTA. Lanes 1 and 6 are total testis (C57/BL6) RNA as controls.

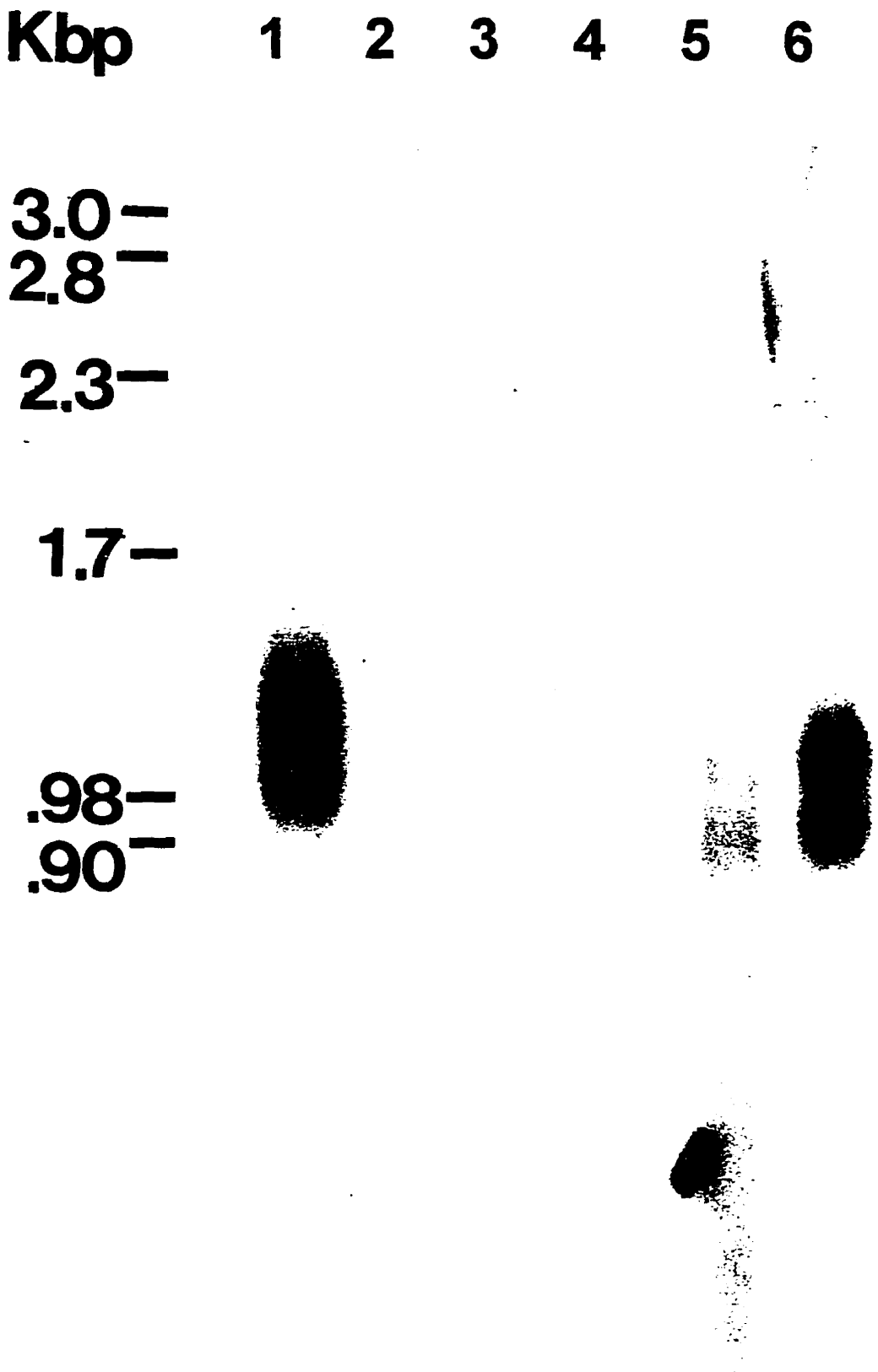

Kbp
1
2
3
4
5
6
3.0
2.8
2.3
1.7
.98
.90

Figure 11: Light micrograph of adjacent seminiferous tubules of mouse testis immunostained with a polyclonal antibody to GST-*Pom-1*. Figure 11a and b show a strong positive reaction over the Leydig cells. Control experiments (c) using pre-immune serum did not show any sign of reactivity over any part of the mouse testis.

GST protein gave a weak, although significant, reaction in the Golgi apparatus (data not shown), and almost completely eliminated the strong Leydig cell reaction (seen earlier with the GST-*Pom-1* antibody in figure 11). When 10 μg of GST-*Pom-1* protein was used to saturate the GST-*Pom-1* antibody, all reactions were abolished. Once again, control experiments using pre-immune serum did not show any sign of reactivity over any part of the mouse testis (data not shown). Thus, the immunolabelling reaction using GST-*Pom-1* antibody saturated with 10 μg of GST protein seems to show a specific reaction of *Pom-1* in the Golgi apparatus.

The GST-*Pom-1* antibody was affinity purified in order to eliminate the problem of cross-reactivity of the GST portion of the antibody and to eliminate the reaction in the Leydig cells. This antibody was shown to be specific for *Pom-1* sequences by immunoprecipiating *in vitro* translated *Pom-1* protein (data not shown). The affinity-purified *Pom-1* antibody was used in a further round of experiments in order to support the earlier findings. Under the light microscope, a stronger positive reaction was observed over the Golgi apparatus (figure 12) in a stage specific manner. In stage VII, pachytene spermatocytes were weakly positive. Step 7 round spermatids were positive while step 16 elongated spermatids were negative for *Pom*-1 protein expression. Stage VIII pachytene spermatocytes were weakly positive, while step 8 spermatids were positive and step 16 spermatids were negative. In stage IX, pachytene spermatocytes and step 9 spermatids were both positive. Stage X shows strong reaction over pachytene spermatocytes and a reaction over step 10 spermatids as well. In stage XI, diplotene spermatocytes were positive as well as step 11 spermatids. Control experiments using pre-immune serum did not show any sign of reactivity over any part of the mouse testis (figure 12). This data

supports the earlier experiment and reveals *Pom-1* protein expression in the Golgi apparatus.

Figure 12: Light micrograph of adjacent seminiferous tubules of mouse testis immunostained with an affinity purified GST-*Pom-1* polyclonal antibody. (A) stage VIII tubule showing pachytene spermatocytes weakly positive, step 7 round spermatids positive and step 16 elongated spermatids negative; (B) stage IX tubule indicating pachytene spermatocytes and step 9 round spermatids both positive; (C) stage X tubule showing pachytene spermatocytes and step 10 spermatids both positive. Arrows indicate different cell types; (D) stage VIII tubule and stage XI tubule. All cell types are positive except for step 16 elongated spermatids in stage VIII tubule; (E) stage IX tubule showing a positive reaction in pachytene spermatocytes and step 9 spermatids; (F) control experiments using pre-immune serum did not show any sign of reactivity over any part of the mouse testis.

C
B
A

## 3.8 Structural components of the *Pom-1* protein

The predicted *Pom-1* protein is a small, basic (lysine and arginine (~12%)) protein, which is also rich in phosphoacceptor amino acids (~20%), mainly threonine and serine (~12%) (Figure 13). We examined *Pom-1* for consensus phosphorylation sequences most frequently recognized by various serine/threonine protein kinases (Kennelly & Krebs, 1991; Pearson& Kemp, 1991). Three protein kinases were noted to have the potential to phosphorylate *Pom-1* protein (Table 1), as several serine and threonine residues lies within such consensus sequences (Table 1). This would argue in favor of *Pom-1* being phosphorylated especially at residue $T_{42}$ which could potentially be phosphorylated by 2 different kinases: PKC and cGMP-PK.

An additional structural characteristic of the predicted *Pom-1* protein is that the basic residues, mainly lysine and arginine, are predominantly distributed along the protein in pairs ($R_{12,13}$; $K_{21}R_{22}$; and $K_{51,52}$). Pairs of basic amino acids have been shown to play an impotant role in the recognition of specific sequences by various serine/threonine protein kinases (Kennelly & Krebs, 1991).

M L P D T S L T T V H R R L **P A G P** Q L
K R M G I G D S L Q L T Y F L D F I S R
V T F S Q L L T M M K K T I P P V *

Figure 13: Deduced amino acid sequence of the short orf of *Pom-1*. Bold residues indicates SH3 binding region; protein kinase sequences are underlined; and pairs of basic amino acids are marked with dash lines.

**Table 1: Consensus sequences for serine and threonine phosphorylation sites located within the predicted Pom-1 protein**

| Kinase | Consensus sequence | Potential phosphoacceptor residue within Pom-1 | | | | |
|---|---|---|---|---|---|---|
| | | $T_9$ | $T_{42}$ | $S_{44}$ | $T_{48}$ | $T_{53}$ |
| casein kinase I | S(P)XXS*/T* | SLTT* | | | | |
| protein kinase C | K/RXS*/T* | | RVT* | | | KKT* |
| | S*/T*$X_{0-2}$(R/K)$_{1-3}$ | | | | T*MMKK | |
| cGMP-dependent protein kinase | R/KXXXS*/T* | | | RVTFS* | | |
| | R/KXS*/T* | | RVT* | | | |

Modified from Kennelly and Krebs (1991) and Pearson and Kemp (1991). X, any amino acid; *, the phosphoacceptor serine or threonine; /, interchangeable amino acid; (P), denotes aphosphorylated residue which acts as a substrate specificity determinant.

## REFERENCES

Kennelly, P.J. & Krebs, E.G., (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatses, J. Biol. Chem., 266:15555-15558.

Kierszenbaum, A.L., and Tres, L.L., (1975) Structural and Transcriptional features of the mouse spermatid genome. J. Cell. Biol. 65:258-270.

Monczak, Y., (1990) Ph.D. Thesis, U. of Montreal, Montreal, Canada

Oakberg, E.F., (1956) A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am. J. Anat. 99:391-413.

Pearson, R.B. & Kemp, (1991) B.E. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations, Methods Enzymol. 200:62-81.

Villemur, R., Monczak., Rassart, E., Kozak, C., and Jolicoeur, P., (1987) Identification of a new common provirus integration site in Gross Passage A Murine Leukemia Virus-Induced Mouse thymoma DNA, Mol. Cell. Biol. 7:512-522.

# CHAPTER 4

# DISCUSSION

## 4.1 The identification of the *Gin-1* gene and its role in tumorigenesis

Insertional activation of cellular genes by replication-competent or replication-defective retroviruses is a common pathway to malignant transformation and tumor progression. This discovery led to the use of provirus-tagging as a method to identify genes in this process. In an effort to understand the role of genes in oncogenesis, we identified a new gene and were able to assess its participation in GPA-MuLV induced thymomas. The results here have shown that *Pom-1* is a novel testis-specific gene which encodes a protein rich in serine and threonine residues, but is not involved in tumorigenesis. This *Pom-1* gene has not yet been described in the scientific literature.

### 4.1.1 Identification of the *Pom-1* gene

The *Pom-1* gene was initially identified (from the *Gin-1* region) as a common provirus integration site in 11 of 44 GPA-MuLV induced thymomas, and has been mapped to mouse chromosome 19 (Villemur et al., 1987). All proviruses were found to be in the same orientation, which suggests a specific molecular requirement for this genetic event to be significant for malignant transformation. Such a clustering of provirus integration sites may reflect the chromatin structure, favoring a provirus integration, or more likely, a selection process for activated genes conferring a growth advantage to tumor cells.

Identifying genes located in common proviral integration sites by using genomic probes that map around the integration site ('proviral tagging') led to the identifcation of new oncogenes for the past 15 years.

Although successful, several factors can contribute to make the identification of putative genes a difficult task. First, the proviruses may be integrated far upstream or downstream of the gene and influence its transcription over a great distance. It is known that cellular enhancer sequences can influence gene transcription over a long distance (van Lohuizen and Berns, 1990; Kung et al., 1991; Peters, 1990). Therefore the genes may be located beyond the cloned sequences. Many genes have been shown to have very large introns. For example, introns over 200 kbp have been described for c-*abl*, *bcl-2*, and Duchenne muscular dystrophy genes (Holland et al., 1989; Seto et al., 1988; Koeing et al., 1987). Finally, exons can be very short, sometimes only 50-100 bp. The use of genomic probes having an average length of 1-2 kbp may not be sensitive enough for detection of a very short exon, particularly if the gene is expressed at very low levels.

By using single copy probes spanning over 60 kbp of cloned sequences around the integration site (Villemur et al., 1987; and Monczak, 1990), a variety of murine tissues were screened for the detection of a transcriptionally active locus. One exon ("R") was identified, as it resulted in a positive doublet (1.0 and 1.2 kbp) signal in testis. A C57/BL6 testis cDNA library was screened, and a portion (648 bp) of the gene was isolated (Monczak,1990). Many additional cDNA libraries were screened and RT-PCR was tried in order to obtain the missing exons. Only a small exon of approximately 100 bp was identified which yielded the complete full-legth 1.0 kbp cDNA. As many testis specific genes have been known to have long poly ($A^+$) tails, RNase H was used to digest the tail and to determine its new length. This method verified that the additional sequences isolated gave rise to the complete full-length 1.0 kbp cDNA.

A similar example has been found in the literature. Superoxide dismutase (SOD) is an essential enzyme in the cellular pathway that inactivates free radicals. Several isozymes have been detected (McCord & Fridovich, 1969; Marklund, 1982; Barra et al., 1984), including the copper-zinc SOD (SOD-1) (Crapo et al., 1992) which is the primary SOD isozyme expressed in a wide variety of cells including testes. Although SOD-1 is encoded by a single copy gene (Levanon et al., 1982), two SOD-1 mRNAs (0.77 and 0.94 kbp) differing by about 200 nucleotides have been found in human tissue culture cells (Lieman-Hurwitz et al., 1982). The two mRNAs have identical 5' untranslated regions (UTRs) and coding regions, but they differ in their 3' UTRs (Sherman et al., 1984). However, in this example the two genes have been identified and the difference is in the 3' UTR. In other examples, significant differences occur in the 5' UTR. The 5' UTR of mRNAs performs important roles in regulating their translation. Translation is inhibited by 96% by placing a stem-loop structure in the 5' UTR of yeast mRNAs (Laso et al., 1993). Certain testicular mRNAs such as the 1.7 kbp cytochrome $c_S$ and proenkephalin mRNAs contain additional sequences in their 5' UTR and are not actively translated (Garrett et al., 1989; Hake and Hecht, 1993). The translation inefficiency of proenkephalin transcripts is directly related to an open reading frame in the 5'UTR (Rao and Howells, 1993). Since the 0.77 kbp SOD-1 mRNA is preferrentially translated over the 0.93 kbp SOD-1 mRNA *in vitro*, the 0.93 kbp may serve as a backup system to synthesize SOD-1 when late stages of spermatids are in free radical stress (Gu et al., 1995). A similar situation may be occuring with *Pom-1*, where the 1.2 kbp is transcribed and only translated if something goes wrong with translation of the 1.0 kbp mRNA.

To date, we have been unable to clone the longer (1.2 kbp) *Pom-1* cDNA. The screening of 6 different testis cDNA libraries never resulted in the cloning of the additional sequences. The factors described above may contribute to make the identification of the putative gene located within the *pom-1* region a difficult task. Moreover, secondary structures within these complementary stretches of cDNA may be present, causing improper reverse transcription may also explain this problem. Other alternative techniques are presently being employed to isolate these remaining sequences.

In conclusion, we have successfully identified the 1.0 kbp*Pom-1* cDNA. Other techniques should help to isolate the 1.2 kbp cDNA, and the two cDNAs will be compared.

4.1.2 The role of *Pom-1* in tumorigenesis

Activation of a cellular oncogene by retroviral integration can occur by several known mechanisms, mainly due to promoter insertion and enhancer activation. Promoter insertion requires that the transcriptional orientation of the proto-oncogene and the provirus be parallel and that the provirus be located 5' of the start site of transcription, whereas enhancer activation can occur even if the provirus is in the transcriptionally opposite orientation and 3' of the proto-oncogene (van Lohuizen and Berns, 1990; Kung et al., 1991; Peters, 1990). Comparison of the cDNA sequences with genomic sequences shows one cluster 3' of the gene and in the same orientation. Since the integrations occurred approximately 20 kbp from the gene, it is likely that provirus integration in this region may activate gene expression through LTR enhancer.

Enhancer insertion is the most commonly found type of activation, probably because enhancers are capable of acting over large distances. This allows a much larger DNA domain as a target for proviral integrations. Extensive studies of naturally occurring tumors reveals that proviruses integrated on the 3' side of the gene are usually in the same transcriptional orientation, which suggests a model in which the bidirectional viral enhancer acts primarily on the nearest promoters. Additionally, it may occur 5' of the gene and in the opposite orientation. In the *Pom-1* region, only one proviral integration cluster was found spanning 5 kbp of genomic DNA. Whether this region harbors a second insertional cluster remains to be seen.

The *Pom-1* gene encodes two major RNA species of 1.0 and 1.2 kbp and is expressed in mouse and rat testis using a mouse cDNA clone as a probe. Same results are also confirmed by *in situ* hybridization, indicating that the *Pom-1* gene is transcriptionally active.

However, despite the data mentioned above, there is sufficient data to suggest that this *pom-1* gene is not involved in tumorigenesis. A recombinant retroviral vector harboring *Pom-1* coding sequences was unable to transform rat-1 fibroblasts using the pBABE/PURO retroviral vector. There were no morphological changes seen in the rat-1 cells as compared to the control. Furthermore, although several oncogenes work together to induce transformation and to confer a tumorigenic potential, as it was demonstrated with *myc* and *ras*, and *myc* and *bcr-abl* (Hunter, 1991; Lugo and Witte, 1989), this was not the case for *Pom-1*. Moreover, the ability for *Pom-1* to function as an anti-oncogene was tested, and this gene was unable to block transformation. It should be noted that although only rat-1 fibroblast cells were tested, other cell lines should also be checked for transforming potential of the *Pom-1* gene as several oncogenes can

eliminate the requirement of growth factors in fibroblasts and/or lymphoid cells.

Furthermore, RNA isolated from rearranged and non-rearranged GPA-MuLV induced thymomas were screened with the *Pom-1* cDNA and no signal was detected, even after long exposures.This data revealed that there is no clear evidence for activation of *Pom-1* in the tumors we have tested.

As mentioned above *Pom-1* is expressed in testis but not in thymus. One possibility was that *Pom-1* was expressed in a subpopulation of cells not seen in adult thymus, but in embryonic thymus. At day E15, 97% of the cells are $CD4^+$ $CD8^+$ and 3% are $CD4^-$ $CD8^+$; at day E16, 63% are $CD4^+$ $CD8^+$, 22% are $CD4^-$ $CD8^+$, 13% are $CD4^-$ $CD8^-$. By birth, 85% of the cells are $CD4^-$ $CD8^-$, 10% are $CD4^+$ $CD8^+$ and 5% are $CD4^-$ $CD8^+$. As the proportions of the cell populations change from E15-birth, it is therfore possible that a gene expressed in $CD4^+CD8^-$ or $CD4^-CD8^+$or $CD4^+CD8^+$ cell populations would be missed if screening adult thymus only. The hypothesis was that *Pom-1* was strongly expressed in embryonic thymus and its expression in adult thymus was in the smaller subpopulation of cells and turned off in the thymoma. Thus, *Pom-1* would act as an anti-oncogene. However, *in situ* hybridization on thymus E15-adult using the *Pom-1* cDNA as a probe gave no signal above background. Therefore, *Pom-1* does not act as an anti-oncogene.

In short, all the data suggest that this novel transcript, originally detected with the R probe, was unlikely to be the gene affected by provirus insertion. There is, therefore, an additional gene in this region that may activate a novel gene involved in tumor formation. As mentioned earlier, enhancer insertion is capable of acting over very large distances, up to 200 kbp (van Lohuizen and Berns, 1990; Kung et al., 1991; Peters, 1990).

Isolating additional novel genomic DNA around the integration sites and screening for the novel putative oncogene would provide insights into the mechanism of GPA-MuLV induced thymogenesis.

## 4.2 *Pom-1* - the testis-specific gene

### 4.2.1 Temporal expression of the *Pom-1* gene

In this study, we describe the identification and isolation of a novel mouse gene, *Pom-1*, which exhibits a tightly regulated pattern of expression during male germ cell differentiation. Transcripts of *Pom-1* constitute a doublet (1.0 and 1.2 kbp) detected only in total testis RNA from adult mice, and not observed in RNA from any other tissues tested or in RNA from mid or late gestation embryos. Furthermore, *Pom-1* expression begins on day 20 at the onset of sexual maturation in the testes.

### 4.2.2 Spatial expression of the *Pom-1* gene

Analysis of RNA from enriched populations of spermatogenic cells revealed that *Pom-1* was weak in pachytene spermatocytes while most abundant in postmeiotic cells (round spermatids, elongated spermatids, and residual bodies), a pattern that was confirmed by *in situ* analysis. Therefore, *Pom-1* exhibits a clear stage-specific activation, related to its regulation at the level of transcription. Isolation of the *Pom-1* promoter will be useful in studying the tissue specific regulation of this gene.

In most mammalian tissues, gene expression is believed to be primarily regulated at the level of transcription with occassional modulation at the translational level. This is also likely to be true for pre-meiotic and

meiotic stages of spermatogenesis. However, chromatin changes in the developing male gamete lead to loss of nucleosomes with a concommitant cessation of RNA synthesis during mid-spermiogenesis. By necessity, many of the proteins of the spermatozoon must be either synthesized before mid-spermiogenesis and stored or their transcripts stored and translationally regulated. It is likely both mechanisms are used. Studies with three DNA-binding proteins, transition protein 1 and the two protamines, that are transcribed in round spermatids indicate translational regulation plays a primary role in their expression. This may also be true for *Pom-1*, as it follows a similar expression pattern.

Regulation at the level of transcription, resulting in the stage-specific activation of specific genes, is clearly occurring for many genes (Wolgemuth and Watrin, 1991). Transcription of a group of genes during spermiogenesis provides an excellent way to examine programmed gene expression in the testis. The regulated transcription of a novel testicular gene at a specific interval of spermatogenesis is presumably dependent upon specific regulatory signals operative in the testis. In addition to testis specific genes, other genes coding for proteins, such as smooth muscle $\gamma$ actin (SMGA) are also post-meiotically expressed during spermtogenesis (Kim et al., 1989). The fact that SMGA is also expressed in the intestine and uterus suggests it should contain regulatory signals recognized by additional tissues. The existence of meiotically expressed genes such as PGK2 (Erickson et al., 1980; Gold et al., 1983) and LDH C4 (Fujimoto et al., 1988) provide a second group of testis-specific genes that are also temporally regulated, albeit at a different stage of spermatogenesis.

## 4.3 Structural components of the *Pom-1* protein

Several significant structural features within the *Pom-1* protein have been identified. Although speculative, comparison of *Pom-1* with other germ cell specific, size related proteins, might however give a clue as to its possible function. The predicted *Pom-1* peptide is a small, basic (lysine and arginine (~12%)) protein, which is also rich in phosphoacceptor amino acids (~20%), mainly threonine and serine (~12%), and proline-rich (~12%) (figure 13). Furthermore, an SH3 binding domain has also been identified.

The identification of SH3 binding proteins by expression cloning and affinity chromatography demonstrated that SH3 domains bind to proline-rich sequences. The structural analysis of these SH3 domains reveals generally a relatively flat and mostly hydrophobic peptide-binding region on the surface of these globular domains. SH3 binding motifs appear commonly to contain several prolines and to form a left handed helical structure (polyproline helix type II). A PXXP motif has evolved as a preliminary consensus for SH3-binding sequences (Ren et al.,1994). Since a helical turn of the SH3-binding peptide helix consists of three amino acidss, the ring structures of the two prolines in the PXXP motif have a parallel orientation in space. They interact with highly conserved aromatic residues of the peptide-binding region within the SH3 domain. Based on these findings, an SH3-binding site has been identified in the *Pom-1* protein. The fact that this small protein is proline-rich may increase affinity or stabilize the conformation of binding complex. Feng et al (1994) have reported two binding orientations for peptides to the Src SH3 domain by nuclear magnetic resonance method. The peptide orientation is determined by a salt bridge formed by the terminal arginine residues of the ligand and a conserved aspartate-99 of the SH3

domain. Two classes of peptides have been used to determine orientation of ligand binding. Class I peptides contain a conserved NH2-terminal arginine which is critical for SH3 binding, whereas class II peptides contain a conserved COOH-terminal arginine. We have found that the potential SH3 binding site of the *Pom-1* protein contains a short proline rich motif RRLPAGP, which contains the arginine in the NH2-terminal domain. Therefore, this motif could be predicted to bind SH3 domain in the 'plus' orientation. However, this potential SH3 binding site in the *Pom-1* protein needs to be tested for specifically binding to cellular proteins. Clearly, a better understanding of the role of *Pom-1* would require study on the proteins that regulate the *Pom-1* or are regulated by it. An antiserum specific for *Pom-1* can be used to investigate possible *Pom-1* binding proteins and perhaps screening an expression library using the yeast two hybrid system will be useful to find these relevant cellular proteins.

As mentioned above, p6$^{Pom-1}$ is relatively rich in potential phosphoacceptor amino acids (~20%). We examined *Pom-1* for consensus phosphorylation sequences most frequently recognized by various serine/threonine protein kinases (Kennelly & Krebs, 1991; Pearson& Kemp, 1991). Three protein kinases were noted to have the potential to phosphorylate *Pom-1* protein (Table 1), as several serine and threonine residues lies within such consensus sequences (Table 1). This would argue in favor of *Pom-1* being phosphorylated especially at residue $T_{42}$ which could potentially be phosphorylated by 2 different kinases: PKC and cGMP-PK. The predicted *Pom-1* protein contains two potential protein kinase C phosphorylation sites, two cGMP-dependent protein phosphorylation sites, and one casein kinase protein phosphorylation site. *In vivo*, protein kinase C exhibits a preference for the phosphorylation of serine or threonine residues

close to a C-terminal residue. Interestingly, all normal and altered *abl* proteins are phosphorylated on serine following *in vivo* labeling, treatment of NIH 3T3 cells with protein kinase C activators resulted in a four to eight fold increase in the phosphorylation of murine c-*abl* due to modification of two serines on the c-*abl* protein (Pendergast et al., 1987). The sites of protein kinase C phosphorylation on the *abl* protein were found to be near the C-terminal and conserved in mouse and human cells, suggesting that phosphorylation of c-*abl* at these sites may be important to modulate c-*abl* functions *in vivo*. In contrast, for the EGF receptor (Hunter et al., 1984) and $pp60^{src}$ (Gould et al., 1985), the sites of protein kinase C phosphorylation are amino-terminal to the kinase domain. Studies on the oncogene Lck have shown that signal from the antigen receptor induces rapid phosphorylation of Lck and that this process depends on two different serine kinases (Veillette et al., 1988a; Veillette et al., 1988b).

Interestingly, 2 isozymes of cAMP-PK have been reported in mouse spermatogenic cells: type I is present in pachytene spermatocytes and in round spermatids, whereas type II is present in round and elongating spermatids (Conti et al., 1979; Conti et al., 1983). It is therefore likely that other protein kinases may be identified in the testes. Furthermore, the *Pom-1* protein is reminiscent of the transition proteins TP1 and TP2, which are germ cell specific, small (54 and 117 aa, respectively), basic (lysine and arginine rich), DNA associated proteins that are believed to function in DNA condensation (Hecht, 1990). Phosphoacceptor amino acids present within the transition proteins have been shown to intercalate between the DNA bases, possibly inducing conformational changes as well as nicks in native DNA (Sing and Satanarayana, 1987). Such interactions might be of significance during chromatin remodeling of postmeiotic spermatids. Of

weaker reactivity with respect to the Golgi apparatus in comparison to the reaction in the round spermatid. Interestingly, pachytene spermatocytes show weak *Pom-1* RNA expression, but a strong reaction is seen in these cells in the Golgi apparatus by immunolabelling with an affinity purified *Pom-1* antibody. It might be possible that the antibody is reacting to two potential isoforms that is not detected by our *Pom-1* cDNA probe.

The main function of the Golgi apparatus is to glycosylate proteins that are delivered to it from the overlying ER cisternae (Bennett, 1984, 1988). Clermont and Tang (1985) suggested that such glycoproteins are possibly destined to the plama membrane where they continue to undergo important modifications during spermiogenesis. Clermont and Tang (1985) have also indicated that the medulla constitutes a distinct compartment of the Golgi apparatus where glycoproteins continue to acumulate before being delivered, depending on the step of spermiogenesis, either to the acrosomic system or to the plasma membrane. The role of the Golgi apparatus in the construction of the acrosomic system has been well documented (Susi et al., 1971; Mollenhauer et al., 1977). It appears that glycoproteins are segregated and concentrated within the Golgi networks; these elements break down into tubules and vesicles that then migrate toward and merge with the outer acrosomal membrane thereby emptying its contents into the growing arosomic system (Thorne-Tjomsland et al., 1988). The acrosome is known to contain more than twenty hydrolyzing enzymes, such as hyaluronidase, acid phosphatase, and acrosin (Morton, 1976; Yanagimachi, 1981; Guraya, 1987; Eddy, 1988).

Although speculative, it is possible that *Pom-1* protein is expressed in the Golgi apparatus to feed the growing acrosome with the necessary signals for its development. It may also send the necessary enzymes to the

acrosome for its role in fertilization. This is supported by the fact that there is no *Pom-1* protein seen in the early spermatids, a time when there is no acrosome as well. *Pom-1* may have a different role in pachytene spermatocytes.

As the number of genes known to be expressed during spermatogenesis or spermiogenesis in a stage-specific manner is rapidly growing, a classification of these genes into three groups has been proposed (Hecht et al., 1990):

1) exclusive expression during spermatogenesis

2) testis-specific expression of an isozyme, and

3) expression of somatic genes with increasing levels in testis

*Pom-1* belongs to the first group as does the protamines and the transition proteins and others (Wolgemuth and Watrin, 1991). Although angiotensin-converting enzyme (ACE) and hemiferrin (a transferrin-like protein similar to ACE) belong to the second group, their protein expression is most similar to *Pom-1*. All three are expressed at the haploid stages of spermatogenesis during spermatid differentiation corresponding to the Golgi, cap, and acrosome phases (Sibony et al., 1994). The growing number of genes expressed after meiosis implies that spermatids do not simply continue transcribing genes activated during meiosis but initiate the transcription of genes programmed for specific activation during spermatogenesis. These results on the *Pom-1* protein emphasize the importance of the elongation phase of spermatids in the differentiation process of the male germ cells with respect to gene activation and not only morphologocal modifications. Further studies are now necessary to examine the mechanism of regulation and to determine the function of *Pom-1*.

# REFERENCES

Bennett, G. (1984) Role of the Golgi complex in the secretory process, *in* Cell Biology of the secretory Process (Cantin, M., ed) pp. 102-147, Karger, Basel

Bennett, G. (1988) Radioautographic and cytochemical studies on the synthesis and intracellular transport of glycoproteins. Acta Histochemica. Suppl.-B and XXXVI, S. 9-49

Clermont, Y., and Tang, X.M. (1985) Glycoprotein synthesis in the Golgi apparatus of spermatids during during spermiogenesis of the rat. Anat. Rec. 213, 33-43

Conti, M., Geremia, R., and Monesi, V. (1979) 3',5'-cyclic monophosphate-dependent protein kinase activity in differentiating germ cell of mouse testis. Mol. Cell. Endocrinol. 13, 137-148

Conti, M., Adamo, S., Geremia, R., and Monesi, V. (1983) Developmental changes of cyclic adenosine monophosphate-dependent protein kinase activity during spermaatogenesis in the mouse. Biol. Reprod. 28, 260-269

Don, J., and Wolgemuth, D.J. (1992) Identification and characterization of the regulated pattern of expression of a novel mouse gene, meg 1, during the meiotic cell cycle. Cell Growth and Differentiation 3, 495-505

Eddy, E.M. (1988) The spermatozoon. *in* The physiology of reproduction (Knobil, E. and Neill, J. eds) Raven Press New York, pp.27-68

Erickson, R.P. Kramer, J.M., Rittenhouse, J., and Salkeld, A. (1980) Quantitation of mRNAs during mouse spermatogenesis: protamine-like histone and phosphoglycerate kinase 2 mRNAs increase after meiosis. Proc. Natl. Acad. Sci. USA 77, 6086-6090

Feng, S., Chen, J.K., Yu, J., Simon, J.A., and Schreiber, S.L. (1994) Two binding orienations for peptides to the Src SH3 domain development of a general model for SH3-ligand interactions. Science 266, 1241-1247

Fujimoto, H., Erickson, R.P., Tone, S. (1988) Changes in polyadenylation of lactate dehydrogenase-X mRNA during spermatogenesis in mice. Mol. Reprod. Dev. 1, 27-34

Garrett, J.E., Collard, M.W., and Douglass, J.O. (1989) Translational control of germ cell-expressed mRNA imposed by alternative splicing: opoid peptide gene expression in rat testis. Mol. Cell Biol. 9, 4381-4389

Gold, B., Fujimoto, H., Kraner, J.M., Erickson, R.P., and Hecht, N.B. (1983) Haploid accumulation and translational control of phosphoglycerate kinase 2 messenger RNA during mouse spermatogenesis. Dev. Biol. 98, 392-399

Gould, K.L., Woodgett, J.R., Cooper, J.A., Buss, J.E.,Shalloway, D., and Hunter, T. (1985) Protein kinase C phosphorylates pp60src at a novel site. Cell 42, 849-857

Gu, W., Morales, C., and Hecht, N.B. (1995) In male mouse germ cell, copper zinc superoxide dismutase utilizes alternative promoters that produce multiple transcripts with different tranlation potential. J. Biol. Chem.

Guraya, S.S. (1987) Biology of spermatogenesis and spematozoa in mammals. Springer, Berlin Heidelberg New York.

Hake, L.E. and Hecht, N.B. (1993) Utilization of an alternative transcription initiation site of somatic cytochrome c in the mouse produces a testis-specific cytochrome c mRNA. J. Biol. Chem. 268,4788-4787

Hecht, N.B. (1990) Regulation of "haploid expressed genes" in male germ cells. J. Reprod. Fertil. 88,679-693

Hunter, T., Ling, N., and Cooper, J.A. (1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311, 480-483

Hunter, T., (1991) Cooperation between oncogenes. Cell 64, 249-270

Kennelly, P.J. & Krebs, E.G., Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatses, J. Biol. Chem., 266:15555-15558, 1991

Kilpatrick, D.L., Zinn, S. A., Fitzgerald, M., Higuchi, H., Sabol, S. L., and Meyerhardt, J., Transcription of the rat and mouse proenkephalin genes is initiated at distinct sites in spermatogenic and somatic cells. Mol. Cell Bio., 10:3717-3726, 1990

Kim, E., Waters, S.H., Hake, L.E. and Hecht, N.B. (1989) Identification and development expression of smooth muscle γ actin in post-meiotic male germ cells of mice. Mol. Cell Biol. 9, 1875-1881

Kung, H.J., Boerkoel, C., and Carter, T.H. (1991) Retroviral mutagenesis of cellular oncogenes: a review with insights into the mechanisms of insertional activation. Current Topics in Microbiology and Immunology 171, 1-25.

Lazo, P.A., Lee, J.S., and Tsichlis, P.N. (1990) Long distance activation of the myc proto-oncogene by provirus insertion in Mlvi-1 or Mlvi-4 in rat T-cell lymphomas. Proc. Natl. Acad. Sci. USA 87, 170-173.

Levanon, D., Lieman-Hurwitz, J., Dafni, N., Wigderson, M., Sherman, L., Bernstein, Y., Laver-Rudich, Z., Danciger, E., Stein, O., and Groner, Y. (1985) Architecture and anatomy of the chromosomal locus in human chromosome 21 encoding the Cu or Zn superoxide dismutase. EMBO J. 4, 77-84

Lieman-Hurwitz, J., Dafni, N., Lavie, V., and Groner, Y. (1982) Human cytoplasmic superoxide dismutase cDNA clone: a probe for studying the molecular biology of Down syndrome. Proc. Natl. Acad. Sci. USA 79,2808-2811

Lugo, T.G., Pendergast, A.M., Muller, A.J., and Witte, O.N. (1990) Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science, 247, 1079-1082

Marklund, S.L. (1982) Human copper containing superoxide dismutase of high molecular weight. Proc. Natl. Acad. Sci. USA 79,7634-7638

McCord, J.M. and Friedovich, I. (1969) The utility of superoxide dismutase in studying free radical reactions. 1. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. J. Biol. Chem. 244,6049-6055

Mollenhauer, H.H., Hass, B.S., and Morre, D.J. (1977) Membrane transformations in Golgi apparatus of the rat spermatids. A role for thick cisternae and two classes of coated vesicles in acrosome formaton. J. Microsc. Biol. Cell 27, 33-36

Moncak, Y., (1990) Ph.D. Thesis, U. of Montreal, Montreal, Canada

Morton, D.B. (1976) Lysosomal enzymes in mammalian spermatozoa. *in* Lysosomes in biology and pathology (Dingle, J.T. and Dean, R.T., eds), North-Holland, Amsterdam, vol. 5, pp.205-255

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N., and Numa, S. (1979) nucleotide sequence of cloned cDNA foe bovine corticotropin-β-lipotropin precursor. Nature 278, 423-427

Pendergast, A.M., Traugh, J.A., and Witte, O.N. (1987) Normal cellular and transformation-associated abl proteins share common sites for protein kinase C phosphorylation. Mol. Cell Biol. 7, 4280-4289

Peters, G. (1990) Oncogenes at viral integration sites. Cell Growth and Differentiation 1, 503-510.

Rao, S.M., and Howells, R.D. (1993) cis-acting elements in the 5' untranslated region of rat testis proenkephalin mRNA regulate translation of the precursos protein. J. Biol. Chem. 268, 22164-22169

Ren, R., Ye, Z.E., and Baltimore, D. (1994) Abl protein-tyrosine kinase selects the Crk adapter as a substreat using SH3-binding sites. Genes & Dev. 8, 783-795

Sherman, L., Levanon, D., Lieman-Hurwitz, J., Dafni, N., and Groner, Y. (1984) Human Cu superoxide dismutase gene: molecular characterization of its two mRNA species. Nucleic Acids Research 12, 9349-9365

Sibony, M., Segretain, D., and Gasc, J.-M. (1994) Angiotensin-Converting enzyme in murine testis: Step specific expression of the germinal isoform during spermiogenesis. Biol. of Reprod. 50, 1015-1026

Sing, J., and Satayanarayana Rao, M.R. (1987) Interaction of rat testis protein, TP, with nucleic acids *in vitro*. J. Biol. Chem. 262, 734-740

Susi, F.R., Leblond, C.P., and Clermont, Y. (1971) Changes in the Golgi apparatus during spermiogenesis in the rat. Am. J. Anat. 130, 251-267

Thorne-Tjomsland,G., Clermont, Y., and Hermo, L. (1988) Contribution of the Golgi apparatus components to the formation of the acrosomic system and chromatoid body in rat spermatids. Anat. Rec. 221, 591-598

Van Der Hoorn, F.A., Tarnasky, H.A., and Nordeen, S.K. (1990) A new rat gene RT7 is specifically expressed during spermatogenesis. Dev. Biol. 142, 147-154

Van Lohuizen, M., and Berns, A. (1990) Tumorigenesis by slow-transforming retroviruses -- an update. Biochem Biophys Acta 1032, 213-235.

Veillette, A., Horak, I.D., and Bolen, J.B. (1988a) Post-translational alterations of the tyrosine kinase p56lck in response to activators of protein kinase C. Oncogene Research 2, 385-401

Veillette, A., Horak, I.D., Horak, E.M., Bookman, M.A., and Bolen, J.B. (1988b) Alterations of the lymphocyte-specific protein kinase (p56lck) during T cell activation. Mol. Cell. Biol. 8, 4353-4361

Villemur, R., Monczak., Rassart, E., Kozak, C., and Jolicoeur, P., Identification of a new common provirus integration site in Gross Passage A Murine Leukemia Virus-Induced Mouse thymoma DNA, Mol. Cell. Biol. 7:512-522, 1987

Wolgemuth, D.J., and Watrin, F., List of cloned mouse genes with unique expression patterns during spermatogenesis. Mamm. Gen. 1:283-288, 1991

Yanagimachi, R. (1981) Mechanisms of fertilization in mammals *in* Fertilization and embryonic development in vitro (Mastroianni, L., and Biggers, J.D. eds) Plenum Press, New York, pp. 81-98

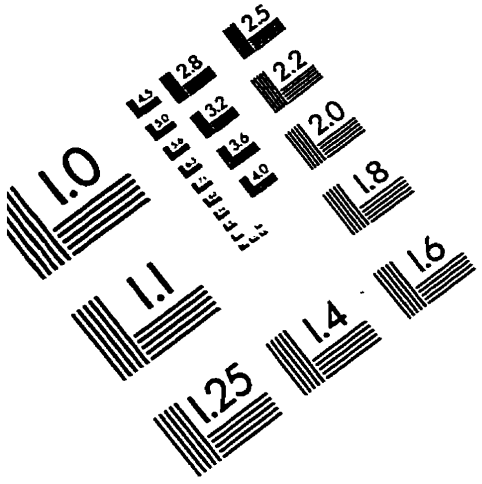
# CHAPTER 5

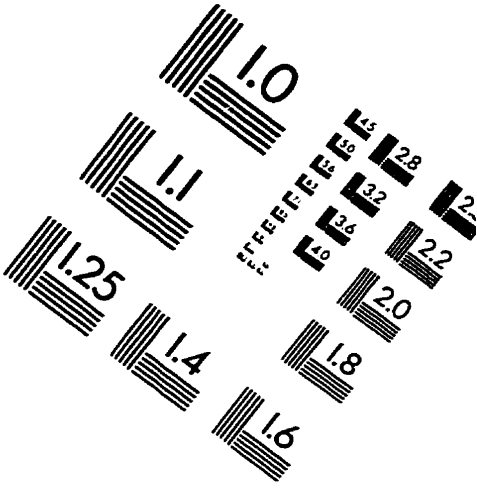
# CLAIMS FOR ORIGINAL RESEARCH

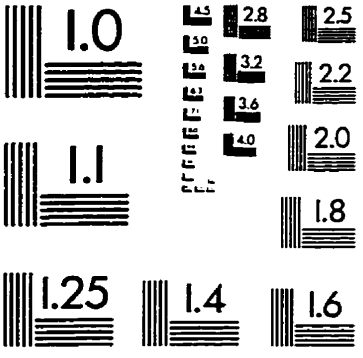
## Claims for Original Research

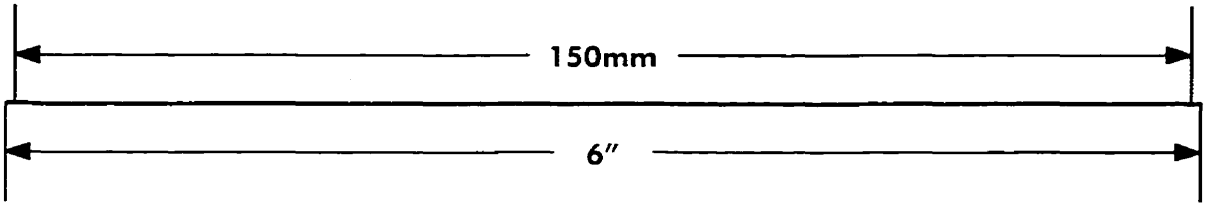
It is expected that this work would make original contributions in several aspects. The molecular cloning and characterization of the *Pom*–1 gene has demonstrated that it is a novel gene implicated in spermatogenesis and has not been described in the scientific literature. The *Pom*-1 gene encodes two major RNA species of 1.0 and 1.2 kbp and the testis is the only tissue where it is transcriptionally active as well as transcriptionally regulated. It is expressed in late pachytene spermatocytes, round spermatids and elongated spermatids. The predicted *Pom*-1 protein is 6 kd and is rich in phosphoacceptor amino acids (serine and threonine) which argues that it may be phosphorylated at these residues in the phosphorylation consensus sequences. The predicted 6 kd protein also has basic residues occurring in pairs which has been shown to play a role in the recognition of specific sequences by various serine/threonine protein kinases. The *Pom*-1 protein is expressed in the Golgi apparatus in the late pachytene spermatocytes, round spermatids and early elongated spermatids. This work therefore directs future experiments to study the structure and biological function of the *Pom*-1 gene, and to determine its function in spermatogenesis.

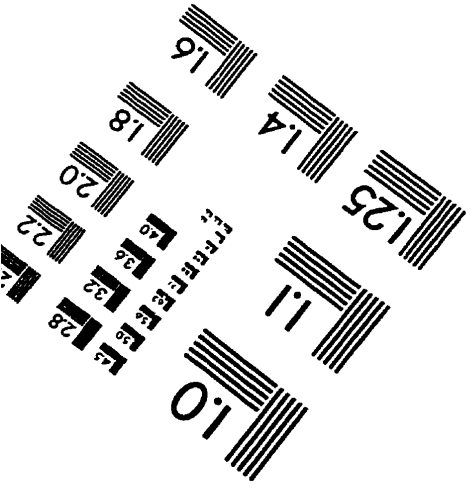
# IMAGE EVALUATION TEST TARGET (QA-3)

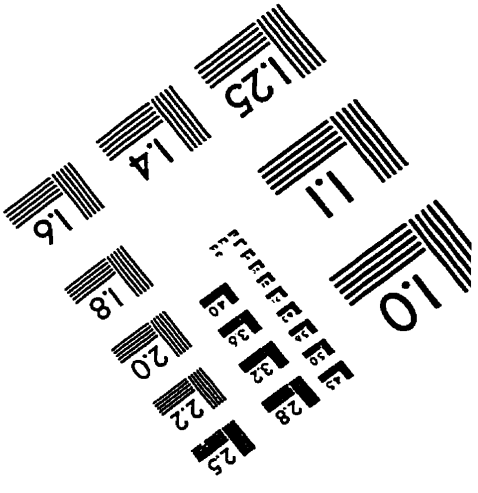



APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989