

STUDIES OF A SPERM ACROSOMAL ANTIGEN RECOGNIZED BY HS-63 MONOCLONAL
ANTIBODY

By

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ABSTRACT

A sperm specific and species conserved monoclonal antibody (HS-63) was shown to inhibit in vitro fertilization of mouse oocytes and human sperm penetration to zona-free hamster ova. The sperm antigen (SA-63) which reacts with HS-63 was found to be localized on the sperm acrosome. Following sperm capacitation, this antigen becomes exposed and is shed after the acrosome reaction. SA-63 may be involved in the sperm acrosome reaction during the initial fertilization process.

Sperm antigen (SA-63) from mouse (MSA-63) was purified from mouse testes with soluble and detergent extraction procedures followed by immunoaffinity chromatography. The purified MSA-63 antigen was shown to be a group of proteins with a size ranging from 25 Kd to 50 Kd and pIs of about 4.2 when analyzed by two dimensional gel electrophoresis. MSA-63 antigen may be associated with actins in its native form. A proteolytic activity was found in the solution of purified MSA-63 preparation.

Purified MSA-63 was used for immunization of mice and rabbits. Following successive immunizations, antisera of high

titres were raised and reacted specifically with sperm acrosome. The isoimmune sera from immunized mice exhibited significant inhibition on in vitro fertilization of mouse oocytes.

Complementary deoxyribonucleic acid (cDNA) fragments encoding the MSA-63 were cloned from a mouse testis cDNA library by using an immunoscreening method with rabbit antisera against MSA-63 as the detecting probe. When a specific cDNA probe was used for Northern blot analysis, an mRNA of 1.5 Kb in size was detected only in the adult mouse testis, but not in any other somatic tissues. By Southern blot analysis, it was also demonstrated that the gene encoding for SA-63 protein is conserved among different mammalian species. The location of SA-63 antigen gene was determined to be on human chromosome 11 when analyzed with a blot of a human-hamster somatic cell hybrid panel.

By DNA sequence analysis, a protein of 28 Kd in size was deduced from the MSA-63 cDNA. The amino acid sequences of trypsin-digested peptide fragments of MSA-63 were used to verify that deduced amino acid sequence from the cDNA.

The recombinant fusion proteins containing MSA-63 protein

fragment were produced in E. coli and used to immunize female mice. Similar to the original HS-63 monoclonal antibody, the antisera thus produced reacted only with the sperm acrosome and revealed significant inhibition of the in vitro fertilization of mouse oocytes.

In the developing mouse testis, the expression of MSA-63 gene was found to be post-meiotic. Protein and mRNA of MSA-63 were not produced until day 20 after birth.

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List of Abbreviations

AR	Acrosome Reaction
BSA	Bovine Sera Albumin
BWW	Biggers, Whitten, and Whittingham medium
cpm	Counts Per Minute
cDNA	Complementary Deoxyribonucleic Acid
Con A	Concanavalin A
CTC	Chlortetracycline
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleoside triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FA-1	Fertilizing-associated sperm antigen recognized by monoclonal antibody MA-24 (Naz et al., 1984a, 1986)
FITC	Fluorescein-isothiocyanate
GA-1	A rabbit germ cell antigen involves in fertilization (Naz et al., 1984b)
HCG	Human chorionic gonadotrophin
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
HS-21	A monoclonal antibody generated against human sperm (Wolf et al., 1983, 1985)

HS-63	A monoclonal antibody generated against human sperm (Lee et al., 1984a)
IEF	Isoelectric Focusing
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IMDM	Iscove's Modified Dulbecco's Media
IPTG	Isopropyl-Beta-D-Thiogalactopyranoside
IVF	In Vitro Fertilization
IU	International Unit
Kb	Kilobase Pair
Kd	Kilodalton
LB	Luria-Bertani
LDH-C4	Lactate dehydrogenase-C4, a sperm specific isozyme of LDH (Goldberg, 1973)
M29	A monoclonal antibody generated against mouse sperm (Saling et al., 1986)
M42	A monoclonal antibody generated against mouse sperm (Saling et al., 1986)
MA24	A monoclonal antibody generated against human sperm (Naz et al., 1984a)
ml	Milliliter
mCi	Milli Curies
moi	Multiplicity of Infection
mRNA	Messenger RNA
MSA-63	Mouse sperm antigen specific to HS-63 monoclonal antibody (Lee et al., 1984a)
MS-204	One of the monoclonal antibodies generated against mouse sperm antigen (Lee et al.,

	1984b, 1986b)
MS-207	One of the monoclonal antibodies generated against mouse sperm antigen (Lee et al., 1984b, 1986b)
O.D.	Optical Density
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PGK-2	3-phosphoglycerate kinase-2
pfu	Plaque Forming Unit
PH-20	A guinea pig sperm antigen defined by Primakoff (Primakoff et al., 1988b)
pI	Isoelectric Point
PMSF	Phenylmethanesulfonylfluoride
PMSG	Pregnant mare's serum gonadotrophin
PNA	Peanut agglutinin.
PSA	Pisum sativum lectin
PVP-40	Polyvinylpyrrolidone (M.W. 40,000)
RCA-II	Ricinis communis agglutinin-II
RIA	Radioimmunoassay
RISA	Radioimmunosorbent assay
RNA	Ribonucleic acid
RNase	Ribonuclease
RSA-1	Rabbit sperm acrosomal antigen defined by O'Rand (O'Rand et al., 1984)
SA-63	Sperm antigen of various species cross-reactive to HS-63 monoclonal antibody (Liu et al., 1990)
SDS	Sodium Dodecyl Sulfate

SP-10	Human sperm acrosomal antigen 10 defined by Herr (Herr et al., 1990a)
SPA	Sperm Penetration Assay
PAGE	Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris	Tri(hydroxymethyl)aminomethane
tRNA	Transfer RNA
ul	Microliter
X-Gal	5-Bromo-4-Chloro-3-Indoyl-Beta-D-Galactopyranoside
ZP-3	Zona pellucidae protein-3 with molecular weight of about 8.5-9 Kd on SDS-gel

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I. INTRODUCTION

A. HISTORICAL BACKGROUND AND RATIONALE

During the last decade, a number of monoclonal sperm antibodies have been generated in our laboratory (Lee et al, 1984a,b,c). The inhibitory effects of these antibodies on sperm function and fertilization have also been assessed (Lee et al, 1987; Menge et al, 1987). Among these antibodies, HS-63 was shown to react with an acrosomal antigen of sperm from several mammalian species including human, rabbit, and mouse, but did not react with any somatic tissues (Anderson et al., 1987). This highly sperm-specific and species-conserved antibody has been demonstrated to inhibit both in vivo and in vitro fertilization of mouse (Lee et al, 1986b), and human sperm penetration of zona-free hamster ova (Menge et al, 1987). According to the report of inter-laboratory evaluations sponsored by the World Health Organization in 1986, HS-63 has been recommended with high priority for the future development of immunocontraceptive vaccines (Anderson et al., 1987).

Judging from the background information, in the first part of my thesis, the HS-63 monoclonal antibody was characterized and used as a probe to investigate the location and possible physiological function of the corresponding sperm antigen, SA-63, in the processes of fertilization.

In the second part, detailed procedures of purification and characterization of the mouse sperm antigen (MSA-63) were worked out. In addition, the antifertility effects of active and passive immunizations of MSA-63 in mice were also evaluated. These data are considered to be essential for the future development of sperm antigen-based immunocontraceptive vaccines.

In the third part, cloning of cDNA fragments encoding the MSA-63 and its characterization were carried out. The recombinant fusion proteins carrying MSA-63 protein fragments produced by such techniques were used to generate antisera for the assessment of antifertility effect.

In the fourth part, the corresponding HS-63 monoclonal antibody, polyclonal antisera and cDNA probes were used as probes for developmental studies of SA-63 antigen synthesis during

spermatogenesis.

B. FORMATION OF SPERM ACROSOME.

The sperm acrosome is a membrane-bound, lysosome-like organelle localized in the anterior region of sperm head, just above the nucleus and beneath the plasma membrane (Mann, 1964, Philips, 1972). Between the outer and inner acrosomal membranes, a variety of hydrolytic enzymes including proteinases, glycosidases, phosphatases, arylsulfatases, and phospholipase are present in the acrosome. Some of these enzymes may be integral or associated components of the acrosomal membranes (Hartmann, 1983).

The acrosome first appears as a product of the Golgi complex in spermatids during spermiogenesis (Fawcett and Bedford, 1979). According to Leblond and Clermont (1952), spermiogenesis of rats is subdivided into 19 stages of development based on the changes of the acrosomic system and nucleus of the spermatid. In stage 2 of spermiogenesis, the hemispherical Golgi apparatus contains several proacrosomic granules in its medulla. These granules fuse into a single acrosomic granule which migrates towards the

nucleus and becomes attached to it while further material is added from the Golgi apparatus. The acrosome then spreads over part of the nuclear surface.

C. ROLE OF SPERM ACROSOME DURING FERTILIZATION

When released from the testis, spermatozoa are not competent to fertilize eggs (Austin and Short, 1982). They must undergo a process of maturation during the transit in the epididymis to gain their progressive motility (Hamilton, 1977, Hoskins et al., 1978).

Besides the epididymal maturation, mammalian sperm require an the capacitation process which occurs within the female reproductive tract, to prepare them for the acrosome reaction and fertilization (Chang, 1951; Austin, 1951; Yanagimachi, 1981; Moore and Bedford, 1983). A number of changes of sperm have been reported to relate to sperm capacitation (Ahuja, 1985). These changes include increase of motility, rearrangements of intramembranous particles, removal of sperm surface components and a decrease in net surface negative charge (Longo, 1987).

After the capacitation process, a spermatozoon with an intact acrosome cannot penetrate an egg. The acrosome must 'react' or break down, to release its contents prior to sperm penetration. The successive stages of this event are generally called the acrosome reaction (AR) (Austin and Short, 1982).

It is generally believed that the acrosome undergoes changes when the spermatozoon comes into contact with the egg (Dan, 1967; Clowin and Clowin, 1967). In mammals, the plasma membrane overlaying the acrosome and the outer acrosomal membrane, fuse at multiple sites and form an array of vesicles during the acrosome reaction (Barros et al., 1967). The vesicles thus formed have been shown to be mosaics consisting of membranes derived from both the plasma and the outer acrosomal membrane (Russell et al., 1979). At the level of the equatorial segment, the plasma membrane and the acrosomal membrane are fused to maintain a continuous membrane that delimits the contents of the spermatozoon.

As a result of the acrosome reaction, the vesicles are derived from the fusion of plasma and outer acrosomal membranes,

and subsequently the contents of the acrosome are released to the surrounding environment. Therefore, the release of acrosomal contents is considered to be an exocytotic event. About a dozen of the enzymes known to exist in the acrosome are released during the acrosome reaction (Mack et al., 1983). Apparently, the function of these enzymes is to assist the sperm to penetrate the egg. Inhibitors and antibodies to these sperm acrosomal enzymes are known to block fertilization at the level of the zona pellucida (Dunbar et al., 1976; Perreault et al., 1979).

The timing of the acrosome reaction in the normal course of mammalian fertilization is controversial (Moore and Bedford, 1983). It is generally believed that sperm should complete the acrosome reaction prior to penetration through the zona pellucida. The most likely sites for this reaction are within the cumulus and at the surface of the zona pellucida (Florman and Storey, 1982; Saling and Storey, 1979). A number of studies indicated that sperm should first penetrate the intact cumulus of the oocyte followed by binding to the zona pellucida and then undergo the acrosome reaction (Florman and Storey, 1982; Storey et al., 1984). Capacitated hamster sperm with intact acrosome can bind to the zona pellucida whereas sperm which have undergone the

acrosome reaction fail to do so. Furthermore, zona proteins which recognize mouse sperm plasma membrane receptors have been identified from the zona pellucida of mouse eggs (Bleil and Wassarman, 1980a,b, 1983). One of the zona proteins, ZP-3, has also been proven to induce the acrosome reaction in vitro (Bleil and Wassarman, 1983, 1986; Wassarman, 1987a,b). However, recent studies indicate that the sperm acrosome reaction may not be induced by zona pellucida only (Bavister, 1982; Lenz et al., 1982; Reyes et al., 1984; Siiteri et al., 1988; Tesarik, 1985).

In view of the complicated processes involved in the sperm acrosome reaction, several techniques have been developed to analyze this event (Soupart and Strong, 1974; McMaster et al., 1978; Saling and Storey, 1979; Talbot and Chacon, 1981; Wolf et al., 1985). Methods are now available to differentiate acrosome-intact from acrosome-reacted sperm at the light microscopic level

Several fluorescein isothiocyanate (FITC)-labeled lectins are widely used to demonstrate the presence or absence of sperm acrosome during the acrosome reaction. These included Ricinus communis agglutinin-II (RCA-II) (Talbot and Chacon, 1980), Pisum sativum agglutinin (PSA) (Cross et al., 1986), peanut agglutinin

(PNA) (Takamune, 1987), and concanavalin A (Con A) (Holden et al., 1990). Although methodologies and tools are available to study the sperm acrosome reaction, little is known about the detailed molecular events associated with this process. This is simply because of the lack of biochemical markers for such studies.

D. TESTICULAR SYNTHESIS OF SPERM PROTEINS IN SPERMATOGENESIS

Generally, genes expressed during spermatogenesis can be classified into three major categories: (a) genes that are expressed exclusively during spermatogenesis, (b) genes encoding proteins for which there is switching of isotopes or isozymes, (c) genes whose expression is somehow altered, either quantitatively or qualitatively, during differentiation (Willison and Ashworth, 1987).

Postmeiotic gene expression during spermatogenesis has been demonstrated by several known biochemical markers such as 3-phosphoglycerate kinase-2 (PGK-2) (Erickson et al., 1980), protamine (Kleene et al., 1984), α -tubulin (Distel et al., 1984;

Hecht et al., 1984) and various unidentified mRNAs in spermatids (Fujimoto and Erickson, 1982). Since acrosome formation is initiated immediately after the completion of meiosis, acrosomal proteins could be synthesized either in the pre- or post-meiotic stage.

Recently, a number of acrosomal proteins were found to appear first in the postmeiotic stages (e.g. spermatids) of spermatogenesis (Lee and Wong, 1986a). It remains to be determined whether the postmeiotic expression takes place at transcriptional or translational levels, or is simply due to the post-translational modifications of earlier expressed sperm proteins (Kleene et al., 1984). To clarify this point, it is essential to have biochemical markers which are associated with acrosome formation or the acrosome reaction.

G. APPLICATION OF MONOCLONAL ANTIBODIES IN SPERM RESEARCH

Mammalian spermatogenesis is an event in which numerous biochemical markers are turned on and off to fulfill structural, functional and developmental requirements of spermatozoa. Prior

to sperm penetration and sperm-egg membrane fusion, spermatozoa have to go through maturation in the epididymis, so that they gain motility, the ability to undergo capacitation and the acrosome reaction (Bedford and Millar, 1979; Feuchter et al., 1981).

To analyze such a complicated event, it is essential to have specific antibodies that can react with different antigenic components of spermatozoa. Since the advent of hybridoma technology, it has become possible to generate monoclonal antibodies that react with various antigenic determinants of sperm surface antigens (Lee et al., 1984a,b; Lee and Wong, 1986a). These monoclonal antibodies can be valuable tools to follow the development and the location of particular sperm antigens in the germ cells at different stages of spermatogenesis.

The generation and characterization of monoclonal antibodies against spermatozoa from several mammalian species were reported by many investigators. They were from guinea pigs (Primakoff et al., 1988a; Myles, 1987), from rabbits (O'Rand and Irons, 1984; Lee et al., 1984c), from mice (Saling et al., 1983; O'Brien et al., 1988; Lee et al., 1984b), from hamsters (Moore and Hartman,

1984), and from humans (Naz et al., 1984a; Wolf et al., 1983; Lee et al., 1984a; Herr et al., 1990a).

Monoclonal antibody HS-21 which was generated against human spermatozoa recognized target antigens restricted to the acrosomal cap determined by indirect immunofluorescent assay (Wolf et al, 1985). It was used as a marker for the evaluation of the acrosomal status of spermatozoa.

Monoclonal antibody 1D4 reacts with a glycoconjugate antigen of the developing mouse spermatid acrosome until the terminal steps of spermiogenesis (Gerton et al., 1988). It can not bind to the acrosome of mouse epididymal spermatozoa. However, 1D4 reacts with the acrosomal region of guinea pig epididymal sperm. From the immunoblots of extracts of guinea pig spermatocytes, round spermatids, condensing spermatids and sperm with this monoclonal antibody, the corresponding antigen changes during germ cell differentiation can be demonstrated. Thus 1D4 can be used as a marker for studies of the synthesis, structure, and assembly of sperm organelles, such as the acrosome.

Monoclonal antibody M42, which reacts with a sperm acrosome

antigen of 220 KD, was shown to inhibit fertilization in mice in a dose-dependent manner (Saling et al., 1986). This antibody specifically inhibits physiologically zona-induced (ZP-3), but not pharmacologically ionophore (A23187)-induced acrosome reaction in mouse sperm. When the fluorescent antibiotic chlortetracycline (CTC) was used to visualize the three different patterns during the acrosome reaction (B, S, and AR), M42 was shown to arrest the zona-induced sperm acrosome reaction in the B-pattern. Therefore, it was concluded that M42 blocks the early step in the acrosome reaction cascade and is unable to prevent subsequent events of the cascade once they have been initiated (Leyton et al., 1989).

Monoclonal sperm antibodies were also used as probes to analyze the developmental expressions of relevant sperm surface antigens during different stages of spermatogenesis (Lee and Wong, 1986a). By immunofluorescent assay, monoclonal sperm antibodies were used as probes to stain freshly isolated testicular cells and tissue sections from mouse testes of different ages. It was generally observed that a significant number of these antibodies reacted with cytoplasmic components of the spermatogenic cells in the testis at the postmeiotic stages

(e.g. day 22 after birth). After meiosis, the percentage of seminiferous tubules stained by indirect immunofluorescence was found to increase with the age of mice. Therefore, it was suggested that some cytoplasmic components (especially acrosomal) of spermatogenic cells are expressed postmeiotically in the testis and later translocated to the sperm surface during late stages of spermiogenesis (Lee and Wong, 1986a). The purification and characterization of these corresponding sperm antigens are essential steps for the evaluation of their functional roles during the fertilization processes. However, very few sperm antigens have been purified and characterized. The main difficulties for such studies arise from the fact that the majority of these sperm surface antigens are membrane-associated or integral membrane proteins (Primakoff and Myles, 1983).

F. DEVELOPMENT OF SPERM ANTIGEN-BASED IMMUNOCONTRACEPTIVE VACCINE

Isoimmunization with sperm or testis extract causes significant antifertility effects in animals (Menge et al., 1979). Therefore, the development of sperm antigen-based vaccines

represents a promising approach to contraception (Talwar, 1986; Anderson et al., 1987). However, crude extracts of sperm or testis are not appropriate to be used directly as a vaccine due to the presence of numerous components which may cross-react with somatic tissues, causing undesirable immunopathological consequences (Mathur et al, 1981; Schachuer et al, 1975). Therefore, it is essential to identify sperm-specific auto- and iso-antigens which may properly serve as effective contraceptive vaccines.

It has been known for decades that certain sperm surface components are auto- or iso-immunogenic (Tung, 1980; Menge et al, 1979). In view of the known effects of sperm antibodies on sperm functions and their association with human infertility, sperm antigen-based contraceptive vaccines appear to be an ideal choice for pre-fertilization contraception in humans and animals (Anderson and Alexander, 1983). From clinical observations, the individuals who suffer from sperm antibody-related infertility do not exhibit other health problems compared to the normal subjects (Mathur et al, 1981; Schachuer et al, 1975). Since the contraceptive effect is associated with the titres of circulating and/or locally secreted sperm antibodies, the reversibility of

this contraceptive method has been reported in animal studies (Primakoff, 1988b).

Desirable sperm antigens could be identified and obtained for contraceptive vaccine developments from several sources. These include (a) sperm antigens which elicit naturally occurring sperm antibodies from sera of infertile patients (Jackson et al, 1975; Teuscher et al, 1982 and 1983), (b) sperm proteins of known sperm-specific enzymes such as lactate dehydrogenase-C4 (LDH-C4) (Goldberg, 1973) and (c) sperm surface antigens reactive to monoclonal antibodies which affect normal sperm functions (Naz et al, 1984b and 1986; O'Rand et al, 1984; Sailing et al, 1986; Primakoff et al, 1988a).

Using hybridoma techniques, monoclonal antibodies, each of which reacts with a single determinant of a given sperm antigen, can be generated. These monoclonal antibodies were used to investigate the developmental and functional aspects of complementary sperm antigens (Lee et al, 1984a,b,c; Lee and Wong, 1986b; and Lee et al, 1987). Some monoclonal antibodies which react mainly with the sperm acrosome were found to inhibit the fertilization of mouse oocytes in vitro and in vivo and

implantation of embryos (Lee et al, 1986a). Therefore, the corresponding sperm antigen which reacts with each of these antibodies may be a suitable choice for the development of immunocontraceptives, if the antigens are identified, purified and characterized.

Some of sperm-specific antigens which react with polyclonal or monoclonal antibodies have been purified and evaluated for their antifertility effects. Lactate dehydrogenase-C4 is a sperm-specific enzyme which mainly attaches to the sperm tail. Active immunization of this sperm-specific antigen was shown to partially suppress fertility in experimental animals (Goldberg, 1973 and 1975; Kille and Goldberg, 1979 and 1980; Goldberg et al, 1981). Menge and his coworkers used a monoclonal sperm antibody to purify and characterize GA-1, a germ cell antigen of the rabbit. The antisera raised against this antigen revealed partial inhibition on post-fertilization events but not on initial fertilization steps (Naz et al, 1984b). Likewise, an acrosomal antigen (RSA-1) of rabbit sperm, which was involved in sperm-egg interaction and fertilization was purified (O'Rand et al, 1984). A membrane antigen of human and murine sperm, FA-1, is recognized by a monoclonal antibody, MA-24 which inhibits the

initial fertilization process (Naz et al, 1984a and 1986). A sperm surface antigen, PH-20, was purified from the octylglucose extracted from guinea pig sperm (Primakoff, 1988a). Active immunization of this sperm antigen resulted in a complete and reversible suppression of fertility in guinea pigs (Primakoff, 1988b).

From numerous previous studies by other investigators, it is apparent that the most suitable sperm antigens for contraceptive vaccines must be sperm-specific and readily accessible to binding by IgG or IgA antibodies (Tung, 1980; Anderson and Alexander, 1983). The most desirable ones are those of which the function can be blocked by bound antibodies to interfere with the fertilization process. However, information concerning the structural aspects of sperm surface antigens appear to be very limited. Therefore, my work not only dealt with the basic study of the sperm antigen, SA-63, but also its application to the development of an immunocontraceptive vaccine.

II. MATERIALS AND METHODS

(The formula of solutions in this section are summarized in Appendix.)

A. IMMUNOLOGICAL TECHNIQUES

1. CHEMICALS

Fluorescein-isothiocyanate (FITC), peroxidase (type VI, from Horseradish), Diethylaminoethyl (DEAE)-cellulose and ammonium sulfate, complete and incomplete Freund's adjuvant were from Sigma (St. Louis, MO). FITC-labeled goat anti-mouse/rabbit IgG+M+A was from GIBCO/BRL (Burlington, Ontario, Canada). Microtiter plates for immunoassays (Immunlon I) were from Dynatech (Arlington, VA).

2. ANIMALS

Randomly bred CD-1 female mice of 8-10 weeks old were used for active immunization. New Zealand white female rabbits between 2 and 3 months of age were used for immunizations.

3. PURIFICATION OF ANTIBODY FROM ASCITES FLUID AND ANTISERA

The HS-63 monoclonal antibody (IgG 1 subclass) in this study

was secreted by a hybrid cell line generated by means of cell fusions between NS-1 myeloma cells and spleen cells of BALB/C mice immunized with human sperm antigen preparation (Lee et al., 1984a). Generally, HS-63 monoclonal antibody could be obtained and purified from the ascites fluid of BALB/C mice which were injected peritoneally with the established hybrid cells secreting this antibody. The titres of this antibody in ascites fluid were determined by indirect immunofluorescent assay and usually in the order of 1:100,000 dilution.

Monoclonal antibodies from ascites fluid or antisera were purified by ammonium sulfate fractionation and DEAE-cellulose chromatography (Heide and Schwick, 1973). Sufficient ammonium sulfate was added to the antibody solution (either ascites fluid, culture supernatant or antisera) to give a final concentration of 313 gram per liter (50 % saturation). The solution was slowly stirred for 30 minutes and centrifuged at 10,000 X g for 10 minutes. The pellet was then recovered and washed at 0°C with a solution of 1.75 M ammonium sulfate until the solution turned milk white (Harboe and Ingild, 1973). After centrifugation, the pellet was dissolved in 10 mM Tris buffer pH 8.0 and dialyzed overnight against the same buffer. The dialyzed immunoglobulin solution was then loaded to a DEAE column (e.g. 5 ml DEAE-

cellulose per ml of ascites fluid) which was equilibrated in the same buffer. The column was washed with two bed volumes of the buffer and the immunoglobulin was eluted by using a linear gradient of 0 to 0.3 M NaCl in the same buffer. The total volume of the salt gradient was 5 to 7 times of the column bed volume. The immunoglobulin (IgG) was eluted as a sharp peak, and the purity was tested by using SDS-PAGE. With this purification method, the purified immunoglobulin was usually 90 % pure.

4. LABELING OF ANTIBODY

a. IODINATION OF ANTIBODY (Greenwood and Hunter, 1963)

Ten ug of purified antibody in 80 ul of 0.1 M phosphate buffer, pH 7.1 was mixed with 0.5 mCi of ^{125}I in a test tube. Twenty ul of Chloramine T (2.5 mg/ml) was then added to the mixture. After 30 seconds of incubation, 100 ul of NaHSO_3 (1.2 mg/ml) was added and the mixture was allowed to stand for an additional 30 seconds. Finally, 100 ul of NaI was added to the reaction mixture. Free ^{125}I was separated by gel filtration on a Sephadex G-25 column (10 ml) which was equilibrated by the same reaction buffer containing 0.5 % BSA. The specific radioactivity of the ^{125}I -labeled antibody was determined by using an LKB

minigamma counter.

b. PREPARATION OF FITC-LABELED ANTIBODY

One mg of the purified antibody in 0.5 ml of 0.1 M NaHCO_3 , pH 9.5 was mixed with 12.5 ug Fluorescein-isothiocyanate (FITC). After overnight shaking at 4°C in the dark, the free FITC was separated from the FITC-labeled antibody by a Sephadex G-25 gel filtration column (10 ml) which was equilibrated by PBS containing 0.5 % BSA. The first peak which eluted from the Sephadex G-25 column was the FITC-labeled antibody.

c. PREPARATION OF HORSE RADISH PEROXIDASE (HRP)-LABELED ANTIBODY

(Nakane and Kawaoi, 1974)

Four mg horse radish peroxidase in 1 ml deionized water was incubated with 0.2 ml of 0.2 M NaIO_4 at room temperature for 20 minutes. The reaction mixture was then dialyzed against 1 mM sodium acetate, pH 4.5 at 4°C for 3 hours with 5 changes of buffer every 30 minutes. The dialyzed HRP was incubated with 10 mg of monoclonal antibody in 2 ml of 0.2 M NaHCO_3 , pH 9.5. After one hour incubation at room temperature, the reaction was stopped by adding NaBH_4 to a final concentration of 0.2 mg/ml. The HRP-labeled antibody was dialyzed against PBS overnight at 4°C and

was then stored in PBS with 0.5 % BSA and 0.1 % Thimerosal at 4°C.

5. INDIRECT IMMUNOFLUORESCENT ASSAY

The indirect immunofluorescent assay was used to determine the binding of antibodies to sperm surface antigens and used especially for determinations of antibody titres (Lee et al, 1984a,b,c).

a. PREPARATION OF SPERM SLIDES

Human sperm were obtained from the ejaculates of healthy donors. After 30 minutes for liquefaction, the sperm were washed twice by centrifugation at 500 X g for 10 minutes with 10 volumes of PBS. The washed sperm was adjusted to 5×10^6 / ml in PBS and then mounted on slides. Air-dried sperm slides were stored at -20°C.

Mouse sperm were obtained from the cauda epididymidis and from the vas deferens of mature male mice. The dissected cauda epididymidis and vas deferens were cut into small pieces, and then incubated at 37°C in a CO₂ incubator for 15 minutes in BWW medium. The tissue debris was removed by a cotton fiber column. The sperm which swam out were prepared like human sperm for sperm

slides. The acrosome-reacted sperm were prepared following the method described by Jamil and White (1981).

b. IMMUNOFLUORESCENT ASSAY

The slides of sperm were fixed in absolute methanol for 10 minutes at room temperature, dried, and 0.5 % BSA in PBS was applied for 10 minutes to block nonspecific binding. The sperm slides were incubated with an antibody solution of serial dilutions in PBS with 0.5 % BSA for 30 minutes at room temperature. After incubation, the sperm slides were washed with PBS twice. Affinity-purified goat anti-mouse or rabbit IgG+M+A conjugated with FITC diluted in PBS containing 0.5 % BSA was added as the second antibody and incubated for an additional 30 minutes. Following the three washes with PBS solution, the immunofluorescent staining was examined by fluorescence microscopy.

Indirect immunofluorescent inhibition assay was usually used for quantitative determination of immunoactivity of the sperm antigen.

The antibody was first diluted to its end point. The end point was defined as the maximum dilution of antibody which still stained the methanol-fixed sperm in the indirect

immunofluorescent assay. The end point of HS-63 from ascites fluid was about 1:100,000. In the inhibition assay, an equal volume of antigen preparation and HS-63 (1:50,000 from ascites fluid) were incubated at 37°C for one hour. The solution was then applied to the air-dried and methanol-fixed sperm for half an hour at room temperature. Following three washes with 0.5% bovine serum albumin in phosphate-buffered saline (PBS-BSA), FITC-labeled goat anti-mouse IgG+M was added to sperm as the second antibody. The absence of the immunofluorescent staining of sperm indicated the presence of antigen. Mouse testes and liver homogenates served as the positive and negative controls, respectively.

An indirect immunofluorescent inhibition assay was also used to determine the tissue-specificity of monoclonal antibodies and isoimmune antisera according to the method of Gaunt (Gaunt, 1982). Briefly, antibodies from either ascites fluid or isoimmune antisera (dilution factor, 1:100 to 1:10,000) were incubated with an equal volume of fresh whole homogenates of testis, brain, heart, kidney, spleen or liver (protein concentration of all homogenates was adjusted to 30 mg/ml). After one hour incubation at room temperature, the mixture was centrifuged at 27,000 x g for 10 minutes. The supernatant was recovered for the indirect

immunofluorescent assay with methanol-fixed mouse sperm.

6. DIRECT IMMUNOFLUORESCENT ASSAY

The direct immunofluorescent assay was used to evaluate the immunofluorescent reaction of FITC-labeled antibody on live sperm.

Preparation of live sperm is described in section D.3.b. from mice and in section D.4.b. from humans. 50 ul of live sperm were incubated with 50 ul of FITC labeled HS-63 which was diluted in PBS in a 1.5 ml microcentrifuge tube for 1 hour at 37°C. After incubation, the immunofluorescent staining was examined by fluorescence microscopy.

In the experiment of chemically treated live sperm, 50 ul of live sperm were incubated separately with equal volumes of 1 M KCl, 0.4 % glutaraldehyde or 0.2 % Triton X-100 which were prepared in BWW medium for 5 minutes at room temperature. After centrifugation, the sperm were resuspended in 50 ul of BWW medium and then incubated with FITC labeled HS-63 as described previously.

7. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

(The formular of solutions are summarized in Appendix 1)

a. PREPARATION OF ANTIGEN-COATED MICROWELLS

Purified antigen (about 1 ug/ml), the soluble extract of human or mouse sperm in 0.1 M Tris-HCl buffer, pH 8.0 was coated on microtiter plates overnight at 4°C. After three washes with distilled water and blocking with blocking buffer for 1 hour, the coated microtiter plates were dried at room temperature and were stored at 4°C. Prepared microwells can be kept at 4°C for several months.

b. ASSAY PROCEDURES

One hundred ul of HS-63 monoclonal antibody, isoimmune sera or other unrelated antibodies in a series of five or ten fold dilutions were incubated with antigen-coated microwells for a given period of time at room temperature. The unbound antibodies were removed by washing three times with 0.02 % Tween-20 in PBS (PBS-Tween). Goat anti-mouse IgG+M labeled with horse radish peroxidase (from BRL) at a dilution of 1:3,000 in PBS with 0.5 % BSA was added for another 1 hour of incubation at 37°C. After three washes with PBS-Tween and one wash with water, the antibody binding was visualized by the colorimetric enzymatic reaction in the presence of 200 ul of substrate solution. After color development in the dark for a suitable period of time, the

reaction was stopped by adding an equal volume of 1 M H_2SO_4 . The result was measured by a microtiter plate reader at 450 nm.

8. RADIOIMMUNOSORBENT ASSAY (RISA)

The specificity of purified sperm or testis antigens and their cross-reactivity with other antibodies were evaluated by ELISA and RISA (Lee et al, 1984a; Engvall and Perlmann, 1971).

For the RISA, purified HS-63 monoclonal antibody was labeled with ^{125}I according to the previously described procedure of protein iodination. The dose-dependent antigen-antibody binding assays were performed by incubating labeled HS-63 of serial dilutions with antigen-coated microwells at room temperature for one hour. Following three washes with PBS-Tween, the residual radioactivity in microwells was determined by an LKB minigamma counter. The RISA was also used to demonstrate specific inhibition of HS-63 binding to sperm coated on microwells by the antigen solution.

9. PRODUCTION OF POLYCLONAL ANTISERA

The purified sperm antigen (MSA-63) from mouse was used to isoimmunize mice and rabbits. Briefly, 10 ug of purified antigen

in 50 ul PBS were emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into each mouse for the primary immunization. Subsequent immunizations (10 ug/mouse) were performed at biweekly intervals using incomplete Freund's adjuvant. Seven days after each immunization, animals were bled and the antisera titres were determined by the indirect immunofluorescent assay. Rabbits were immunized with the same protocol, except that 50 ug of antigen was used for each immunization. The titre and specificity of the antisera from mouse or rabbit were determined by either immunofluorescent assay or by ELISA.

C. PROTEIN CHEMISTRY

1. CHEMICALS

Sephacryl S-300 and Ampholine were from Pharmacia/LKB (Piscataway, NJ). Trypsin was from Sigma (St. Louis, MO). Affi-gel 10, protein assay reagent and all the analytical grade reagents required for polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). Nitrocellulose filter paper was from Millipore (Mississauga, Ontario, Canada). ^{125}I (1 mCi/mmol) was from Amersham (Oakville, Ontario, Canada). N-glycosidase and O-glycosidase were from Boehringer Mannheim Biochemica (Dorval, Quebec, Canada). Centriprep 10 and Centricon 10 concentrator was from Amicon (Danvers, MA).

2. DETERMINATION OF PROTEIN CONCENTRATION

The protein concentration was routinely estimated by measuring optical density (OD) at 280 nm. However, Bio-Rad dye reagent was used to determine the protein concentration in microscale (Bradford, 1976). In this method, 0.2 ml of Bio-Rad stain was added to 0.8 ml of sample solution containing 1 to 10 ug protein. The protein concentration was determined by reading the OD at 595 nm after 2 minutes. Bovine serum albumin was used

as a standard.

Amido black staining method was used to determine the protein concentration in sample solutions containing detergent (Schaffner and Weissman, 1973).

3. DETECTION OF ANTIGEN IMMUNOACTIVITY

The indirect immunofluorescent inhibition assay and RISA were used to determine antigen immunoactivity during various purification stages. The relative specific immunoactivity of purified antigen was defined as the inverse of protein concentration of a given antigen preparation that was required to inhibit the immunofluorescent staining of sperm by HS-63 at its end point dilution.

For the indirect immunofluorescent inhibition assay, HS-63 ascites fluid which had been diluted to 1:10,000 with PBS-BSA, was incubated with an equal volume of antigen solution for 1 hour at 37°C. The procedures of indirect immunofluorescent assay was described previously in the section of immunofluorescent assay.

4. PREPARATION OF IMMUNOAFFINITY GEL

Purified HS-63 monoclonal antibody (50 mg in 50 ml of 0.1 M NaHCO_3) was mixed with 5 ml Affi-gel 10 which had been washed

with distilled water for the removal of isopropanol in the gel suspension. After overnight shaking at 4°C, the gel was washed with water and then incubated with 1 M Tris-HCl, pH 8.0 for 1 hour at room temperature. Finally, the gel was washed with a solution of 6 M urea and 2 M NaCl and equilibrated with PBS. The coupled gel was stored with 0.01 % NaN₃ at 4°C .

5. PURIFICATION OF MSA-63 ANTIGEN FROM MOUSE TESTES

Sperm or testis antigen specifically recognized by HS-63 was purified from mouse testes by using two different procedures described as follows:

a. CONVENTIONAL METHOD COMBINED WITH IMMUNOAFFINITY

CHROMATOGRAPHY

Ten grams of frozen testes were homogenized in 100 ml PBS containing 1 mM phenylmethylsulfonylfluoride (PMSF) with a polytron homogenizer (Brinkmann) at 4°C. After centrifugation at 27,000 X g for 20 minutes, the supernatant was collected and subjected to ammonium sulfate fractionation. In this procedure, solid ammonium sulfate (390 mg/ml) was slowly added to the supernatant, and then stirred for 1 hour at 4°C. After centrifugation again, the pellet was dissolved in 50 mM phosphate

buffer (potassium salt) pH 7.2 and dialyzed overnight against the same buffer. The dialyzate was then applied onto a DEAE-cellulose column (2.5 x 40 cm) which had been equilibrated with the same phosphate buffer. The adsorbed proteins were eluted with a 0 to 1 M NaCl linear gradient (500 ml:500 ml). The antigen containing fractions detected by using indirect immunofluorescent inhibition assay were pooled and dialyzed against PBS overnight. The solution was then loaded on an immunoaffinity column (10 ml) using purified HS-63 monoclonal antibody as the affinity ligand. After the application of the sample, the column was washed extensively with 0.5 M NaCl in PBS until adsorbance of the eluent at 280 nm was 0.001 or less. The antigen was eluted with 0.1 M glycine-HCl buffer at pH 2.2. The fractions containing most of the antigen immunoactivity were pooled, and concentrated by centrifugation using Centricon-10.

b. IMMUNOAFFINITY CHROMATOGRAPHY WITH DETERGENT EXTRACTION

Ten grams of mouse testes were homogenized in PBS with 2 % Triton X-100 and 2 mM PMSF at 4°C. After centrifugation at a speed of 27,000 X g for 10 minutes, the supernatant was collected and passed through a column of cotton fibers to remove the tissue debris. The supernatant was diluted to 100 ml and then applied to

an immunoaffinity gel (10 ml) using HS-63 as the affinity ligand. After repeated loadings for 2 hours at 4°C, the affinity gel was washed with 500 ml of PBS containing 0.5 M NaCl and 0.1% Triton X-100. The bound antigen was then eluted with 0.1 M glycine-HCl, pH 2.2 containing 0.1 % Triton X-100 and 2 mM PMSF. The antigen-containing fractions were pooled, and concentrated by centrifugation using a Centriprep concentrator.

6. GEL FILTRATION

Sephacryl S-300 gel filtration chromatography was used to determine the native molecular weight of sperm antigen in the purified state and in the homogenate. Swollen Sephacryl S-300 was packed into a 30 cm X 0.9 cm glass column and was equilibrated with PBS containing 0.1 % Triton X-100. Supernatant of testes homogenate or purified MSA-63 antigen in a volume of 0.5 ml containing 10 % glycerol was loaded on to the column. At the same time, fractions of 1 ml were collected. The immunoactivity of antigen was detected by using the indirect inhibition immunofluorescent assay as described above.

7. SODIUM DEDECYL SULFATE (SDS) POLYACRYLAMIDE GEL

ELECTROPHORESIS (SDS-PAGE)

(The formular of solutions are summarized in Appendix 2)

A. PROCEDURE OF ELECTROPHORESIS

A vertical slab gel apparatus for SDS-PAGE was obtained from Albergene Co. Generally, 3 % acrylamide stacking gel and 10 % acrylamide running gel were used (Laemmli, 1970).

The running gel was prepared from a mixture containing 10 ml running gel acrylamide solution, 7.5 ml running Tris buffer, 12 ml water, 0.5 ml of 2 % potassium persulfate and 25 ul N,N,N',N'-tetramethylenediamine (TEMED). Immediately after mixing, the running gel was filled between the glass plates to a height of 10 cm, water was overlaid on top of the gel to ensure a level surface for the running gel.

Stacking gel was prepared from a mixture containing 1 ml stacking gel acrylamide solution, 2.5 ml stacking Tris buffer, 6.34 ml water, 0.1 ml of 2 % potassium persulfate and 5 ul TEMED.

When the running gel was formed, the water on top of the gel was discarded. The stacking gel was overlaid on top of the running gel. A slot comb was placed at the upper margin of glass plate to form wells for the loading of the protein samples.

The protein sample was mixed with equal volumes of sample

buffer, boiled for 5 minutes to denature the proteins, and then applied to the gel. The voltage of electrophoresis was first set at 150 V. After the protein had entered the running gel, the voltage was then switched to 250 V. The electrophoresis was run until the dye front reached about 10 cm from the top of running gel.

After the electrophoresis, the gel was removed from the glass plates carefully for protein staining or for Western blot analysis.

b. PROTEIN STAINING BY COOMASSIE BRILLIANT BLUE

For Coomassie brilliant blue staining, the gel was immersed in a solution containing 0.2 % Coomassie brilliant blue R in 10 % acetic acid, 50 % methanol, and 40 % dH₂O for 4 hours or more. The gel was destained by repeated washings in 50 % methanol, 10 % acetic acid, and 40 % dH₂O until the protein bands on the gel were visualized. Finally, the gel was kept in 10 % acetic acid.

c. PROTEIN STAINING BY SILVER REAGENT

Silver staining kit from Bio-RAD was used for protein staining. After electrophoresis, the gel was fixed in a solution of 40 % methanol and 10 % acetic acid for 30 minutes followed by

a solution containing 10 % ethanol and 5 % acetic acid for two changes at 30 minutes interval. After incubation with oxidizer for 5 minutes, the gel was washed with dH_2O for three changes of 5 minutes. The gel was then incubated with silver reagent for 20 minutes. After a rinse in dH_2O , developer solution was added and the gel was incubated for 30 seconds or until the solution turned dark. The protein bands on the gel were visualized by continual incubation in the developer solution. The stained gel can be stored in 5 % acetic acid solution for up to several months.

8. TWO-DIMENSIONAL GEL ELECTROPHORESIS

(The formular of solutions are summarized in Appendix 3)

Two-dimensional gel electrophoresis was performed by first running isoelectric focusing gel electrophoresis and then by slab SDS-PAGE according to the published procedures (O'Farrell et al., 1977).

For the isoelectric focusing gel electrophoresis, five ml of gel mixture was made and filled into a 10 cm glass tube (made from a 1 ml serological pipette). A layer of water was carefully overlaid on top of the gel. 50 μl of purified protein was mixed with sample buffer of the same volume. 0.2 % H_3PO_4 was the anode buffer and 0.1 % NaOH was the cathode buffer. The electrophoresis

was carried out at a voltage of 350 V for overnight and then 500 V for 1 hour at room temperature. After the electrophoresis, the gel was removed and immersed in a SDS sample buffer for 1 hour in order to remove urea from the gel. The gel was then placed on top of a slab SDS gel. The two gels were sealed together by using 1 % agarose. The second dimension SDS-PAGE was performed on gel slabs as previously described.

9. IMMUNOBLOTTING ANALYSIS

a. DOT BLOT ASSAY

(The formular of solutions are summarized in Appendix 4)

The dot blot assay was modified from the method described by Towbin (Towbin et al., 1979). This method gives rapid detection of antigen in various samples and is especially useful for testing column eluents during antigen purification.

Five ul of sample was dotted onto nitrocellulose filter paper and allowed to dry completely. The nitrocellulose filter was treated with blocking solution for 30 minutes and then incubated with rabbit anti-MSA-63 sera with a dilution of 1 to 200 for 1 hour at room temperature. After washing three times with TBST HRP-labeled goat anti-rabbit IgG+M antibody (from BRL) with a

dilution of 1 to 200 was added and incubated for 1 hour. Following three washes with TBST and once with TBS, the nitrocellulose filter paper was incubated with a peroxidase substrate solution for color development until clear signal appeared. The color development was then stopped by water.

Dot blot assay was also used for stability evaluation of the HS-63 epitope. The supernatant of mouse testes homogenate was treated by the following enzymes and chemicals: (a) 2 % SDS, (b) 2 % Triton X-100, (c) 0.4 % NP-40, (d) 0.2 % trypsin, (e) 0.2 % pronase, (f) 40 mM sodium periodate, and (g) 0.2 unit/ml neuraminidase. After incubation at 37°C for one hour, the treated supernatant was dotted on a nitrocellulose filter and the stability of the HS-63 epitope was analyzed by using HRP-labeled HS-63 monoclonal antibody (0.5 mg/ml) as a detecting probe.

b. WESTERN BLOT ASSAY

(The formular of solutions are summarized in Appendix 5)

This method was used to identify the specific protein bands separated by SDS-PAGE in the presence of antibodies. The details of this method are described in the instruction manual supplied by BIO-RAD (Towbin et al., 1979).

After electrophoresis, the gel and nitrocellulose filter

paper were rinsed in transfer buffer. The nitrocellulose filter paper was placed against the gel on a flat surface and was laid smoothly over the gel by rolling a 5-ml glass pipette on top to remove all air bubbles. Two pieces of 3 MM filter paper and sponge (prewetted with transfer buffer) were used to clamp the gel/filter to make a sandwich. The whole sandwich unit was placed in electrophoretic transfer apparatus with the gel facing the cathode. The buffer tank was filled with transfer buffer to cover the gel. After 2 hours of electrophoretic transfer at a constant voltage of 50 V, the nitrocellulose filter paper was placed in blocking buffer for immunoblotting analysis which was described previously. The protein pattern can be visualized by using amido black staining.

10. DEGLYCOSYLATION OF PROTEIN

Deglycosylation of MSA-63 protein using N-glycosidase and O-glycosidase was modified from the instruction manual supplied with the enzymes and the method described by Steube et al. (1985).

One μ l of iodinated MSA-63 protein (specific activity 0.1 mCi/ μ g) was diluted with 22.5 μ l of 0.1 M phosphate buffer pH 8.3 containing 1 % Triton X-100 and 0.1 % SDS and then incubated with

N-glycosidase (0.4 unit) and O-glycosidase (1 mU), respectively. After overnight incubation at 37°C, the reaction mixtures were analyzed by SDS-PAGE. HCG protein served as a positive control.

11. AMINO ACID SEQUENCE ANALYSIS

After the electrotransfer, the protein spot of the purified MSA-63 antigen on the 2D-gel was cut from nitrocellulose filter paper which had been stained with amido black. The protein spot (about 10 ug) was placed in a 1.5 ml centrifuge tube and incubated with 0.1 M Acetic acid containing 1 % PVP-40 at 37°C for 30 minutes. After rinsing ten times with water and once with 0.1 M NaHCO₃ pH 8.2, 50 ul of 0.1 M NaHCO₃ containing 5% acetonitrile and 2 ul of trypsin (1 mg/ml) were added for overnight tryptic digestion at 37°C. Following digestion, peptide fragments were separated by high performance liquid chromatography (HPLC) with C-18 reverse phase column (Millipore Co.) and individually analyzed using the method of Edman degradation by a protein sequenator (Applied Biosystems Inc.).

D. EVALUATION OF ANTIBODIES BY SPERM FUNCTIONAL ASSAYS

The antisera were heat-inactivated at 56°C for 45 minutes prior to use for the sperm functional assay. The antifertility effects of these isoimmune antisera on mouse in vitro fertilization (Lee et al, 1985; and Biggers et al, 1971) and human sperm penetration of zona-free hamster eggs (Yanagimachi, 1976) were evaluated. HS-63 monoclonal antibody and normal mouse or rabbit sera served as the positive and the negative control for each experiment, respectively. Monoclonal antibodies, MS-204 and MS-207 generated against mouse sperm were served as positive control in in vivo mating study.

1. CHEMICALS

Calcium ionophore-A23187, Pisum sativum lectin (PSA), pregnant mare's serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), bovine serum albumin (BSA), lacmoid, hyaluronidase and trypsin were from Sigma (St. Louis, MO). Chemicals for culture media were tissue culture grade from GIBCO/BRL (Burlington, Ontario).

2. ANIMALS

Randomly bred CD-1 female mice (4 and 6 weeks old) were used

for in vivo mating, in vitro fertilization and active immunization. Male mice of CD-1 strain (8 and 10 weeks old) were used for mating experiments after they had been proven to be fertile. Inbred male BALB/c mice were used for ascites fluid production. Eight-week-old female golden hamsters were used for the human sperm penetration assay. New Zealand white female rabbits of 2 to 3 months old were used for immunization.

3. IN VITRO FERTILIZATION OF MOUSE OOCYTES (Lee et al., 1985, Biggers et al., 1971).

(The formular of solutions are summarized in Appendix 6)

a. INDUCTION OF SUPEROVULATION

Female mice were injected intraperitoneally with 5 IU PMSG at 6 pm on day 1 and followed by a second injection with 5 IU hCG at 6 pm on day 3. The light cycle of the animal room was set from 5 am to 7 pm.

b. SPERM PREPARATION

On day 4, two male mice were killed at 8 am. The cauda epididymidis and vas deferens were dissected out and placed into a sterilized petri dish with 2 ml BWB medium. Sperm were released

by cutting the tissues into small pieces. After 10 minutes of incubation at 37°C in a CO₂ incubator, the sperm suspension was passed through a cotton fiber column to remove the tissue debris. The sperm suspension was then incubated at 37°C in a CO₂ incubator for 1 hour to ensure sperm were fully capacitated in Biggers, Whitten and Whittingham (BWW) medium.

c. SPERM-EGG INTERACTION

About 30 minutes after the sperm suspension was prepared, female mice were killed and the oviducts were dissected out. Oocytes surrounded with cumulus cells were released by punching holes in the ampulla of the oviduct with a 30 G needle under a dissecting microscope.

For each experiment, 20 ul sperm (1×10^6 sperm/ml) suspension and 20 ul antibody solution of known concentration were added to 140 ul of BWW medium placed under mineral oil in 35 mm petri dishes. Eggs with cumulus cells were added to the sperm-antibody mixture. After a 6 to 8 hour incubation in a CO₂ incubator, the eggs were washed three times by using PBS and then fixed with 2 % glutaldehyde solution overnight. The oocytes were stained by staining solution and then treated with destaining solution. The fertilization of oocytes was judged by the

appearance of two pronuclei, two polar bodies, a swollen sperm head, or a sperm tail.

4. SPERM PENETRATION TO ZONA-FREE HAMSTER OVA (Yanagimachi, 1976)

a. INDUCTION OF SUPEROVULATION

Ten days before the assay, the vaginal discharge of each hamster was checked to determine the exact stage of their oestrous cycle. Hamsters which were on day 1 of the cycle with post ovulatory mucus were injected intraperitoneally with 40 IU PMSG at 9 am on day 1 followed by a 40 IU hCG injection at 4:30 pm on day 3.

b. PREPARATION OF HUMAN SPERM

Human semen was donated by masturbation in the afternoon of day 3 from a healthy donor. After allowing 30 to 60 minutes for complete liquefaction, one ml of semen was placed in a 15 ml sterile culture tube and gently overlaid with 2 ml BWW medium. The tubes were tilted at a 20° angle from the horizontal position to maximize the surface of the semen-medium interface. They were then incubated at 37°C for a period of 1 hour in a CO_2 incubator.

After the incubation, the culture tubes were gently returned

to the vertical position for 15 minutes at room temperature and the top 80 % of the overlaying medium was transferred into another sterile culture tube. The sperm were collected by centrifugation at 500 X g for 5 minutes. The pellet was then resuspended in BWB medium with a final concentration of 10×10^6 sperm/ml and incubated overnight in a CO₂ incubator. The concentration of sperm was finally adjusted to 3.5×10^6 sperm/ml. A solution mixture containing 10 ul of antibody preparation and 100 ul of sperm suspension was then placed under paraffin oil in a 35 mm petri dish.

c. SPERM-EGG INTERACTION

In the morning of day 4, hamsters were killed and eggs were flushed out from the oviduct by using a syringe with a 30 G needle. The cumulus cells surrounding oocytes were removed by the addition of 0.1 % hyaluronidase and the zona pellucidae were digested by using 0.1 % trypsin. About thirty zona-free hamster eggs were pipetted into the sperm-antibody mixture under paraffin oil in petri dishes. The mixture was incubated at 37°C for 3 hours in a CO₂ incubator. After incubation, the eggs were removed and washed to remove the loosely attached sperm from the egg surface. The percentage of eggs penetrated by sperm was recorded

under a phase contrast microscope by observing the presence of a swollen sperm head inside the zona-free eggs.

5. INHIBITION TO ACROSOME REACTION

Mouse sperm were prepared as described in the section on mouse in vitro fertilization. The sperm acrosome reaction could be induced by zona pellucida (Lakoski et al., 1988).

To examine the zona pellucida-induced AR, mouse ova were retrieved from superovulated mice as described in the previous section. Cumulus cells were removed by treatment with 0.1 % hyaluronidase in PBS. The zona pellucida of oocytes were solubilized in 0.1 M phosphate buffer, pH 2.5. The solution was then neutralized with 10 X PBS. The concentration of zona pellucida was adjusted to 40 zona/ul. Ten ul of mouse sperm at a concentration of 1×10^6 sperm/ml was incubated with 1 ul of solubilized zona pellucida to induce acrosome reaction. After 30 minutes of incubation at 37°C in a CO₂ incubator, the sperm were coated on slides. FITC-labeled Pisum sativum lectin was used as a probe to estimate the percentage of spermatozoa with intact sperm acrosome (Cross et al., 1986).

To test the effect of selected monoclonal antibodies on the induced AR, monoclonal antibody of known dilution was mixed with

the sperm solution at a ratio of 1:10 for 30 minutes prior to the treatment of solubilized zona pellucida. Incubation with unrelated antibodies served as the negative control in the experiment.

6. IN VIVO MATING EXPERIMENTS

Female mice were superovulated with 5 IU of PMSG on day 1 between 1 and 2 pm. Heat-inactivated ascites fluid containing a known amount of given monoclonal sperm antibody was injected intraperitoneally between 9 and 10 am on days 2 and 3. Five IU of hCG was administered between 1 and 2 pm on day 3 followed by mating with proven fertile males. In the morning of day 4, the vaginal plugs of females were checked for evidence of mating. In the morning of day 5, antibody-treated female mice were sacrificed, and percentages of recovered embryos in the two-cell stage were recorded.

7. STATISTICAL ANALYSIS

Student's t test or Chi-square test was used for the statistical comparison between control and experimental groups. Differences were considered significant when $p < 0.05$.

E. METHODOLOGY OF MOLECULAR BIOLOGY

1. MATERIALS

Bacterial culture supplies and media were purchased from Difco (Burlington, Ontario, Canada). Kiolbase DNA sequencing kit and Molecular biological supplies were from Gibco/BRL (Burlington, Ontario, Canada). ^{125}I (1 mCi/mmol), ^{32}P -dCTP (1 mCi/mmol) and ^{35}S -dATP were from Amersham (Oakville, Ontario, Canada). The GENE CLEAN kit was from BIO 101 Inc. (La Jolla, CA). Forward and reverse sequencing primers were from Promega (Madison, WI). The chromosome panel blot was from BIOS (New Haven, CT).

2. BACTERIA STRAIN

E. coli of strain Y1090 was used for immunoscreening of mouse testis cDNA library constructed with Lambda gt 11 expression vector. Strain Y1089 was used for the production of recombinant fusion protein. Strain DH5 was used for DNA subcloning and sequencing.

3. DNA ISOLATION

(The formular of solutions are summarized in Appendix 7)

a. TISSUE DNA PREPARATION

High molecular-weight cellular DNA from tissues was extracted by using the phenol and chloroform method (Maniatis et al., 1982). Briefly, 1 gm of tissue sample was homogenized in Polytron in 5 ml of Tris-Na-EDTA buffer. Protease K (100 ug/ ml) and 0.5 % SDS were then added to the tissue homogenate. The mixture was incubated at 50°C for 3 hours. After incubation, the digested homogenate was extracted three times with an equal volume of phenol and once with choloform. The extracted DNA was then precipitated by adding one half volume of 7.5 M ammonium acetate and 2.5 volumes of 95 % ethanol. After centrifugation, the DNA was dissolved in Tris-EDTA buffer. The concentration of DNA was determined spectrophotometrically at absorbance of 260 nm. The quality of DNA was evaluated by agarose gel electrophoresis.

b. BACTERIOPHAGE DNA PREPARATION

(The formular of solutions are summarized in Appendix 8)

Bacteriophage lambda DNA was prepared in small scale according to the plate lysate method (Maniatis et al., 1982). Briefly, a single, well-isolated plaque was picked and transferred into 1 ml SM buffer by using a pasteur pipette. After

vortexing, 100 μ l of bacteriophage suspension was mixed with 100 μ l of E. coli cells and incubated at 37°C for 20 minutes. Three ml of molten top agarose (0.7 %) which was kept at 50°C was added to the mixture of bacteriophage and bacteria cells. It was spread onto the surface of a 85-mm plate containing 30 ml of Luria-Bertani (LB) medium with 1.5 % agarose. The plate was inverted and incubated at 37°C for 12 to 14 hours until the plaques covered almost the entire surface of the plate. Five ml of SM buffer was added onto the plate and shaken for 3 hours at room temperature to elute the bacteriophages from the top agarose. The washed SM buffer was transferred to a centrifuge tube. The bacterial debris was removed by centrifugation at 8,000 X g for 10 minutes at 4°C. RNase A and DNase I were added to a final concentration of 1 μ g/ml to the supernatant. After a 30 minute incubation at 37°C, an equal volume of a solution containing 20 % (w/v) polyethylene glycol (PEG 8000) and 2 M NaCl in SM buffer was added to bacteriophage solution and incubated at 0°C for one hour. The precipitated bacteriophage particles were recovered by centrifugation at 10,000 X g for 20 minutes at 4°C. After removal of the supernatant, the tube was inverted on a paper towel to drain away remaining liquid. The bacteriophage was resuspended by adding 0.5 ml of SM buffer and shaken vigorously

with a vortex meter. The bacteriophage solution was centrifuged at 8,000 X g for 2 minutes at 4°C. to remove debris. The supernatant was transferred to a fresh Eppendorf tube, 5 ul each of 10 % SDS and 0.5 M EDTA (pH 8.0) solutions were added. After incubation at 68°C for 15 minutes, the supernatant was extracted sequentially with phenol, phenol/chloroform (1:1), and then with chloroform. An equal volume of isopropanol was added to the final aqueous phase to precipitate bacteriophage DNA. After storing at -70°C for 20 minutes, the solution was thawed and centrifuged in an Eppendorf centrifuge for 15 minutes at 4°C. The pellet was washed with 70 % ethanol, dried and resuspended in 50 ul of TE buffer.

The extracted bacteriophage DNA was analyzed by restriction enzymes digestion followed by agarose gel electrophoresis.

c. PLASMID DNA PREPARATION (Maniatis et al., 1982)

(The formular of solutions are summarized in Appendix 9)

One single well-isolated colony was picked from the plate and seeded in 10 ml of LB medium for overnight incubation at 37°C (with shaking). 10 ml of the well grown preculture medium was then poured into a 500 ml of LB medium followed by overnight incubation at 37°C. The bacterial cells were harvested by

centrifugation at 4,000 X g for 10 minutes at 4°C. The cell pellet was washed with dH₂O followed by additional centrifugation. The bacterial pellet was then resuspended in 10 ml of solution I containing 5 mg/ml lysozyme and incubated for 5 minutes at room temperature. 20 ml of freshly prepared solution II was added. After an additional 10 minute incubation in an ice bath, 15 ml of ice-cold solution III was added and mixed thoroughly for 10 minute on ice. The precipitated pellet was removed by centrifugation at 4,000 X g for 20 minutes at 4°C. The supernatant was transferred to a fresh tube, the plasmid DNA was precipitated by adding two volumes of 95 % ethanol and placed at -70°C for 15 minutes. The DNA pellet was collected by centrifugation at 10,000 X g for 10 minutes at 4°C and then dried under vacuum. The dry DNA pellet was dissolved in 2 ml of dH₂O and incubated with RNase A at a concentration of 200 ug/ml. After incubation at 37°C for 1 hour, the DNA solution was extracted twice with phenol and once with chloroform. Finally, it was precipitated by adding 1/10 volume of 7.5 M ammonium acetate and 2.5 volumes of 95 % ethanol. The quality and quantity of purified plasmid was determined by restriction enzymes digestion followed by agarose gel electrophoresis.

4. RNA ISOLATION

(The formular of solutions are summarized in Appendix 10)

An extraction method involving the use of acid guanidinium thiocyanate, phenol and chloroform was used for total RNA isolation (Chomczynski and Sacchi, 1987). Briefly, 1 gm of fresh or frozen tissue (stored in a liquid nitrogen tank) was minced on ice and homogenized with 10 ml of solution D in a glass-teflon homogenizer and subsequently transferred to a polypropylene tube. A solution containing 1 ml of 2 M sodium acetate, pH 4, 10 ml of phenol (water saturated), and 2 ml of chloroform-isoamyl alcohol (49:1) was added to the homogenate with thorough mixing. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 minutes. The suspension was then centrifuged at 10,000 g for 20 minutes at 4°C. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube followed by mixing with an equal volume of isopropanol, and then placed at -20°C for 1 hour to precipitate RNA. After centrifugation, the RNA pellet was dissolved in 3 ml of solution D (see Appendix 10) and precipitated with 1 volume of isopropanol. After washing with 75 % ethanol, the RNA pellet was vacuum dried and then dissolved in

0.5 ml of 0.5 % SDS. The solution was then incubated at 65°C for 10 minutes. The concentration and quality of extracted RNA were determined spectrophotometrically by absorbance at 260 nm and 280 nm and agarose gel electrophoresis, respectively.

5. NICK TRANSLATION OF DNA (Maniatis et al., 1982)

(The formular of solutions are summarized in Appendix 11)

The cDNA insert was excised from a vector by restriction enzyme digestion. The fragment was purified on an agarose gel by using a GENECLAN kit. The purified DNA fragment (0.5 ug) was mixed with a solution containing 6 ul of deoxyribonucleoside triphosphate (dNTP) mixture, 2 ul of 10 X nick translation buffer, 8 ul of ^{32}P labeled α -dCTP and 4 ul of dH_2O . After the addition of 1 ul of 100 ng/ml DNase I and 10 units of E. coli DNA polymerase I, the mixture was first incubated at 37°C for 10 minutes and then at 15°C for 2 hours. During incubation, 1 ml of Sephadex G50 was packed in a 1 ml syringe and was washed with STE buffer. The packed column was centrifuged at 1600 X g for 4 minutes for washing and separating the free nucleotide. After incubation, 2 ul of 10 % SDS and 2 ul of 0.5 M EDTA were added to the mixture to stop the reaction. Following the addition of 5 ul

of yeast tRNA (10 mg/ml) and 180 ul of STE buffer, the mixture in a final volume of 200 ul was applied to a Sephadex G-50 column which was then centrifuged. The eluted DNA solution was collected in a 1.5 ml Eppendorf tube. The radioactivity of labeled DNA was detected by a beta scintillation counter. The specific activity was presented in cpm/ug DNA.

6. SOUTHERN BLOT HYBRIDIZATION

(The formular of solutions are summarized in Appendix 12)

a. SOUTHERN TRANSFER

Southern blot analyses of the DNA were performed according to the standard procedures (Southern, 1975). After size-fractionation in agarose gel electrophoresis, the DNA on the gel was denatured in a solution containing 0.5 M NaOH and 1.5 M NaCl for 1 hour and then neutralized in 1 M Tris-HCl pH 8.0 containing 1.5 M NaCl for 1 hour with smooth shaking. Subsequently, the DNA was transferred by pressure to a nylon filter with a transfer buffer (20 X SSC) and then immobilized by baking at 80°C for 2 hours or by UV light (Khandjian, 1987) for 2 minutes. The dry filters were stored in a sealed bag at room temperature.

b. HYBRIDIZATION

The blotted filter was prehybridized in a hybridization solution at 65°C for 30 minutes and hybridized in the same solution with a ³²P-labeled DNA probe overnight at 65°C. After hybridization, the hybridized filter was washed twice in 2 X SSC containing 0.5 % SDS at room temperature for 15 minutes and then once at 50°C for 30 minutes. The blot was then air dried and autoradiographed by using intensifying screens at -70°C.

7. NORTHERN BLOT HYBRIDIZATION

Total RNA from tissues was isolated, denatured by glyoxalation and size-fractionated in 1.2 % agarose gel electrophoresis (Varmus et al., 1981, Maniatis et al., 1982). The RNA was transferred by pressure to nylon filters and hybridized with a ³²P-labeled DNA probe as previously described.

8. IMMUNOSCREENING OF LAMBDA GT 11 cDNA LIBRARY

(The formular of solutions are summarized in Appendix 13)

a. AMPLIFICATION OF cDNA LIBRARY

The mouse testis cDNA library was obtained from Dr. Y.F. Lau's lab at the University of California, San Francisco, and was constructed in a bacteriophage expression vector, lambda gt11 by using purified poly (A)⁺ mRNA from the testes of 3-month old

BALB/C mice. The mouse testis cDNA library was in SM buffer and the titre of the library was determined by a serial dilution of the library which was plated on LB plates as described previously. For the amplification of the library, 10^5 recombinant phages were plated on a 150 mm LB plate. After incubation overnight at 37°C , 10 ml of SM buffer was added to resuspend the phage by shaking at room temperature for 2 hours. The titre of resuspended cDNA library was redetermined prior to use.

b. PREPARATION OF POLYCLONAL ANTISERA

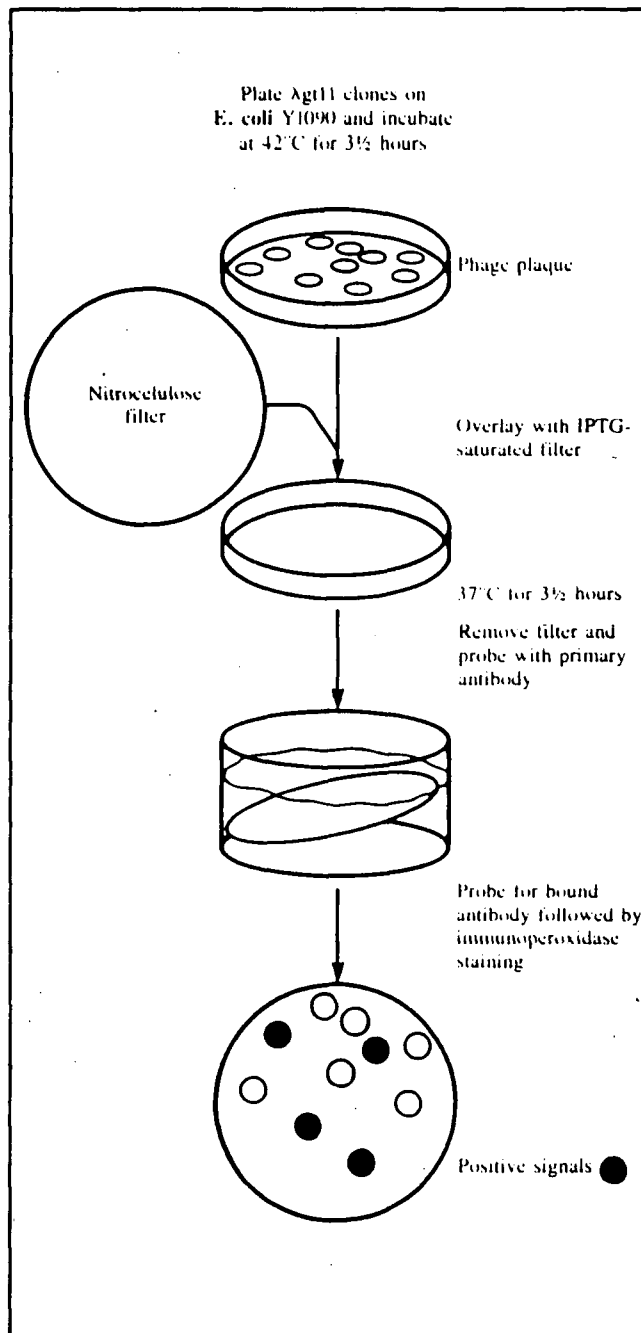
Five ml of rabbit anti-MSA-63 sera were precipitated with 50 % (w/v) ammonium sulfate. After centrifugation, the protein pellet was dissolved in 5 ml of TBS and dialyzed against TBS overnight. The dialyzed antibody solution was used for immunoscreening of the cDNA library.

c. PROCEDURES OF IMMUNOSCREENING OF MOUSE TESTIS cDNA LIBRARY

Immunoscreening was performed according to the instruction of the Clontech Lambda gt 11 Immunoscreening Kit with some modifications. The standard protocol of immunoscreening is presented in Figure 1. Briefly, a single, isolated colony of *E. coli* Y1090 was picked and grown to saturation in LB broth

containing 0.2 % maltose at 37°C with good aeration. 0.2 ml of the Y1090 culture and 0.1 ml of cDNA library containing 0.5×10^5 recombinant phage were mixed and incubated at 37°C for 15 minutes. After the addition of 7.5 ml of molten LB soft agar, the culture was mixed and poured onto a 150 mm LB plate and then incubated at 42°C for 3.5 hours. The cDNA library was poured onto twelve 150 mm LB plates for immunoscreening. After incubation, the plates were transferred to a 37°C incubator and a dry nitrocellulose filter which was previously saturated with 10 mM IPTG was overlayed to each plate and incubated for 3.5 hours. The filters were then removed from the plates and incubated in Tris buffered saline with Tween 20 containing 20 % fetal calf serum for 30 minutes at room temperature for blocking. Rabbit anti-MSA-63 sera at a concentration of 2 ul/ 10 ml TBST was added to filters and incubated overnight at room temperature. After three washes of TBST, these filters were incubated with biotinylated goat anti-rabbit IgG antibody at a concentration of 20 ul/ 10 ml TBST for 30 minutes. The filters were washed by TBST and incubated with avidin-biotinylated horseradish peroxidase complex for 30 minutes. The filters were then washed by TBS (without Tween 20) and incubated in a peroxidase substrate solution for 30 minutes to develop the color. The positive plaques were

Figure 1. Protocol of immunoscreening of cDNA library with antibody probe. (Modified from instruction of Clontech immunoscreening Kit)



identified and picked for a second screening by the same method.

d. IDENTIFICATION OF POSITIVE CLONES

The DNA of positive recombinant phage clones was extracted according to the method described in section E.3.b., and digested by EcoRI restriction enzyme. After the agarose gel electrophoresis, the DNA was transferred to a nitrocellulose filter paper by Southern blotting and hybridized by using one of the cDNA inserts as a probe. Two cDNA inserts were probes for the hybridization. The longest cDNA insert which cross-reacted with the others was selected for subcloning and for further analysis.

9. SUBCLONING OF DNA INSERTS

a. PREPARATION OF RECOMBINANT PLASMID DNA FROM RECOMBINANT BACTERIOPHAGE

The positive clones which were identified from the bacteriophage cDNA library were subcloned into a pUC18 plasmid vector for the further restriction analysis and DNA sequencing.

The DNA insert from the recombinant bacteriophage DNA was completely digested by EcoR I and recovered from the agarose gel using the GENECLEAN kit. The isolated DNA insert was then ligated

with the pUC18 vector which was digested by EcoRI. The DNA ratio of inserted DNA to plasmid was about 1 to 10. After the completion of ligation, the recombinant plasmid was ready to be transferred to the bacterial host.

b. E. coli TRANSFORMATION

To prepare competent cells for transformation, a single colony of host strain DH5 was incubated with 10 ml of LB at 37°C overnight with constant shaking. One ml of the cell culture was added to 50 ml of LB and incubated for 2 to 3 hours until the cell density reached an absorbance of 0.6 at 550 nm. The culture was chilled on ice for 5 minutes and harvested by centrifugation for 5 minutes at 2,500 X g at 4°C. The cell pellet was resuspended in 18 ml of 100 mM CaCl₂ and kept for 15 minutes on ice. After additional centrifugation, the cell pellet was resuspended in 2 ml of 100 mM CaCl₂ and kept for 60 minutes at 4°C. After the above preparation, the cells were ready for DNA transformation. In a sterile Eppendorf tube, 10 ul of ligated recombinant plasmid was mixed with 200 ul of competent cells for 30 minutes at 4°C. The Eppendorf tube containing the mixture was transferred to a 42°C water bath for 2 minutes to heat-shock the cells and then transferred to a 37°C water bath for 1 hour

shaking in the presence of 1 ml of LB medium to allow expression of the antibiotic resistance gene. One hundred ul of the cell mixture was spread over the surface of an LB/agar/ampicillin plate (85 mm) for overnight culture. The positive colony was selected by colony hybridization with a probe of ^{32}P labeled insert DNA.

10. DNA SEQUENCING

The dideoxy sequencing method by means of denatured plasmid templates was used for the DNA sequence analysis (Hattori and Sakaki, 1986).

a. PREPARATION AND ALKALINE DENATURATION OF PLASMID DNA

The EcoRI digested cDNA insert was subcloned to the plasmid pUC18 and this recombinant plasmid was extracted according to the method previously described. Eighteen ul of purified plasmid DNA solution (0.2 ug/ul) was mixed with 2 ul of 2 N NaOH and kept at room temperature for 5 minutes. Then, 8 ul of 5 M ammonium acetate (pH 7.4) was added and the denatured DNA was precipitated by the addition of 100 ul of ethanol and placed at -70°C for 5 minutes. The precipitate was harvested by centrifugation at 12,000 rpm for 5 minutes, rinsed once with 70 % ethanol, and

dried under vacuum. The denatured plasmid DNA pellet was dissolved in 6.5 ul of dH₂O before use.

b. Enzyme Reaction for Sequencing

Enzyme reactions for sequencing including template-primer annealing and labeling-extension reactions were described in the instruction manual of the KiloBase Sequencing System (BRL). Briefly, the mixture of 6.5 ul of denature plasmid (5 ug), 1.5 ul of primer (0.5 pmol) and 2 ul of KiloBase Sequencing Buffer (10 X) was heated to 65°C for 10 minutes. The mixture was allowed to cool slowly to room temperature (about 30 minutes). After the temperature equilibration, 2 ul of diluted (1X) KiloBase Extension Mix, 1 ul of 1 M dithiothreitol, 1 ul ³⁵S-labeled - dATP and water were added to the mixture of denatured plasmid. After incubation for 5 minutes at 37°C, 1 ul of the large fragment of DNA polymerase I (1.5 units/ul) was added with an additional incubation at 37°C for 5 minutes. 3.5 ul of reaction mixture was then transferred respectively to each of the four tubes containing Kilobase Termination Mixes-A, -C, -G, and -T. After an incubation period of 5 minutes at 37°C, the reaction was stopped by adding 4 ul of dideoxy stop buffer. Finally, the reaction mixture was denatured by heating the tubes at 80°C for 5

minutes followed by a rapid transfer to wet ice.

c. SEQUENCING

The sequencing gel (38 X 50 cm, 0.4 mm) was prepared according to the method described in the instruction manual of Sequi-Gen Nucleic Acid Sequencing Cell (BIO-RAD). Three μ l of each reaction solution was applied to one lane of the sequencing gel. After the gel had been run at a constant voltage of 2000 V for 2.5 hours, the gel was fixed in a solution containing 10 % methanol and 10 % acetic acid for 15 minutes. Then, the fixed gel was dried at 80°C for 1 hour by using a gel dryer under vacuum. Finally, the dried gel was placed in a cassette with X-ray film for autoradiography. The result of the sequence was analyzed by using PC-GENE computer program.

11. PRODUCTION OF RECOMBINANT FUSION PROTEIN

Crude lysates of E. coli strain Y1089 which expressed MSA-63 fusion protein were prepared according to the published procedures (Snyder et al, 1987).

a. LYSOGENIC STRAIN PREPARATION

The saturated culture of Y1089 cells in a LB medium

containing 0.2 % maltose and 50 ug/ml of ampicillin was infected with MSA-63 recombinant phage clones using an m.o.i. (multiplicity of infection) equal to 10 for 20 minutes at room temperature. Two hundred cells were plated per LB plate and incubated overnight at 30°C. The individual colonies were picked with a toothpick and tested for growth at 42°C and 30°C. The lysogens selection was based on their growth at 30°C, but no growth at 42°C.

b. PREPARATION OF FUSION PROTEIN

Lysogens were grown in LB medium with vigorous shaking at 30°C until an absorbance of 0.4 at 600 nm. Lysogens were induced by shifting the temperature to 44°C for 15 minutes with vigorous shaking. IPTG was then added to the culture to a final concentration of 10 mM. After incubation at 37°C for 20 minutes, the cells were harvested as quickly as possible by centrifugation at 10,000g for 30 seconds and the cell pellet was frozen immediately at -70°C. For SDS-PAGE, SDS sample buffer was added just prior to freezing. For the radioimmunosorbent inhibition assay, the harvested cell pellet was sonicated and incubated immediately with I¹²⁵ labeled HS-63 containing 2 mM PMSF.

For the isolation of MSA-63 recombinant fusion protein, the LB medium containing lysed lysogens was concentrated to a small

volume by using Amicon pressure cell and applied to a Sephacryl S-300 column. The eluent which had a molecular size of greater than 100 Kd was collected and used to immunize rabbits for the production of polyclonal antisera.

12. SCREENING OF cDNA LIBRARY BY DNA PROBE

The mouse testis cDNA library was plated on a 15 mm LB agar plate at a density of 5×10^4 plaques and grown overnight at 37°C. The plate was then chilled at 4°C for 1 hour to allow the top agar layer to harden. A dry nylon filter was placed onto the surface of the top agar for 1 minute at room temperature to absorb the recombinant plaques. After removing from the plate, the filter was placed in a denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 minute, and then transferred into neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 minutes, and air dried. After the filter was dried, DNA was fixed to the filter by baking or UV light for the hybridization as described previously.

F. IMMUNOHISTOCHEMISTRY

For the preparation of frozen testicular sections, testes were removed from male mice of different ages, (from birth to maturity) and then frozen immediately in liquid nitrogen. Subsequently, they were placed in an embedding medium (Tissue TekII, O.C.T. Compound No. 4583, Lab-Tek) at -60°C . Sections of 10 μm thickness were cut and then placed on slides, air dried, fixed in 100 % methanol, and stored at -20°C .

The indirect immunofluorescent assay was used for immunohistological studies of tissue sections according to the procedures described previously (Lee and Wong, 1986b).

III. RESULTS

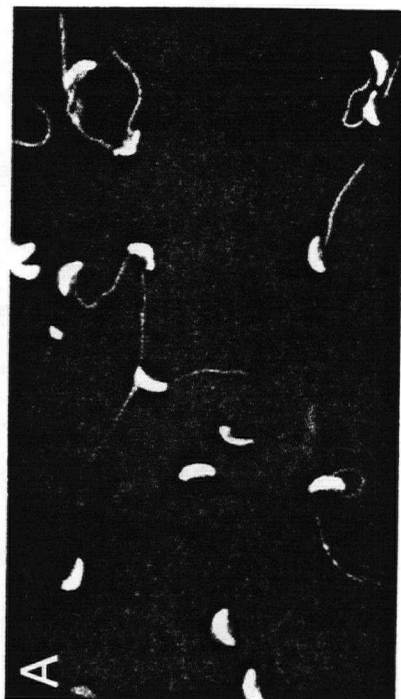
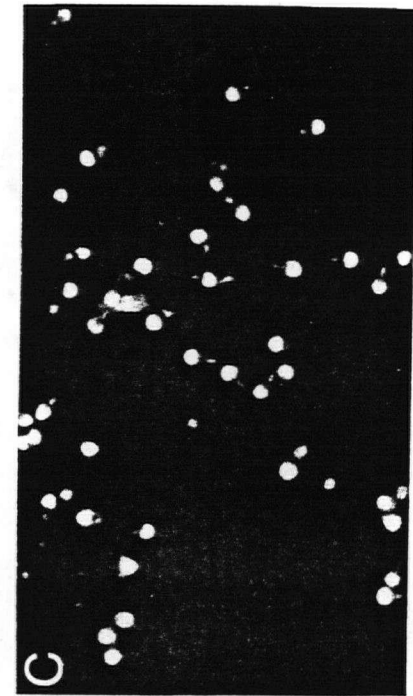
Part I. CHARACTERIZATION OF HS-63 MONOCLONAL ANTIBODY AND EVALUATION OF ITS CORRESPONDING SPERM ANTIGENS

A. LOCALIZATION OF SA-63 ANTIGEN IN SPERM

By using indirect immunofluorescent assay, HS-63 monoclonal antibody was shown to react with the acrosome of sperm among different mammalian species. The indirect immunofluorescent staining patterns of HS-63 to mouse and human sperm are presented in Figure 2. Both air-dried and methanol-fixed acrosome-intact sperm were stained positively in the acrosomal region upon incubation with this antibody.

Reactivity of HS-63 to live sperm was also examined by direct immunofluorescent analysis. Percent immunofluorescent staining of freshly prepared live mouse sperm from cauda epididymidis and vas deferens were found to increase dramatically with time upon 30 minutes incubation with FITC-labeled HS-63. However, FITC-labeled HS-63 was found to have little or nonstaining with freshly prepared live sperm which were fixed with glutaraldehyde.

Figure 2. Indirect immunofluorescent staining of mouse (A, B) and human (C, D) sperm by using HS-63 monoclonal antibody. Sperm under UV light are presented in (A, C) and sperm under visible light are given in (B, D) (X 400).



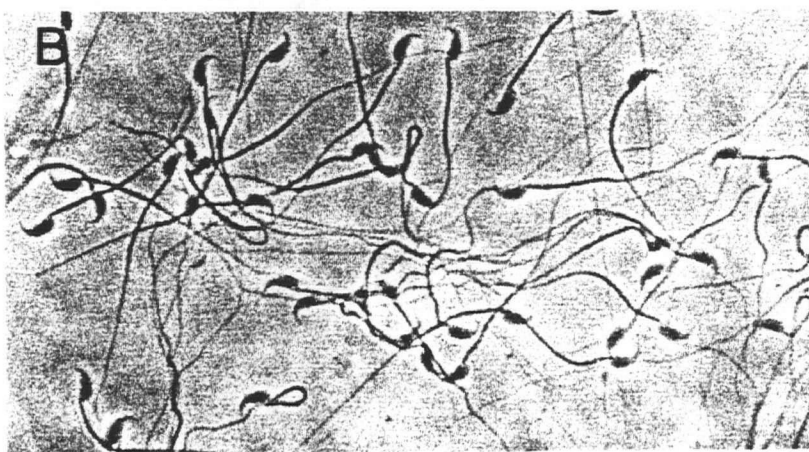
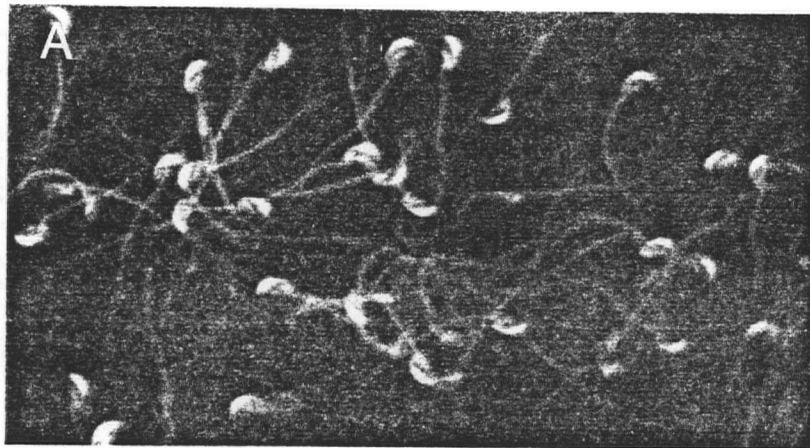
On the other hand, upon 1 hour incubation of live sperm in the BWB medium for capacitation, glutaldehyde-fixed capacitated sperm were stained positively with FITC-labeled HS-63. The immunofluorescent staining pattern of capacitated mouse sperm upon incubation with HS-63 is presented in Figure 3.

When freshly prepared sperm were washed with 0.5 M KCl or 0.1 % Triton X-100, they were readily stained with the FITC-labeled HS-63 (Menge et al., 1987). Following incubation of sperm with calcium ionophore A-23187 to induce acrosome reaction, the acrosome-reacted sperm could no longer be stained with FITC-labeled HS-63.

B. EPITOPE EVALUATION OF HS-63

By using HRP-labeled HS-63 as a probe, the immunoactivity of the antigen in mouse testis homogenate was not significantly decreased following the treatment with Triton X-100 or NP-40. However, no HS-63 immunoactivity was detected when the testis homogenate was prepared in the presence of SDS.

Figure 3. Immunofluorescent staining of capacitated mouse sperm by using FITC-labeled HS-63. Sperm under UV light are presented in (A) and sperm under visible light are given in (B) (X 400).



Treatments with proteolytic enzymes, such as trypsin and pronase, were shown to completely abolish the immunoactivity of the antigen in mouse testis homogenate. However, incubations of testis homogenate with sodium periodate and neuraminidase which either modifies or removes carbohydrate moiety of glycoproteins have little effect to the immunoactivity of the antigen.

C. EVALUATION OF HS-63 ON SPERM FUNCTION (Liu et al., 1989)

1. IN VITRO FERTILIZATION OF MOUSE OOCYTES

Because of the cross-reactivity of HS-63 to sperm of various mammalian species, the inhibitory effect of HS-63 on fertilization was evaluated by using mouse in vitro fertilization experiment. The heat-inactivated ascites fluid containing HS-63 monoclonal antibody (antibody titre > 1:100,000) showed significant inhibitory effect on the fertilization of mouse oocytes at a dilution of 1:10 (Fertilization rate of 24.6 % vs. 57.7 % for the control, $P < 0.001$). The results of this study are presented in Table 1. It was observed during this experiment that HS-63 neither had an effect on sperm motility nor caused any sperm agglutination.

Table 1. Inhibitory effect of HS-63 monoclonal antibody on in vitro fertilization of mouse oocytes.

Antibodies Added	Number of assays	Ova examined (No.)	Ova fertilized (No.)	Fertilization rate (%)	P ^c value
HS-63 ^a	2	57	14	24.6	
Control ^b	2	45	26	57.7	<0.001

^aThe titre of HS-63 was determined by indirect immunofluorescent assay to be 1:100,000 in ascites fluid.

^bAscites fluid derived from hybrid cells secreting unrelated antibodies was used as the control.

^cCompared with the control group.

To evaluate the dose-dependent inhibitory effect of HS-63 on fertilization of mouse oocytes in vitro, purified HS-63 monoclonal antibody of different concentrations was added to sperm-egg incubation mixture. When the concentration of HS-63 in the incubation mixture is higher than 0.2 mg/ml, the fertilization inhibition by the antibody was found to be significant ($P < 0.05$). The inhibitory effect of HS-63 on fertilization was found to decrease gradually with decreasing antibody concentration (from 0.2 to 0.0016 mg/ml). At the antibody concentration lower than 0.04 mg/ml, no significant inhibitory effect was found on fertilization (fertilization rate of 45.6 % vs. 52.9 % for the control group). The results of this dose-dependent assay are presented in Table 2.

Table 2. Inhibitory effect of purified HS-63 on in vitro fertilization of mouse oocytes.

Concentration (mg/ml)	Titer ^a	Ova examined (No.)	Ova fertilized (No.)	Fertilization rate (%)	P Value ^b
1	1:200,000	19	4	21	<0.05
0.2	1: 40,000	55	13	23.6	<0.05
0.04	1: 8,000	46	21	45.6	NS
0.008	1: 2,000	37	15	40.5	NS
0.0016	-	54	28	51.8	NS
0 (control)	-	17	9	52.9	

^aThe titre of antibody was determined by indirect immunofluorescent assay.

^bCompared with the control group. NS: not significant

2. HUMAN SPERM PENETRATION ASSAY

In human sperm penetration assay, the dose-dependent inhibitory effect of HS-63 on human sperm penetration of zona-free hamster eggs was evaluated. In the presence of 1 mg/ml of HS-63, the penetration rate was significantly reduced as compared to that of the control. The presence of this antibody had not only reduced the mean number of swollen sperm head per egg (1.3 ± 1.2 vs. 4.2 ± 2.1 for control group, $P < 0.0001$) but also that of the sperm attached to each egg (6.1 ± 6.3 vs. 63.3 ± 21.6 for control group, $P < 0.0001$). The results of this study are presented in Table 3. Similar to mouse in vitro fertilization, the penetration rate and sperm attachment in this assay were found to decrease gradually with increasing antibody concentration. Little or no effect was observed at an antibody concentration lower than 0.04 mg/ml.

Table 3. Inhibitory effect of purified HS-63 on human sperm penetration of zona-free hamster ova.

Antibody Concentration (mg/ml)	Ova examined (No.)	Fertilization rate (%)	Swollen sperm ^a head (Avg.No./Egg)	Attached sperm (Avg.No./Egg)	P value ^b
1	19	73.6	1.3 \pm 1.2	6.1 \pm 6.3	<0.0001
0.2	18	100	2.7 \pm 1.1	16.1 \pm 10.5	<0.0001
0.04	16	100	4.8 \pm 2.2	70.5 \pm 32.5	NS
0.008	14	100	3.6 \pm 0.8	79.0 \pm 37.0	NS
0 (control)	16	100	4.2 \pm 2.1	63.3 \pm 21.6	

^aAverage number of swollen sperm head per egg = Sperm penetration rate.

^bCompared with the control group. NS: not significant

3. THE EFFECT OF HS-63 ON INDUCED ACROSOME REACTION

The acid solubilized mouse zona protein is known to induce sperm acrosome reaction in a time- and dose-dependent manner (Saling et al., 1986). The effect of HS-63 monoclonal antibody on the zona-induced acrosome reaction was evaluated. It was clearly demonstrated that the percentage of acrosome-reacted mouse sperm was significantly decreased in the presence of HS-63 as compared to that of the control. In a typical experiment, upon 30 minutes incubation with HS-63 (1:10 dilution of heated-inactivated ascites fluid) and solubilized zona, the percentage of acrosome-reacted sperm was 10.7 % as compared to 38.8 % in the control.

4. IN VIVO MATING EXPERIMENT BY PASSIVE IMMUNIZATION IN MOUSE

For comparative purposes, in vivo mating experiments were also performed to evaluate the antifertility effect of HS-63 in mice. The heat-inactivated ascites fluid containing high titres of HS-63 monoclonal antibody (>1:100,000) was injected intraperitoneally into female mice to study their antifertility effects. Unrelated ascites fluid was used as the negative control. In this experiment, ascites fluids containing MS-204 or

MS-207 monoclonal antisperm antibody served as the positive controls. Previous studies in our laboratory (Lee and Wong, 1986a) have shown that these two monoclonal antibodies were mouse sperm specific and capable of inhibiting both in vitro and in vivo fertilization. Results are tabulated in percentages of two-cell embryos recovered in a given mating experiment as the apparent fertilization rates (Table 4). Statistically, the inhibitory effect of HS-63 on the in vivo fertilization of passively immunized mice was not significant (Percentage of two-cell embryos being 87.2 ± 17.3 vs. 97.0 ± 0.05 for the control). As determined by the indirect immunofluorescent assay, the titre of HS-63 in the passively immunized mice was found to be about 1:50,000 to 1:100,000 in the mouse sera, but only 1:2,500 in the fluid of oviduct. In the positive controls, the ascites fluids of MS-204 and MS-207 were shown to inhibit the mouse in vivo fertilization significantly as judged from the relatively low recovery rates of two-cell embryos as compared to the negative control (48.5 ± 37.5 and 59.5 ± 31.6 , respectively vs. 97.0 ± 0.05)

Table 4. Effects of antibodies on the in vivo fertilization of mouse oocytes following passive immunization and mating experiments

Antibodies ^a	No. of Mice	Percentage of two cell ^b embryos (%)	P value ^c
control	12	97 ± 5.9	
HS-63	8	87 ± 17.3	
Rabbit anti- MSA-63	8	67 ± 29.4	<0.05
MS-204	10	48 ± 37.5	<0.001
MS-207	9	59 ± 31.6	<0.01

^aAscites fluids and antisera were heat-inactivated at 56°C for 30 minutes prior to intraperitoneal injection.

^bPercentage of two cell embryos
= number of two cell embryos / total ova recovered.

^cCompared with the control group. NS: not significant

Part II. PURIFICATION AND CHARACTERIZATION OF MSA-63 AND

EVALUATION OF ANTIFERTILITY EFFECTS BY ITS ISOIMMUNE SERA

A. PURIFICATION OF HS-63 SPECIFIC ANTIGEN FROM MOUSE TESTES

(MSA-63) (Liu et al., 1989)

1. DETERMINATION OF SUBCELLULAR DISTRIBUTION OF MSA-63

To determine if the antigen reacting with HS-63 was a soluble or integral membrane proteins, mouse testes were homogenized in an isotonic PBS containing 1 mM PMSF in the absence of detergent. The soluble protein in the supernatant and the integral membrane protein in the pellet were separated by centrifugation. The majority of HS-63-reactive antigen was detected in the soluble fraction when analyzed by indirect immunofluorescent inhibition assay. Further extraction of the pellet (membrane fraction) with the same buffer resulted in a release of more active antigen. After several extractions with the same buffer, the antigen in the membrane fraction could be completely removed. However, the immunoactivity of the antigen in the soluble fraction was not removed by passing through a 0.22 μ m Millipore filter membrane.

2. PURIFICATION OF MSA-63 ANTIGEN

Since the immunoactivity of antigen reactive to HS-63 was shown to be in the soluble fraction of mouse testes homogenate, ammonium sulfate fractionation followed by DEAE ion exchange chromatography were used to purify the corresponding antigen (MSA-63) from mouse testes. The immunoactivity of antigen in each purification step was measured by indirect immunofluorescent inhibition assay. During ammonium sulfate fractionation, the immunoactivity of antigen was precipitated in the presence of 400 mg/ml ammonium sulfate. After dialysis, the fractionated MSA-63 was then applied to a DEAE-cellulose column. The elution profiles of protein and immunoactivity of this antigen in a salt gradient (0 - 1.0 M NaCl) were shown in Figure 4. The highest immunoactivity was eluted at 0.38 M of NaCl. Immunoaffinity chromatography was used as the last step for the purification. The binding and washing conditions between the antigen and immobilized HS-63 antibody were studied prior to the antigen elution. PBS containing 0.5 M NaCl was finally selected as a washing solution to remove all the nonspecifically bound proteins. Results for the purification of MSA-63 from mouse testis were summarized in Table 5. By using the procedures

Figure 4. Fractionation of the soluble supernatant of crude mouse testes homogenate by DEAE-cellulose chromatography. The heavy solid line represents NaCl salt gradient (0-1.0 M). (—) and (----) indicate the protein concentration and relative immunoactivity of antigen, respectively.

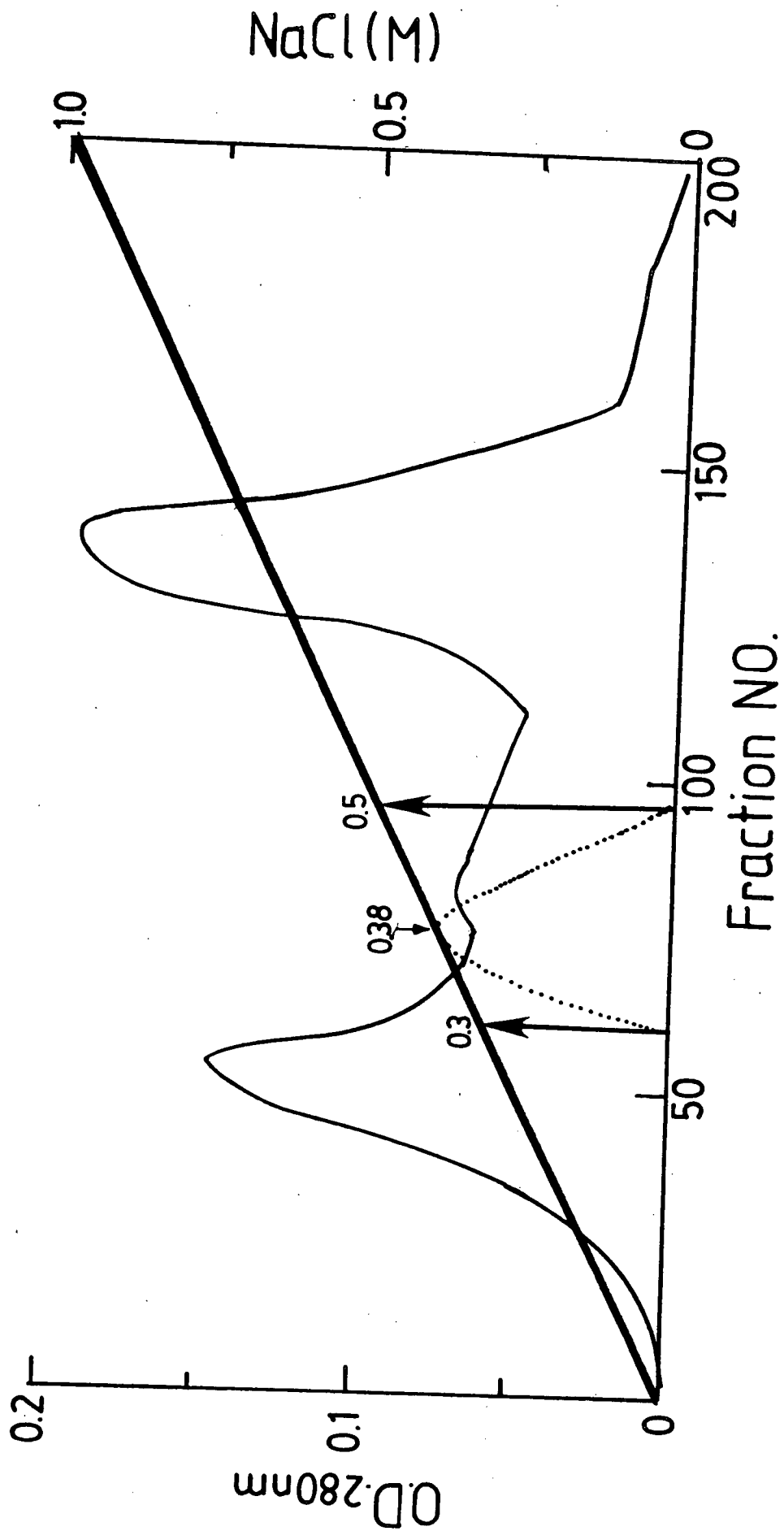


Table 5. Purification of MSA-63 from Mouse Testis by Two Purification Procedures

	Total volume (ml)	Protein concentration (mg/ml)	Specific ^a activity	Total activity	Yield (%)
I. Soluble Extraction					
Supernatant of homogenates	70	26.6	0.6	1117	100
Ammonium sulfate precipitation	60	13.2	1.2	950	85
DEAE ion exchange	200	0.7	2.8	392	35
Immunoaffinity chromatography	1.3	0.23	600	233	21
II. Detergent Extraction					
Supernatant of homogenate	100	100	0.4	4000	100
Immunoaffinity chromatography	1	0.5	320	160	4

^aSpecific immunoactivity of MSA-63 was determined by the indirect immunofluorescent inhibition assay as described in the section of Materials and Methods (Liu et al., 1989).

described, more than a one thousand fold protein purification was achieved. On the average, about 0.3 mg of purified antigen could be obtained from 10 g of mouse testes.

When stored at -20°C , precipitation was observed in the purified MSA-63 antigen preparation. In order to improve the solubility of MSA-63 antigen, Triton X-100 was used during the antigen purification. The crude testes homogenate was first solubilized with 2 % Triton X-100. Following centrifugation, the soluble supernatant was applied directly to the HS-63 immunoaffinity column. The immunoactivity of the purified MSA-63 was also detected by using indirect immunofluorescent inhibition assay. Results of the purification of MSA-63 from mouse testis with Triton X-100 are summarized in Table 5. On the average, about an 800 to 1600 fold protein purification was achieved. The purification data also revealed that about 0.5 mg of purified antigen could be recovered from 10 g of mouse testes.

The purified MSA-63 retained its ability to inhibit HS-63 binding to the methanol-fixed sperm. Following a typical ELISA procedure, the dose-dependent bindings between HRP-labeled HS-63 and MSA-63 coated on microwells were clearly demonstrated. In

contrast, no evidence of such binding was observed between HRP-labeled HS-63 and BSA coated on wells. The specific binding between HRP-labeled HS-63 and purified MSA-63 coated on microwells is shown in Figure 5.

B. CHARACTERIZATION OF MSA-63 ANTIGENS

1. ANALYSIS OF PURIFIED MSA-63 BY TWO-DIMENSIONAL SDS-GEL (2D-GEL) ELECTROPHORESIS

Following isoelectric focusing and SDS gel electrophoresis, the purified MSA-63 antigen from either purification procedures revealed quite a few protein spots on the 2D-gels. By using the Triton X-100 extraction method, purified MSA-63 displayed a group of protein spots with pIs ranging from 4.0 to 6.0 and molecular sizes from 20 Kd to 84 Kd. The 2D-gel of this preparation for purified MSA-63 is shown in Figure 6A. Based on distribution of their respective pI's value and molecular weights, protein spots on 2D-gel were subdivided into five groups (see Figure 6A).

Figure 5. ELISA showing the specific reaction of HRP labeled HS-63 monoclonal antibody to the purified MSA-63. Absorbance at 450 nm was plotted as a function of antibody dilution. (●) and (○) represent the absorbance following binding of HS-63 to microtiter plate coated with purified MSA-63 and BSA, respectively. Detailed experimental conditions are described in the section on Materials and Methods

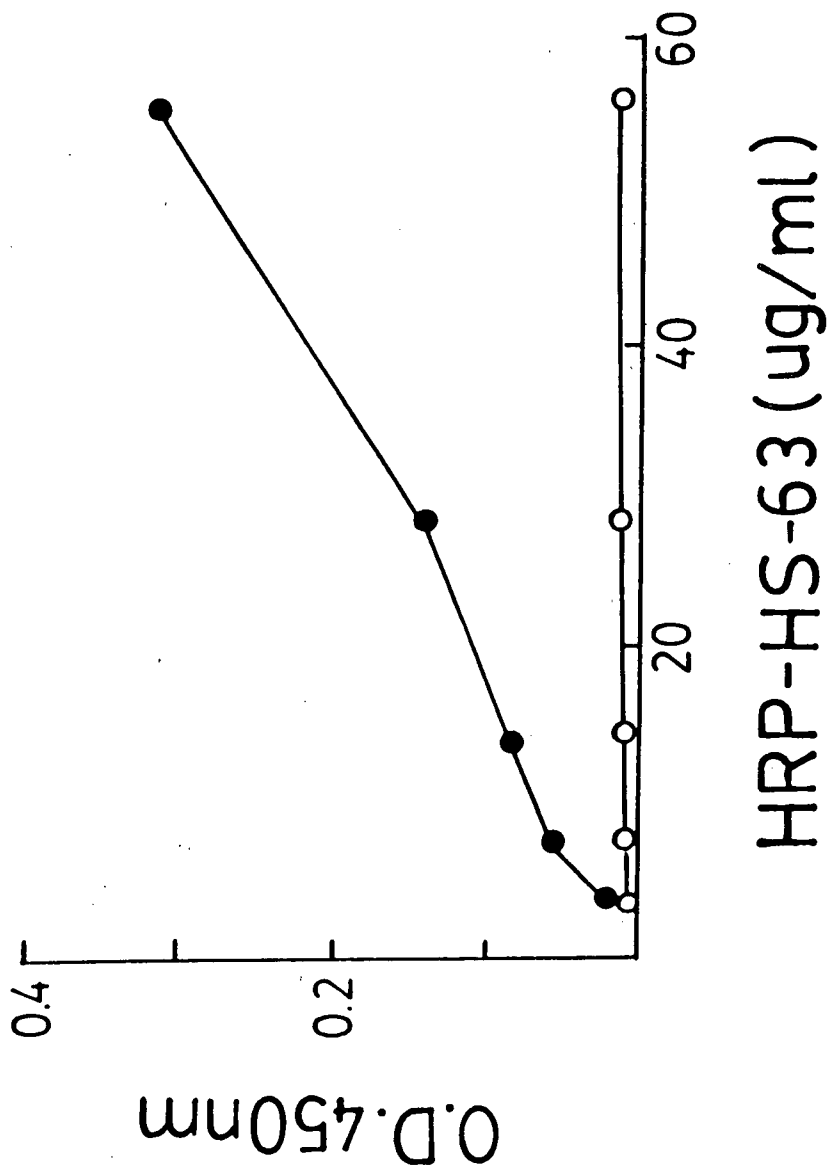
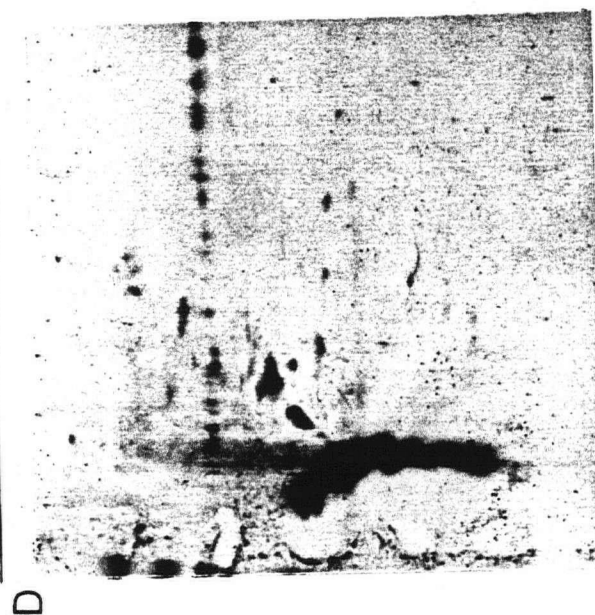
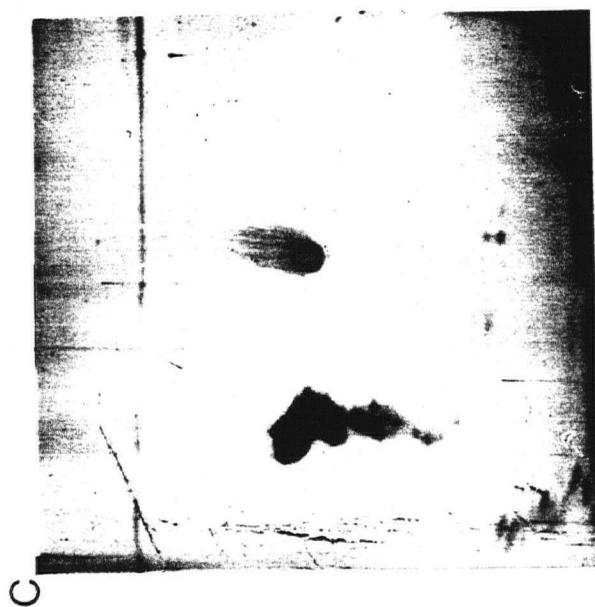
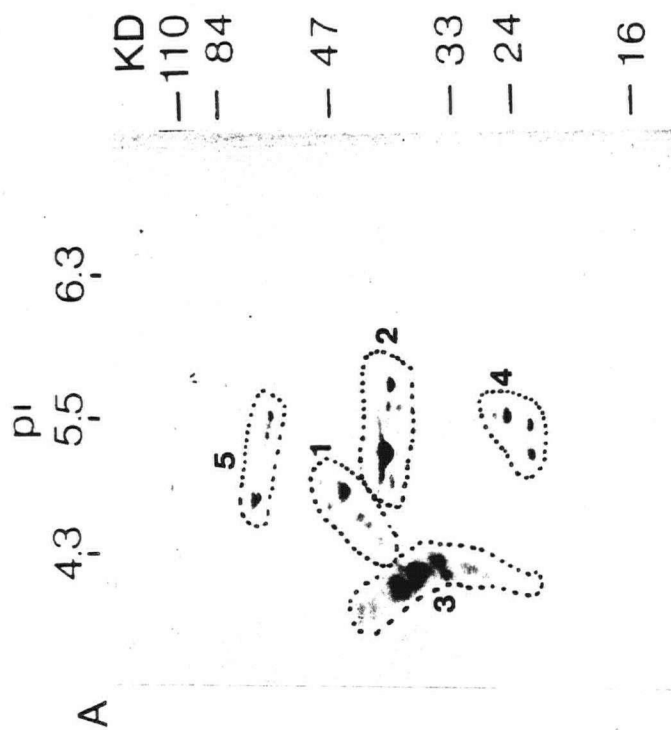


Figure 6. Two-dimensional gel electrophoresis of purified MSA-63 from mouse testes. The isoelectric point (pI) is indicated on the top and molecular weight (KD) is indicated on the right side of the gel. (A). Purified MSA-63 from Triton X-100 extraction was stained by Coomassie blue R-250. The protein spots were subdivided into 5 groups as indicated. (B). Purified MSA-63 from soluble extraction was stained by silver reagent. (C). Silver stained protein distribution pattern of detergent-purified MSA-63 following incubation at 37°C for 5 days with 0.01 % NaN_3 . (D). Western blot analysis of detergent-purified MSA-63 with rabbit antisera against protein spots in group 3 (MSA-63 protein) as a probe.



By using the soluble extraction procedure for MSA-63 purification, fewer protein spots were detected on the 2D-gel. Only protein group 2 and 3 were found on the 2D-gel with pI's ranging from 4.0 to 5.5 and molecular weights ranging from 24 to 45 KD (Figure 6B).

The protein distribution patterns of soluble- and detergent-purified MSA-63 on 2D-gels were found to alter significantly following long incubation at 37°C. After 5 days incubation at 37°C in the presence of 0.01 % NaN_3 , the protein spots in group 1, 2 and 5 were found to disappear on 2D-gel. The intensity of protein spots in group 4 became very weak when detected by silver staining reagent. Several protein spots with size of around 10 to 20 KD appeared on 2D-gel. However, the protein spots in group 3 were not found to change during the incubation. The 2D-gel analysis of this observation is demonstrated in Figure 6C. Following 2 weeks incubation, all protein spots on 2D-gel disappeared except those in group 3 with concomitant increase in staining intensity of those of 10 to 20 KD protein spots. The alteration of gel patterns for purified MSA-63 was not significantly affected by the addition of the following protease inhibitors in the incubation mixture: EDTA (10 mM), iodoacetamide

(70 mM), PMSF (1 mM), leupeptin (1 ug/ml), antipain (2 ug/ml), benzamidine (10 ug/ml), chymostatin (1 ug/ml) and pepstain (1 ug/ml).

2. PRIMARY STRUCTURE ANALYSIS OF PROTEIN SPOTS OF MSA-63 ANTIGEN ON 2D-GELS

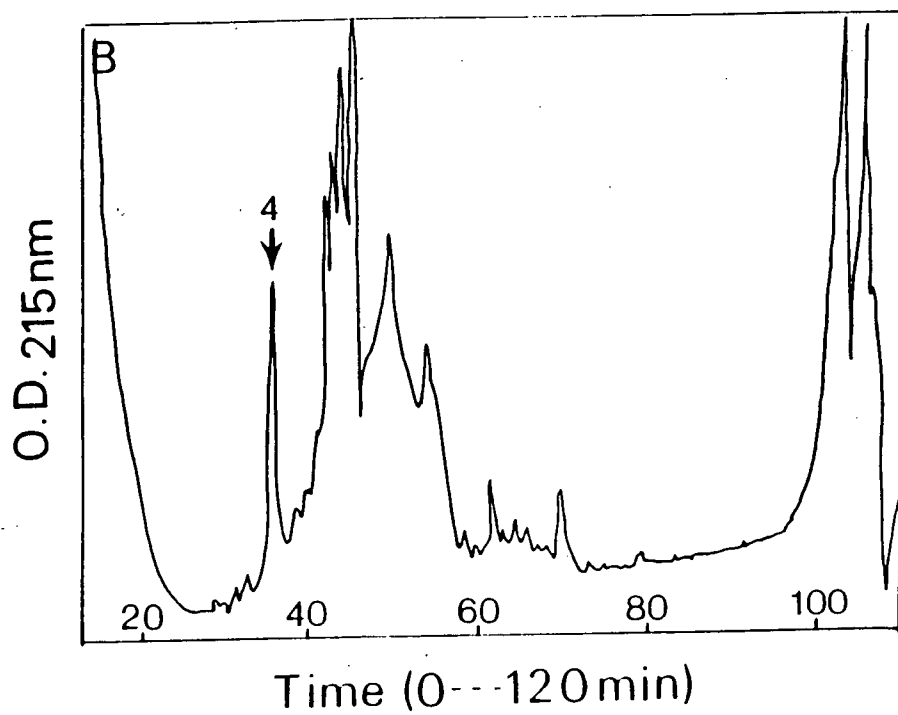
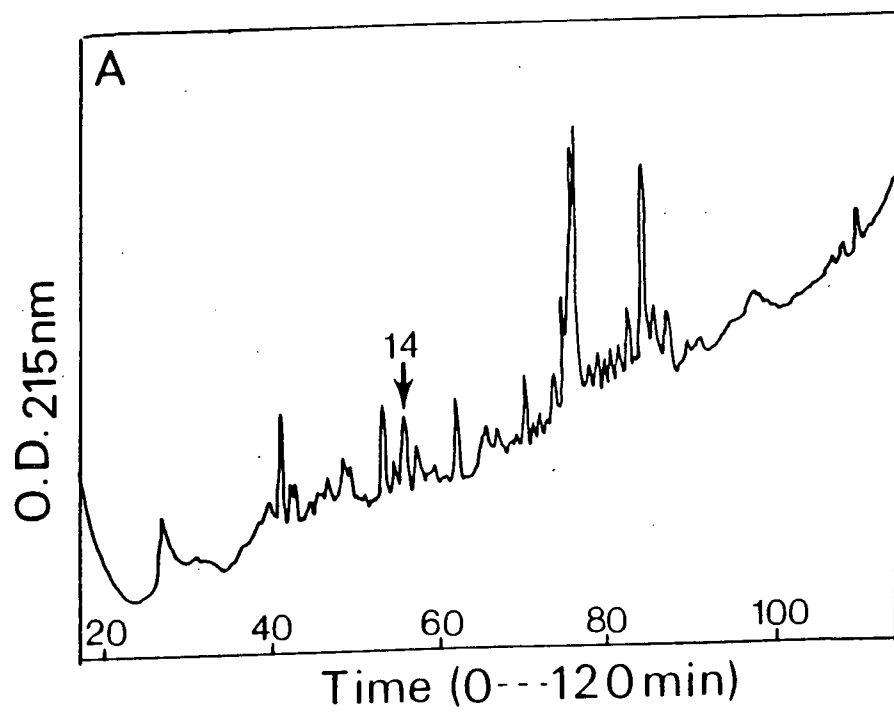
In view of the heterogeneity of purified MSA-63 protein on 2D-gels, it is imperative to analyze the structural and immunological relationships among these protein spots. The protein spots of each group were removed from Western blot of 2D-gels and used for amino acid sequence analysis. Following trypsin digestion of individual protein spots, the peptide fragments were separated by HPLC. The separation profiles of HPLC are shown on Figure 7. The amino acid sequences of the isolated peptides obtained by using Edman degradation sequencing method are summarized in Table 6.

Through computer analysis from the protein data bank, the amino acid sequences from trypsin-digested peptide derived from protein spots in group 1, 2 and 4 showed 100 % sequence homology

Figure 7. High-performance liquid chromatography on reverse phase C-18 column of trypsin-digested purified MSA-63 antigen.

Acetonitrile (0-70 %) gradient in 0.1 % trifluoroacetic acid (TFA) was used as the mobile phase. The column was performed at a flow rate of 2.0 ml/min. The selected peptide peaks for amino acid sequence determination were indicated by arrows. (A) HPLC profile of tryptic peptides from protein spots in group 1 and 2. (B) HPLC profile of tryptic peptides from protein spots in group 3. (C) HPLC profile of tryptic peptides from protein spots in group 4. (D) HPLC profile of tryptic peptides from MSA-63 protein purified according to the soluble extraction procedure.

(Work carried out in Dr. Ruedi Aebersold's lab in Biomedical Research Center at UBC)



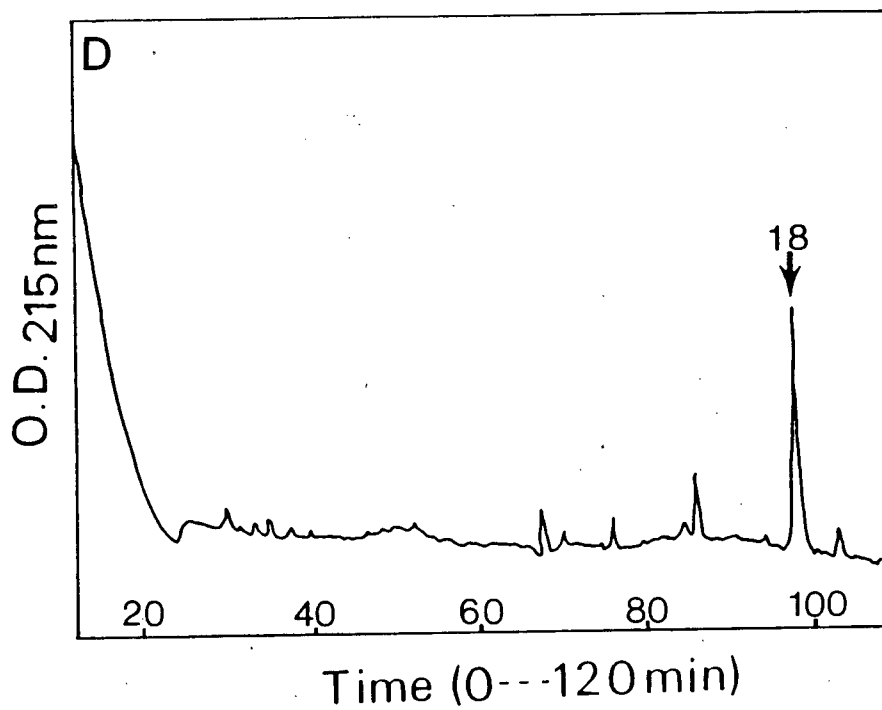
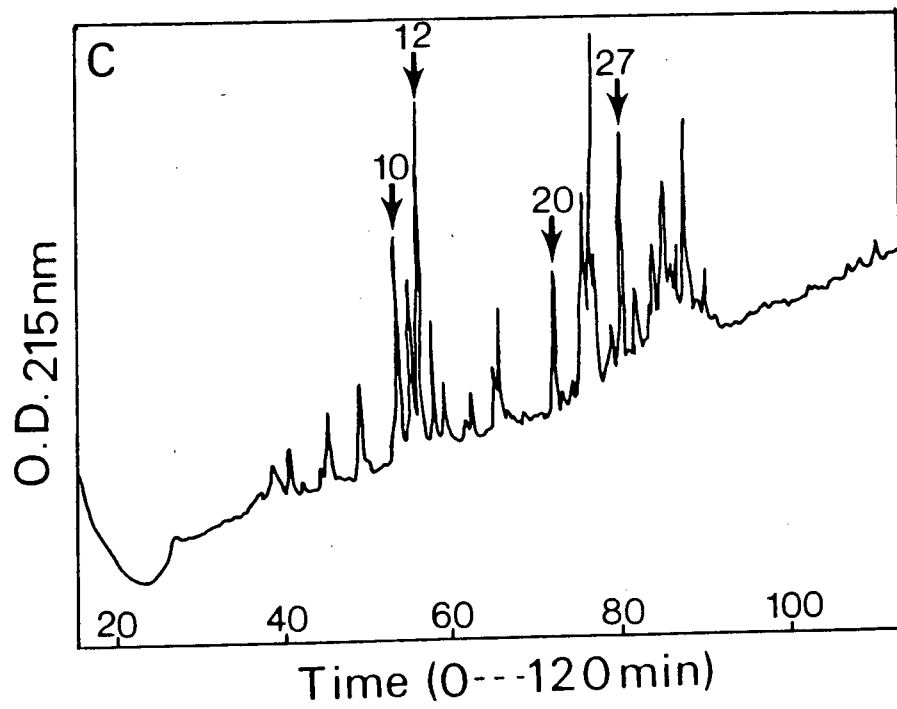


Table 6. Amino acid sequence analysis of purified MSA-63.

Protein spot (Group)	Peak No.	Amino acid sequence ^a	Sequence homology to
1, 2	14	X-Tyr-Ser-Phe-Thr-Thr-Thr-Ala-Glu	α -actin
3	4	Tyr-Glu-Thr-Phe-Leu-Asp-Glu	Nil
		Gly-Glu-Gly-Val-X-Thr-Thr-Gln-Asn-Ser-Gln	Nil
		Met-Gln-Ile-Met	Nil
4	10	Ile-Ile-Ala-Pro-Pro-Glu	α -actin
		Leu-Asp-Leu-Ala-Gly-Arg	α -actin
	12	Tyr-Ser-Phe-Thr-Thr-Thr-Ala-Glu-Arg	α -actin
		X-X-Glu-Ala-Phe-Leu-Asn-Phe-Lys	α -actin
	20	Leu-Thr-Asp-Tyr-Met	α -actin
	27	Tyr-Ala-Asn-Thr-Val-Leu-Ser-Gly-Gly- -Thr-Thr-Met-Tyr-Phe-Gly-Ile-Ala-Asp	α -actin
Soluble- purified MSA-63	18	Phe-Met-Val-Gln-Gly-X-Glu-Asn-Met-Phe- -Pro-Ser-Met-Asn-Leu-Phe	Nil

^aX: unidentified amino acid residue.

to those of the α -actin protein. Three amino acid sequences of peptides obtained from trypsin-digested protein spots in group 3 were shown to be unrelated to any other published proteins or peptides (Table 6).

Purified MSA-63 obtained from the soluble extraction procedure was also employed for amino acid sequence analysis of the peptides. After separation by using HPLC, peak 18 peptides from the trypsin-digested purified MSA-63 was selected for the amino acid sequence analysis (Figure 7D). The amino acid sequence of this peptide was shown to have no homology with those of the published known proteins and peptides.

Immunological relationship of MSA-63 protein spots on 2D-gels was also investigated. The protein spots in group 1, 2 and 3 were also removed separately from the Western blot of 2D-gel and served as immunogen for immunizing rabbits. The raised antisera (after 5 immunizations) were then used as probes for further Western blot analysis. Antisera raised against protein spots in groups 1 and 2 could mutually cross-react with each other, but failed to recognize protein spots in group 3. However, the antisera against protein spots in group 3 could cross-react with

all protein spots on the 2D-gel (Figure 6D).

Furthermore, the antisera against protein spots in group 1 could highly cross-react with α -actin from rabbit muscle (Sigma A2522) by using dot blot assay. However, rabbit antisera against protein spots in group 2 and 3 revealed no cross-reactivity to α -actin. By using an radioimmunobinding method, HS-63 monoclonal antibody was shown to have no significant cross-reactivity to α -actin from rabbit muscle.

By means of indirect immunofluorescent assay, only antisera raised against protein spots in group 3 were shown to react with the acrosome of mouse sperm fixed on slides. The sperm staining pattern was similar to that of HS-63.

3. MOLECULAR WEIGHT ANALYSIS OF PURIFIED MSA-63 PROTEINS

To analyze the molecular size distribution of MSA-63 antigen in native form, the purified MSA-63 antigen obtained from Triton X-100 extraction method was applied to a Sephacryl S-300 gel filtration column. In the presence of 0.1 % Triton X-100, a broad distribution of MSA-63 immunoactivity was observed with a maximum

activity in the molecular weight ranging from 200 Kd to 300 Kd (Figure 8). Antigen fractions with the highest immunoactivity were subsequently analyzed by SDS-PAGE. It was found that the molecular weight of major protein bands on the gel ranged between 24 Kd and 50 Kd (Figure 8).

4. INTERACTION BETWEEN MSA-63 AND ACTIN

In view of high molecular weight distribution of MSA-63 immunoactivity in native form, the interaction between MSA-63 and α -actin (from rabbit muscle, Sigma A2522) was investigated. Following overnight incubation with purified MSA-63 (prepared by Triton extraction method) at 4°C, the molecular weight distribution profile of actin was found to change when analyzed by Sephacryl S-300 gel filtration (Figure 9).

5. ANALYSIS OF PURIFIED MSA-63 PROTEINS BY ENZYMATIC DEGLYCOSYLATION

Attempts were made to analyze the molecular heterogeneity of MSA-63 protein through enzymatic deglycosylation. N-glycosidase and O-glycosidase were used for in vitro deglycosylation of MSA-63 proteins. After overnight incubation at 37°C, the protein

Figure 8. Sephacryl S-300 gel filtration chromatography and SDS-PAGE to reveal the molecular size distribution of purified MSA-63 antigen. Arrows A, B, and C indicate the position of molecular size marker of 670, 158, and 44 KD, respectively. The profile of antigen immunoactivity determined by indirect immunofluorescent inhibition assay indicated the main activity was detected in fractions with molecular size ranging from 200-300 Kd. The corresponding fractions examined by SDS-PAGE are shown on the top. The molecular marker is indicated on the right side of the gel.

Fraction NO.

11 13 15 17 19 21 23 25 27 29

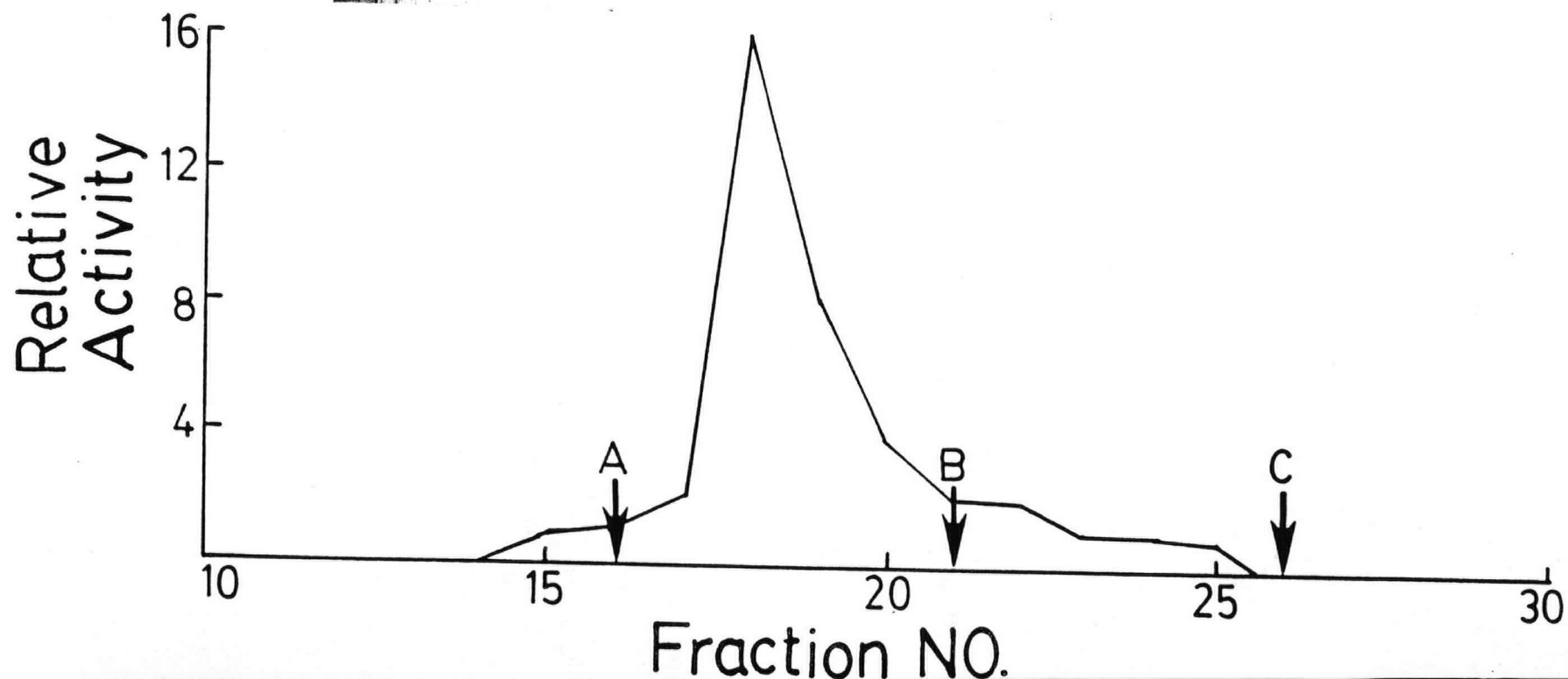
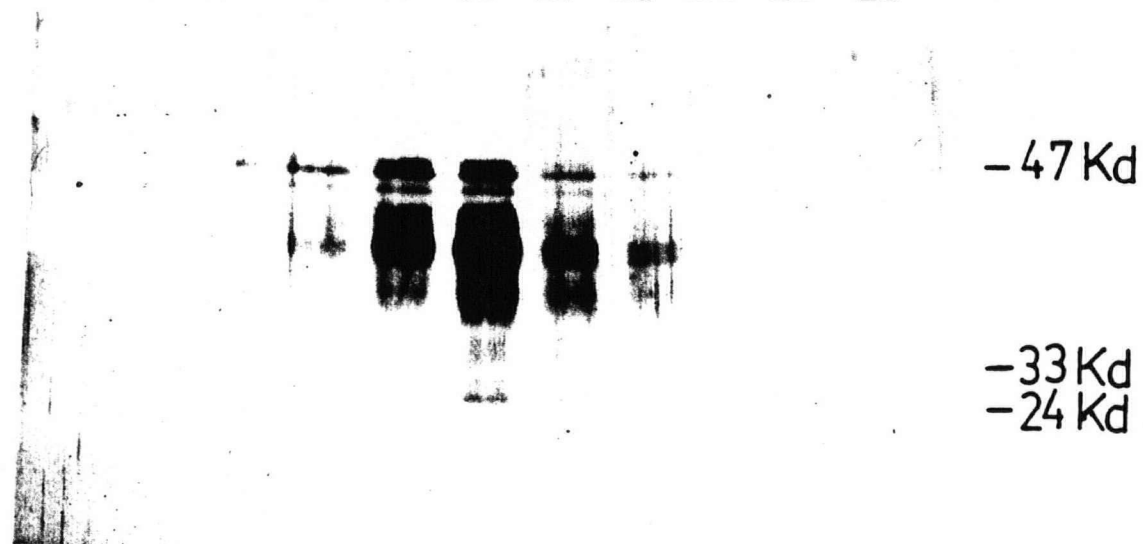
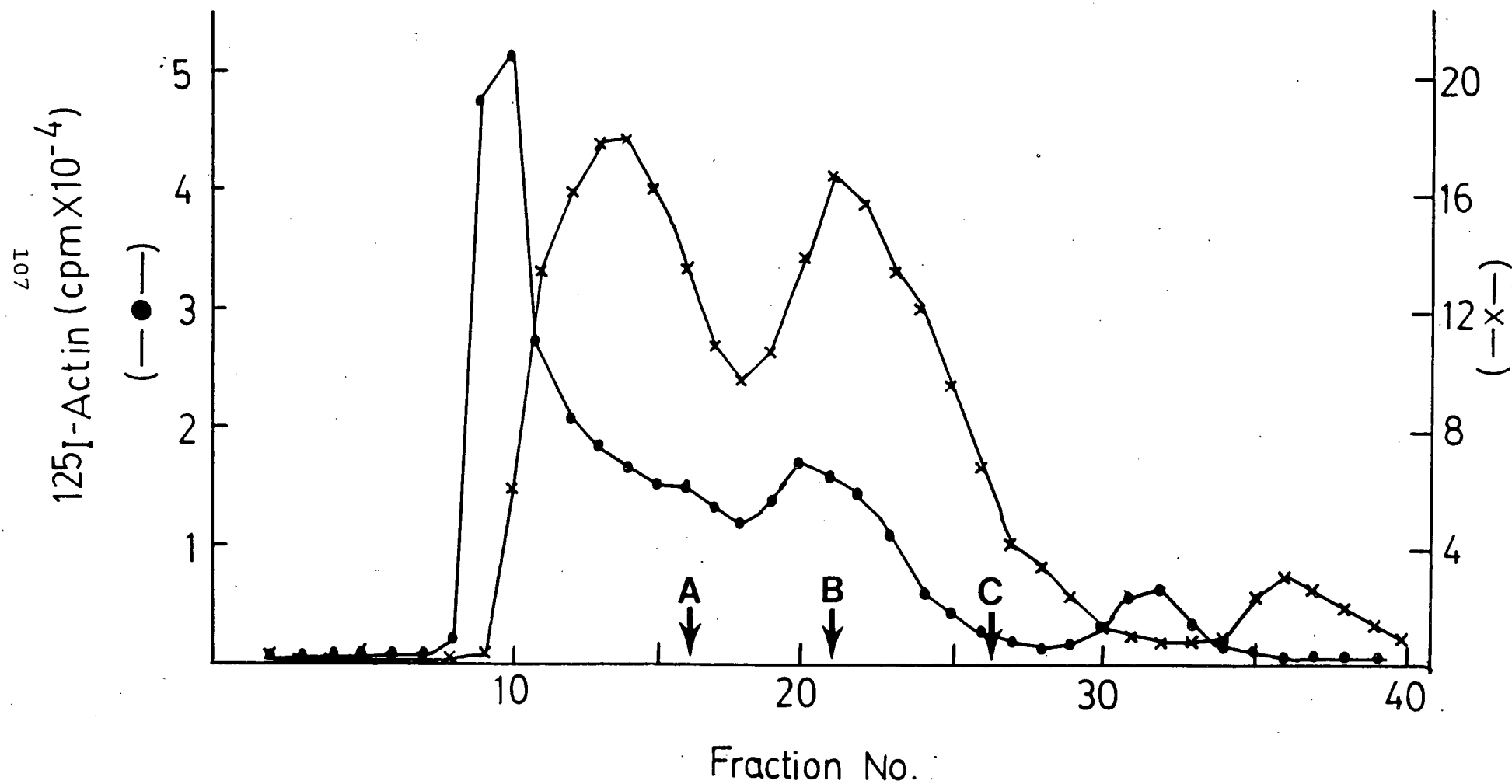


Figure 9. Sephacryl S-300 gel filtration chromatography to reveal the interaction between α -actin and purified MSA-63. (-●-) indicates the molecular weight distribution of ^{125}I -labeled Actin protein (specific activity 0.02 mCi/ug). (-X-) indicates the molecular weight distribution of ^{125}I -labeled actin protein incubated with 10 ug of purified MSA-63 for overnight at 4°C. The fractionated radioactivity is measured by using a γ -counter. Arrows A, B, and C indicate the position of estimated molecular size marker of 670, 158, and 44 KD, respectively.



distribution pattern of MSA-63 on SDS-gel was not changed in the presence of either N-glycosidase or O-glycosidase. Results of this analysis are presented in Figure 10. Human chorionic gonadotrophin (hCG) protein served as positive control to show the enzymatic activity of these two glycosidase.

C. PRODUCTION AND EVALUATION OF ANTISERA ON FERTILIZATION

Female mice and rabbits were immunized with purified MSA-63 antigen to investigate immunogenicity and antifertility effects. MSA-63 antigen prepared by the soluble extraction procedure was used as a immunogen to raise antisera in both mice and rabbits. The titre of antisera increased with increasing number of immunizations. Antisera raised against purified MSA-63 from rabbit and mouse were shown to react with the acrosome of mouse and human sperm when examined by indirect immunofluorescent assay. The specificity of these antisera against MSA-63 was also demonstrated by using ELISA and compared with that of HS-63 and an unrelated antibody. Results of this study are presented in Figure 11.

Figure 10. (A). Enzymatic deglycosylation of MSA-63 proteins using N-glycosidase and O-glycosidase in the presence of 1% Triton X-100 and 0.1 % SDS in 0.1 phosphate buffer pH 7.3. ^{125}I -labeled MSA-63 protein (specific activity 0.1 mCi/ug) was incubated with (1) 0.6 unit of N-glycosidase (2) 1 mU of O-glycosidase (3) 0.6 unit of N-glycosidase plus 1 mU of O-glycosidase (4) no enzyme for overnight at 37°C. The protein pattern of each treatment was analyzed by SDS-PAGE. Molecular Weight (KD) is indicated on the right side of the gel. (B). Enzymatic deglycosylation of hCG protein was treated under the same condition serving as positive control.

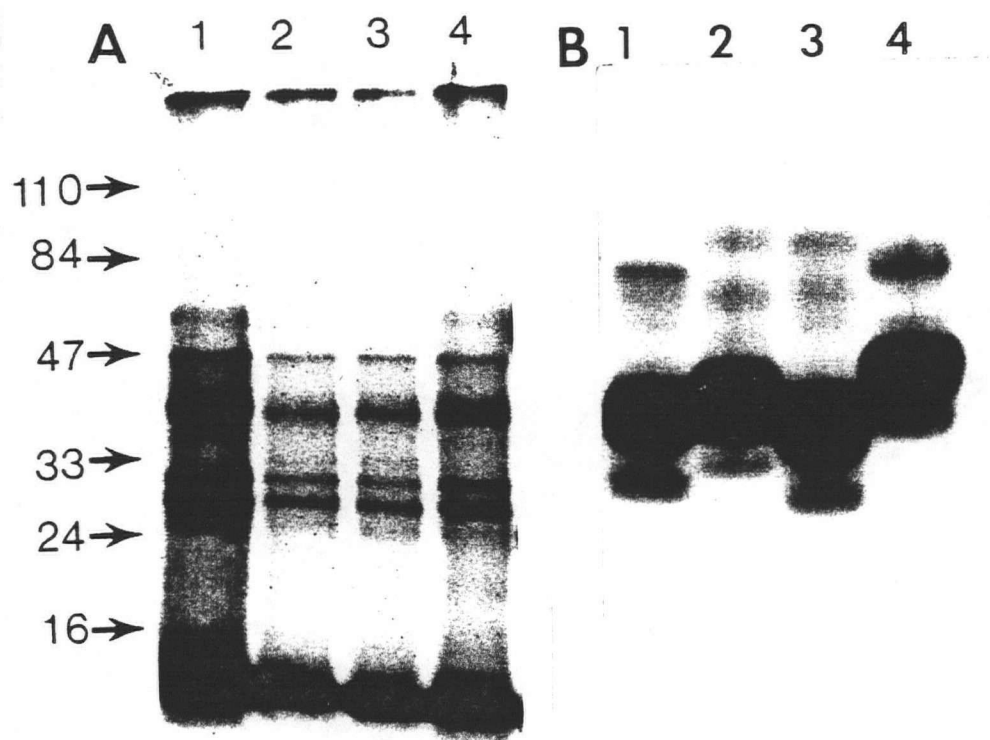
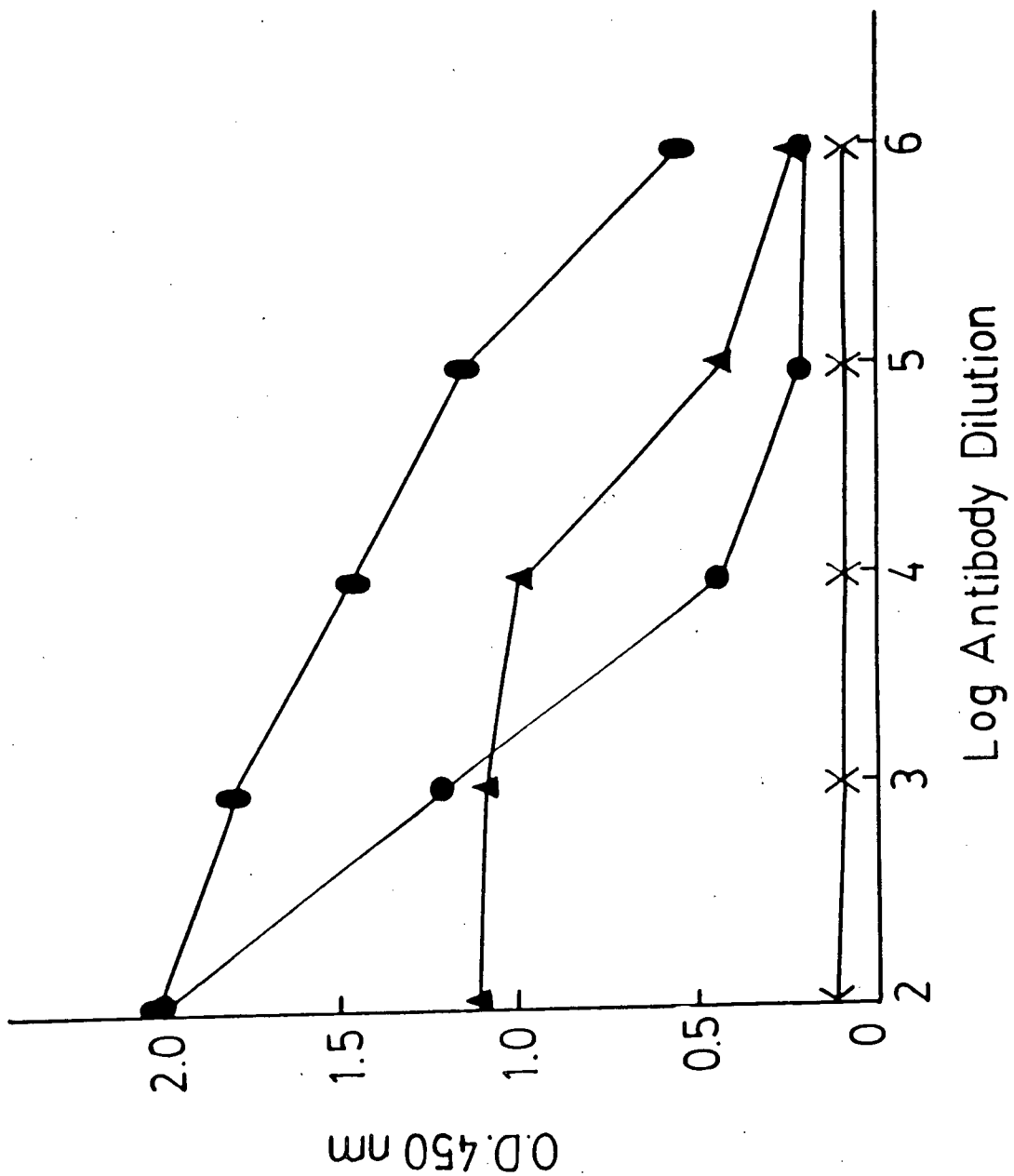


Figure 11. ELISA showing the binding between HS-63, mouse isoimmunesera, rabbit anti-MSA-63 sera or unrelated antibodies and the purified MSA-63 coated on microwells. Absorbance at 450 nm was plotted as a function of antibody dilution in log scale, where (▲) denotes HS-63, (O) is mouse isoimmune sera against MSA-63, (●) denotes rabbit antisera against MSA-63 and (X) is unrelated anti-hCG monoclonal antibody.



The antifertility effect of these antisera was evaluated by using a mouse in vitro fertilization experiment (Table 7). A significantly lower fertilization rate was observed in the presence of mouse isoimmune sera or rabbit anti-MSA-63 as compared to that of the control using nonimmune sera.

The antifertility effect of rabbit antisera raised against MSA-63 was also evaluated by in vivo mating experiments following passive immunizations. Under the same experimental conditions, polyclonal antisera appeared to be more effective than HS-63 monoclonal antibody in terms of in vivo fertilization inhibition. Results of such analysis are presented in Table 4.

Table 7. Inhibitory effect of polyclonal antisera raised against MSA-63 on in vitro fertilization of mouse oocytes.

Antisera ^a	Number of assays	Ova examined (No.)	Ova fertilized (No.)	Fertilization rate (%)	P value
Control (NMS)	3	70	57	81.4	
MSA-63	3	54	16	29.6	<0.001 ^b
Control (NRS)	2	55	28	50.9	
RSA-63	2	61	15	24.6	<0.01 ^c

^aAntisera used for experiments: NMS: nonimmune mouse serum, MSA-63: isoimmune sera against MSA-63, NRS: nonimmune rabbit serum, RSA-63: rabbit anti-MSA-63.

^bCompared with the control group (NMS).

^cCompared with the control group (NRS).

Part III. CLONING OF MSA-63 CDNA AND PRODUCTION OF ITS FUSION
PROTEIN

A. IMMUNOSCREENING OF MOUSE TESTIS cDNA LIBRARY

Rabbit antisera raised against the MSA-63 proteins were used for the immunoscreening of a mouse testis cDNA library to detect the cDNA clones which expressed the corresponding fusion proteins.

A mouse testis cDNA library containing approximately 1×10^6 independent recombinant bacteriophages was constructed in a lambda gt11 expression vector. Through the immunoscreening of 6×10^5 bacteriophages from this library, more than 60 positive primary clones were identified (Figure 12). Twelve of these positive primary clones were selected randomly for secondary immunoscreening. The DNA of these purified recombinant bacteriophages was analyzed by using Southern blot hybridization. Two of the cDNA inserts were randomly selected as probes. Among the positive primary clones, six could cross-hybridize with each other. Under highly stringent washing conditions, four of the cross-hybridized clones were identified (Figure 13).

Figure 12. Immunoscreening of mouse testis cDNA library by using rabbit anti-MSA-63 sera as the detecting probe. Positive clones are circled. (Work carried out in Dr. Chris Lau's lab at University of California, San Francisco)

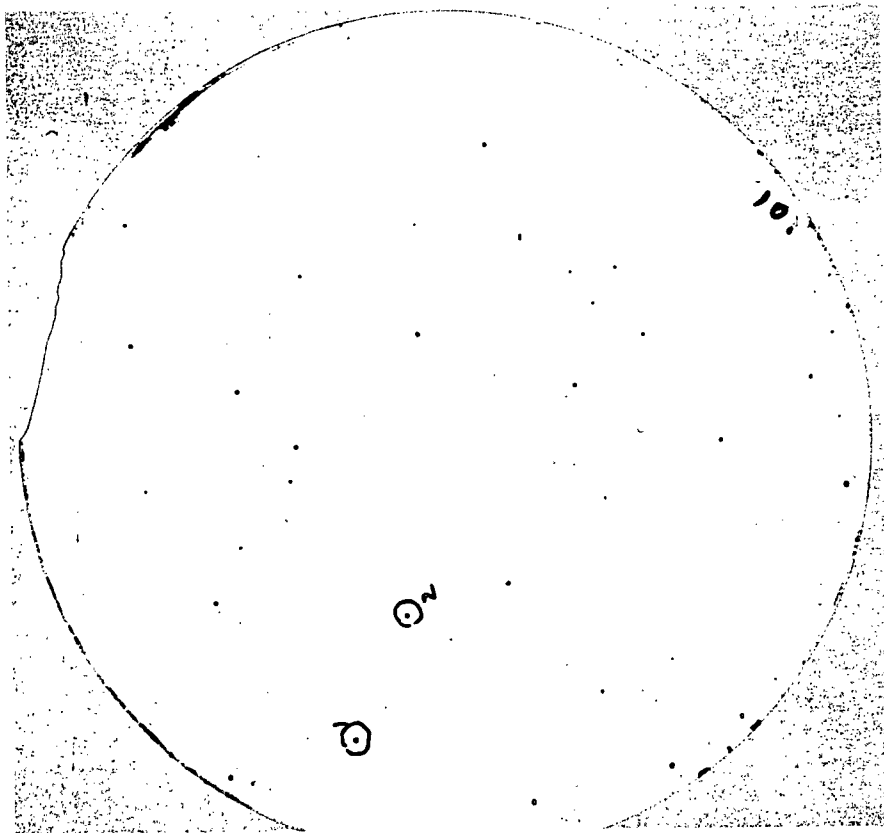
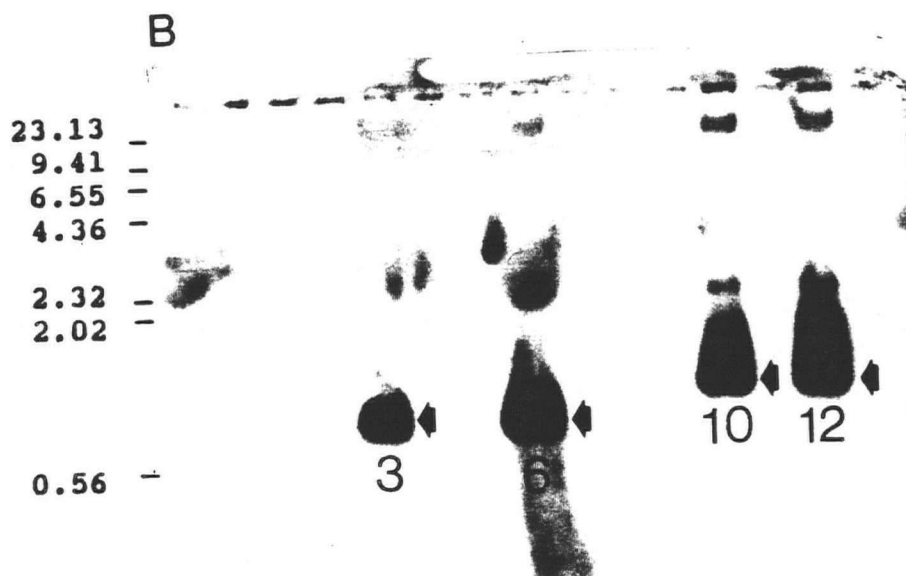
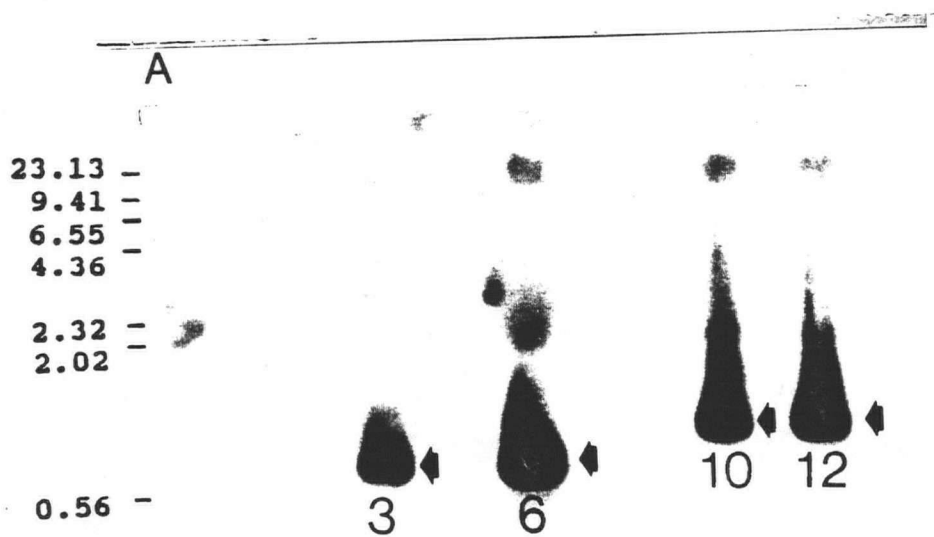


Figure 13. Southern blot analysis to demonstrate the cross hybridization of immuno-screened positive clones. (A). cDNA inserts of Clone 3, 10 and 12 are shown to hybridize with the cDNA probe from clone 6. (B). Using the cDNA insert of clone 12 as a probe, cDNA of clone 3 ,6 and 10 are shown to hybridize with the probe. Hind III digested lambda DNA as size marker shown on the left, are 23.13 Kb, 9.41 Kb, 6.55 Kb, 4.36 Kb, 2.32 Kb, 2.02 Kb and 0.56 Kb, respectively from the top. (Work carried out in Dr. Chris Lau's lab at University of California, San Francisco) (The data were published in Liu et al., 1990)

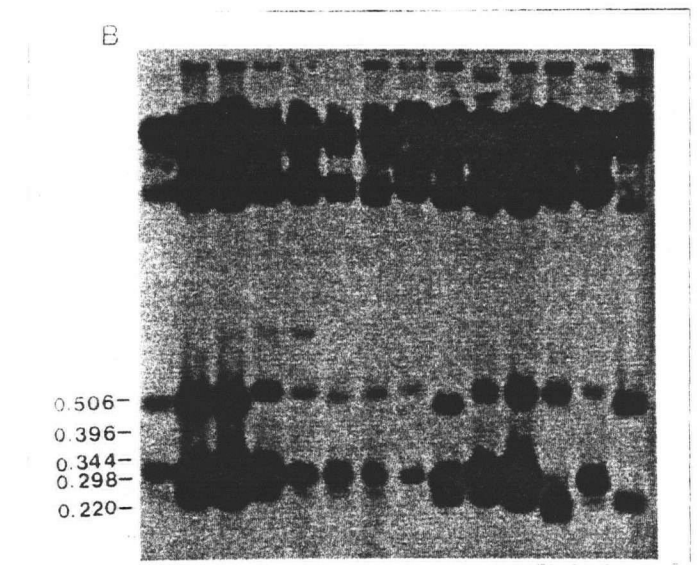
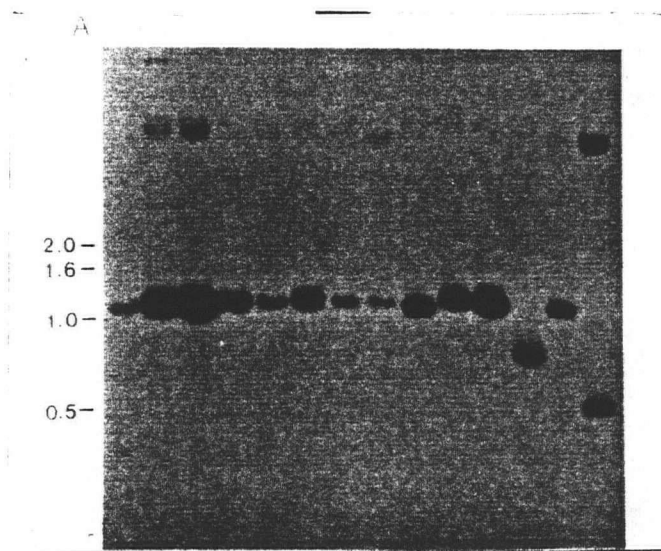


When analyzed by agarose gel electrophoresis, the size of the cDNA insert of clone 6 and 12 were determined to be 0.8 and 1.0 Kb, respectively. These two cDNA clones were selected for production of recombinant fusion proteins.

B. SCREENING OF THE MOUSE TESTIS cDNA LIBRARY BY USING THE CLONED DNA PROBE

The cDNA insert of clone 12 was used as a probe to screen the mouse testis cDNA library in order to isolate larger or full length clones. After screening of 1×10^5 bacteriophages, several positive clones were identified. By Southern blot analysis, the molecular size of many clones was about 1.2 Kb (Figure 14A). The restriction maps of these cDNA clones were found to be identical (Figure 14B). One of these clones with a size of about 1.2 Kb was used for DNA sequence analysis.

Figure 14. (A). Southern blot assay of established positive cDNA clones by using clone 12 cDNA as a probe. (B). The restriction map analysis of established positive cDNA clones digested with EcoRI and PstI. The molecular size markers in Kb are indicated on the left.



C. DNA SEQUENCE ANALYSIS

Following EcoR I digestion, the cDNA insert was obtained from the recombinant phages and then subcloned into plasmid pUC18. The restriction map of the cDNA insert was deduced from the analysis of restriction enzyme digestion. Pst I digested fragments were continuously subcloned into pUC18 and subjected to DNA sequencing analysis. The restriction map and sequencing strategy for the cDNA insert of MSA-63 gene are presented in Figure 15.

The nucleotide sequence of the cDNA insert and the corresponding protein sequence are presented in Figure 16. The cDNA insert was 1067 bp in length and contained an open reading frame of 783 nucleotides (261 amino acids) starting from an initiation codon (ATG) at nucleotides 60-62. The termination codon TGA was located at nucleotides 842-844. The Length of the 5' untranslated region was found to be 59 bp and nucleotides upstream from the ATG initiation codon were AAATCAAA, being similar to most eukaryotic start codons for translation (Kozak, 1984).

Figure 15. Restriction map and sequencing strategy for the cDNA insert of MSA-63. Restriction sites are shown at the top: H: Hind III, P: Pst I, B: Bgl II, X: Xba I. EcoR I and Pst I are used for subcloning into pUC18. Arrows indicate the direction and extent of each sequence determination.

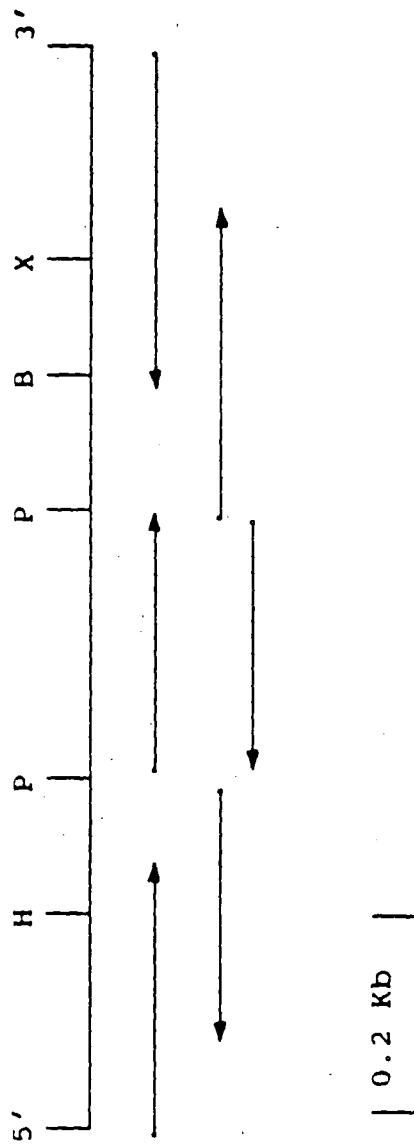


Figure 16. The nucleotide sequence and deduced amino acid sequence of MSA-63. The predicted amino acid sequence is shown below the nucleotide sequence. The numbering to the right indicates the nucleotide and amino acid positions. A poly A addition signal is underscored with the symbol (***) and an mRNA consensus degradation sequence is underscored with the symbol (^^^). The predicted antigenic determinants are underscored with the symbol (...) and a canonical N-linked glycosylation sequences is underscored with the symbol (+). The underlined sequence is identical to that from amino acid sequencing analysis of peptides isolated from trypsin-digested MSA-63 protein.

5'

CAGTTTCTTC TCAGCTCTTG AGTGTGCCAC ATTAGAGATC TTTATTTACC TAAATCAAA ATG AAG GAG TTA ATC TTA CTG GCA 83
 TER TER Met Lys Glu Leu Ile Leu Leu Gly 8

GGT CTT TAT CTG CTT TCT GCC CAA GGA GCA CCA CCA GGT CAG CCT GAG GAG CTT CTT GAC TCT GTA GAC CAA 155
 Gly Leu Tyr Leu Leu Ser Ala Gln Gly Ala Pro Pro Gly Gln Pro Glu Glu Leu Leu Asp Ser Val Asp Gln 32

CAA GCT TCG GTT CAG CAA CTT TCA AGC GAG TAT CTC TCA CTC GCA AAC CCT TCA GAT GCC GAG GCT TTA TAT 227
 Gln Ala Ser Val Gln Gln Leu Ser Ser Glu Tyr Leu Ser Leu Ala Asn Pro Ser Asp Ala Glu Ala Leu Tyr 56
 +

GAA ACT CCT TTA GAT GAG AAG ACT CTG AGT GGT CAT ACT TCA AGT GAA CAG GAA TCA ACT GAG CAT GCT GTA 299
 Glu Thr Pro Leu Asp Glu Lys Thr Leu Ser Gly His Ser Ser Ser Glu Gln Glu Ser Ser Glu His Ala Val 80

GCT GAA CAT TCT GCA GGT GAG CAC TCT TCA GGA GAA CAG TCT TCA GAA CAC ATG TCA GGT GAC CAC ATG TCA 371
 Ala Glu His Ser Ala Gly Glu His Ser Ser Gly Glu Gln Ser Ser Glu His Met Ser Gly Asp His Met Ser 104

GGA GAG CAC TTG TCA GAA CAC ACT TCA GAG GAG CAC TCC TCG GCG GAG CAC ACT TCC ACC GAG CAC ACT TCA 443
 Gly Glu His Leu Ser Glu His Thr Ser Glu Glu His Ser Ser Gly Glu His Thr Ser Thr Glu His Thr Ser 128

GGT GAA CAA CCT GCA ACT GAA CAG TCC TCA AGT GAC CAG CCC TCC GAA GCA TCT TCA GGT GAA GTT TCG GGT 515
 Gly Glu Gln Pro Ala Thr Glu Gln Ser Ser Ser Asp Gln Pro Ser Glu Ala Ser Ser Gly Glu Val Ser Gly 152

GAC GAA GCA GGT GAA CAG GTG TCT AGC GAG ACA AAT GAC AAA GAA AAT GAT GCT ATG AGT ACA CCA CTT CCA 587
 Asp Glu Ala Gly Glu Gln Val Ser Ser Glu Thr Asn Asp Lys Glu Asn Asp Ala Met Ser Thr Pro Leu Pro 176

AGC ACA TCT GCA GCC ATA ACA TTA AAT TGC CAC ACA TGT GCT TAT ATG AAT GAT GAT GCA AAA TGT CTC CGT 659
 Ser Thr Ser Ala Ala Ile Thr Leu Asn Cys His Thr Cys Ala Tyr Met Asn Asp Asp Ala Lys Cys Leu Arg 200

GGA GAA GGA GTA TGC ACC ACT CAA AAC TCC CAG CAG TGC ATG TTA AAG AAG ATC TTT GAA GGT GGA AAA CTC 731
 Gly Glu Gly Val Cys Thr Thr Gln Asn Ser Gln Gln Cys Met Leu Lys Lys Ile Phe Glu Gly Gly Lys Leu 224

CAG TTC ATG GTT CAA GGG TGT GAG AAC ATG TGC CCA TCT ATG ACC CTC TTC TCT CAT GGA ACA AGA ATG CAA 803
 Gln Phe Met Val Gln Gly Cys Glu Asn Met Cys Pro Ser Met Asn Leu Phe Ser His Gly Thr Arg Met Gln 248

ATT ATG TGC TGT CGG AAT GAA CCT CTC TGC AAC AAG GTC TAG ATGCCCGTGC CCTACTTCTT GCTCTGACTT AGGCAGGTTC 885
 Ile Met Cys Cys Arg Asn Glu Pro Leu Cys Asn Lys Val TER 261

ACCACTCTAC TTGGCTCAAT TTATGTTCAA CTTCACAAC TAATCAGATC GGCTCTGCCT GATCACCAGA TAAGAAGCTC AAACCTTGTC 975
 AA AAA

TTTATGATA CCCCAATGCC TAAGTCTCTT GCCTTACTTT GCTCCCATCC TTCCGCAGAT GTTCTTTTTT GCAATAAAAT GCTATTAAAGAA 1067

3'

Through this sequence analysis, a putative eukaryotic mRNA degradation sequence (ATTTA) was also identified at nucleotides 903-907 (Caput et al., 1986; Shaw and Kamem, 1986). A consensus polyadenylation sequence (AATAAA) at nucleotides 1048-1053 was also located at the 3' untranslated region followed by a poly A tail.

The sequence analysis revealed that there was a considerable overlap between cDNA clone 6 and 12. cDNA clones 6 and 12 were found to start at nucleotides 68 and 418, respectively.

The amino acid sequence for MSA-63 deduced from the cDNA sequence predicted a protein of 27.9 Kd. Part of the amino acid sequence was found to be matched with those obtained from the soluble-purified MSA-63 antigen and from protein spots in group 3 on 2D-gel.

The amino acid composition of the deduced MSA-63 protein is presented in Table 8. It is noted that MSA-63 consists a relatively high percentage of serine (15.7 %), glutamic acids (12.2 %), and leucine (8.4 %). There is no tryptophane residue in the entire sequence. The repeating amino acid motifs: Ser-Ser-

Table 8. Amino acid composition of the deduced MSA-63 protein.

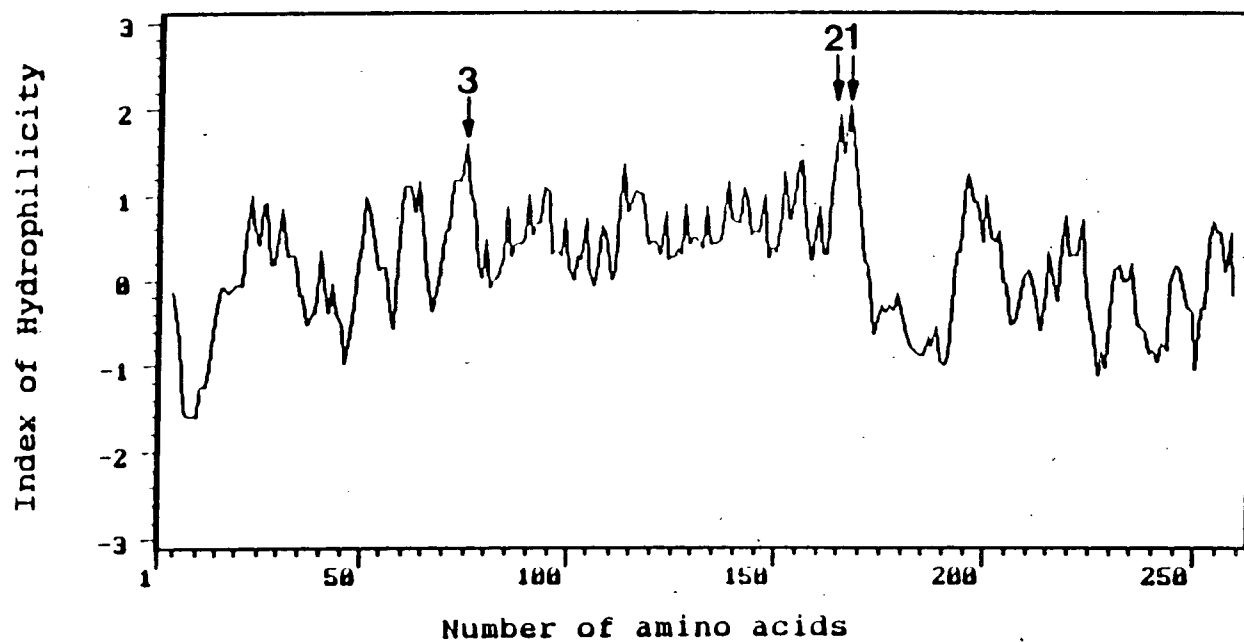
Amino acid residue	Number of residue	Percentage (%)
Ala	17	6.5
Arg	3	1.1
Asn	10	3.8
Asp	11	4.2
Cys	10	3.8
Gln	18	6.8
Glu	32	12.2
Gly	20	7.6
His	13	4.9
Ile	4	1.5
Leu	22	8.4
Lys	8	3.0
Met	11	4.2
Phe	3	1.1
Pro	11	4.2
Ser	41	15.7
Thr	15	5.7
Trp	0	.0
Tyr	4	1.5
Val	8	3.0

Gly-Glu and Ser-Ser-Glu were found in high frequency in the entire amino acid residues. A canonical N-linked glycosylation sequence (Asn-Xaa-Ser/Thr) (Pless and Lennarz, 1977; Hart et al., 1979) was located at amino acid residue 49 (see Figure 16).

According to the PC/GENE computer program analysis, the hydrophilicity plot of deduced MSA-63 protein showed a hydrophobic amino terminus which is a characteristic of a signal peptide (von Heijne, 1986). The main hydrophobic domains were found to be proximal to the carboxy terminus. The result of this analysis is presented in Figure 17. Three antigenic determinants having the highest points of hydrophilicity were: (1) amino acid residues 164-169; Asn-Asp-Lys-Glu-Asn-Asp (2) amino acid residues 162-167; Glu-Thr-Asn-Asp-Lys-Glu (3) amino acid residues 72-77; Glu-Gln-Glu-Ser-Ser-Glu (see Figure 16).

The theoretical isoelectric point (pI) of MSA-63 protein was estimated to be 4.2. This is consistent with the pI value of protein spots in group 3 on 2D-gel.

Figure 17. Hydrophilicity plot for the deduced MSA-63 amino acid sequence. Three of the highest hydrophilic regions assumed to be the antigenic determinants of this protein, are indicated by arrows on the plot.



The search of PC/GENE data base (release 6.25, July 10, 1990) revealed little homology of nucleotide and amino acid sequence to any known genes or proteins. However, about 60 to 70 % homology in nucleic acid and amino acid sequence was observed between MSA-63 and SP-10 antigen of human sperm origin (Wright et al., 1990).

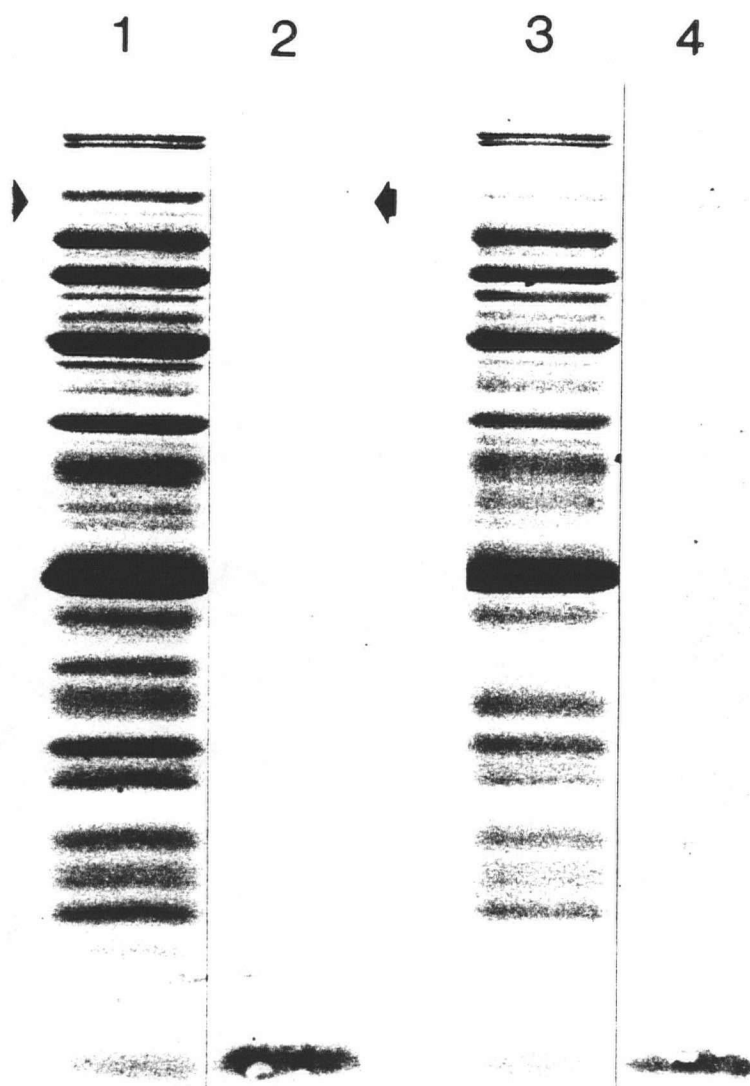
D. PRODUCTION AND CHARACTERIZATION OF MSA-63 FUSION PROTEIN

1. PREPARATION OF RECOMBINANT MSA-63 FUSION PROTEIN FROM LYSOGENIC STRAIN OF E. COLI

Two of the recombinant phages carrying cDNA insert from clones 6 and 12, respectively were chosen for production of MSA-63 fusion protein in a lysogenic strain of E. coli. After infection and selection, several lysogenic bacteria colonies were formed. MSA-63 fusion protein expressed by the recombinant lysogens was detected and characterized by using Western blot and immunobinding inhibition assay.

The SDS gels and the corresponding Western blot assay of lysates of E. coli with or without recombinant MSA-63 cDNA insert are shown in Figure 18. By Western blot analysis, the

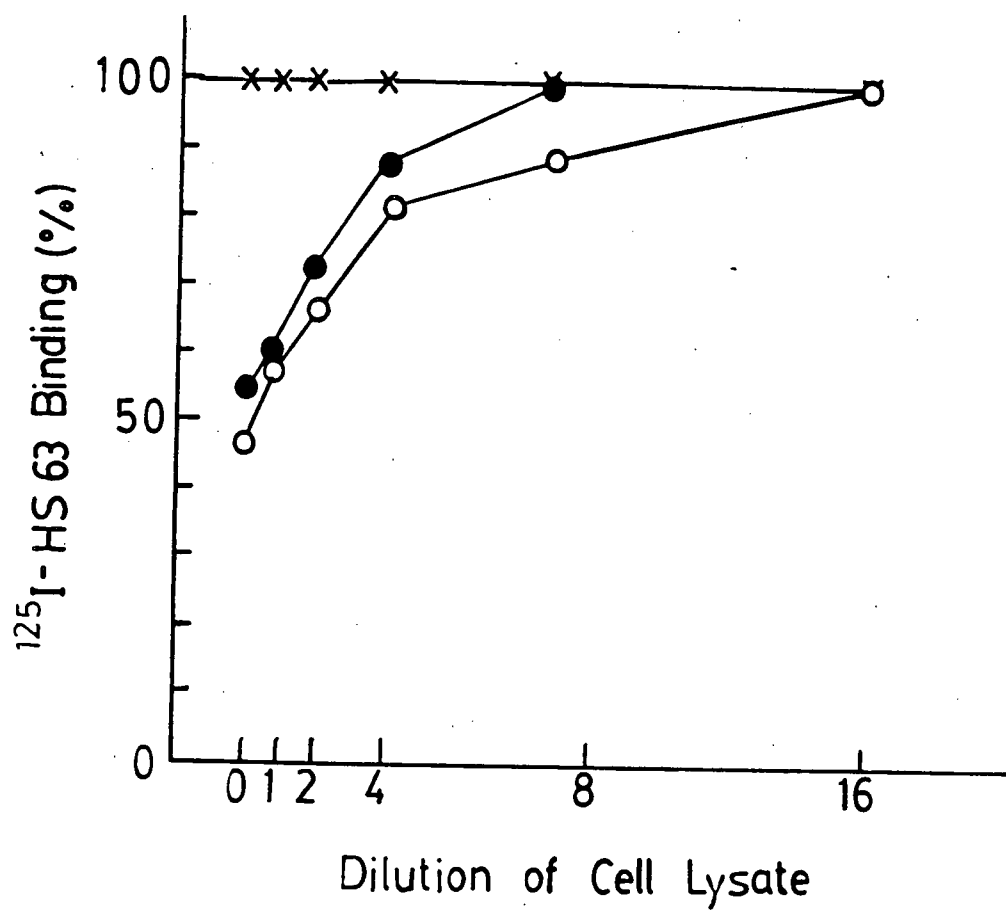
Figure 18. SDS-PAGE and Western blot assay of recombinant MSA-63 fusion protein. Proteins on SDS-gel stained by Coomassie blue are shown on lanes 1 and 3. The results of Western blot are shown on lanes 2 and 4: Lanes 1 and 2 denote the lysates of clone 12 lysogens, respectively. A recombinant fusion protein detected by rabbit anti-MSA-63 sera is shown on lane 2 with the arrow indicating a molecular size of about 150 KD. Lanes 3 and 4 represent those of the control and lysates of E.coli strain Y1089, respectively. (The data were published in Liu et al., 1990).



expressed MSA-63 fusion protein from lysogenic E. coli carrying clone 12 cDNA insert was detected by using polyclonal antisera raised against native MSA-63 protein as the probe. The molecular weight of the expressed fusion protein was estimated to be about 150 KD on SDS gel.

Figure 19 shows the immunospecificity of the MSA-63 recombinant fusion protein to HS-63 as demonstrated by radioimmunosorbent inhibition assay. Bacterial cell lysates containing the fusion protein (from clone 12 and clone 6 lysogens) were found to inhibit the binding of ^{125}I -labeled HS-63 to the mouse sperm coated on microwells in a dose-dependent manner.

Figure 19. Percent inhibition of the binding of ^{125}I -labeled HS-63 (specific activity 0.1 mCi/ μg) to microwells coated with the soluble extract of mouse sperm by the lysates of clone 6 lysogens (\bullet), clone 12 lysogen (O) and Y1089 host (X). 1×10^6 cpm/well is used for each assay. The initial protein concentration of cell lysates was adjusted to 5 mg/ml. (The data were published in Liu et al., 1990)



2. ISOLATION OF FUSION PROTEINS AND EVALUATION OF CORRESPONDING ANTISERA

MSA-63 fusion protein from the recombinant lysogens was partially purified by gel filtration chromatography (Sephacryl S-300). Fractions containing specific immunoactivity to HS-63 were collected and used as immunogens to immunize female mice and rabbits. Following the typical immunization protocols, the mouse and rabbit antisera were raised against partially purified recombinant MSA-63 fusion protein. Antisera raised against the host bacterial extract served as the negative control. By using indirect immunofluorescent assay, the antisera from mouse and rabbit were shown to react only with the acrosome of mouse sperm. This result is shown Figure 20. The titres of mouse antisera were increased to 1:500 after the fourth immunization. Specific binding between the antisera and mouse sperm coated on microwells was also demonstrated by ELISA. Antisera against recombinant MSA-63 fusion proteins showed significant dose-dependent binding to coated mouse sperm. In contrast, antisera against host bacterial extract revealed only nonspecific binding to coated wells. Results of this analysis are given in Figure 21.

Figure 20. Indirect immunofluorescent staining of mouse sperm by using mouse antisera against MSA-63 fusion protein. Mouse sperm under visible light and UV light are presented in (A) and (B), respectively (X 400). (The data were published in Liu et al., 1990)

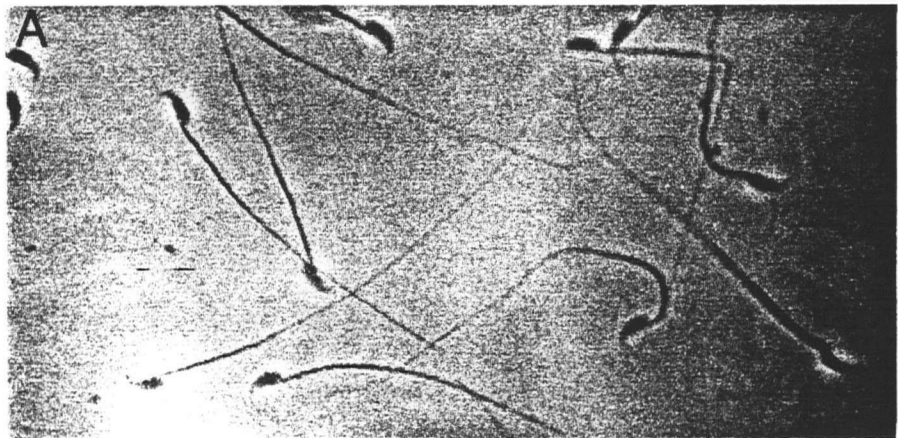
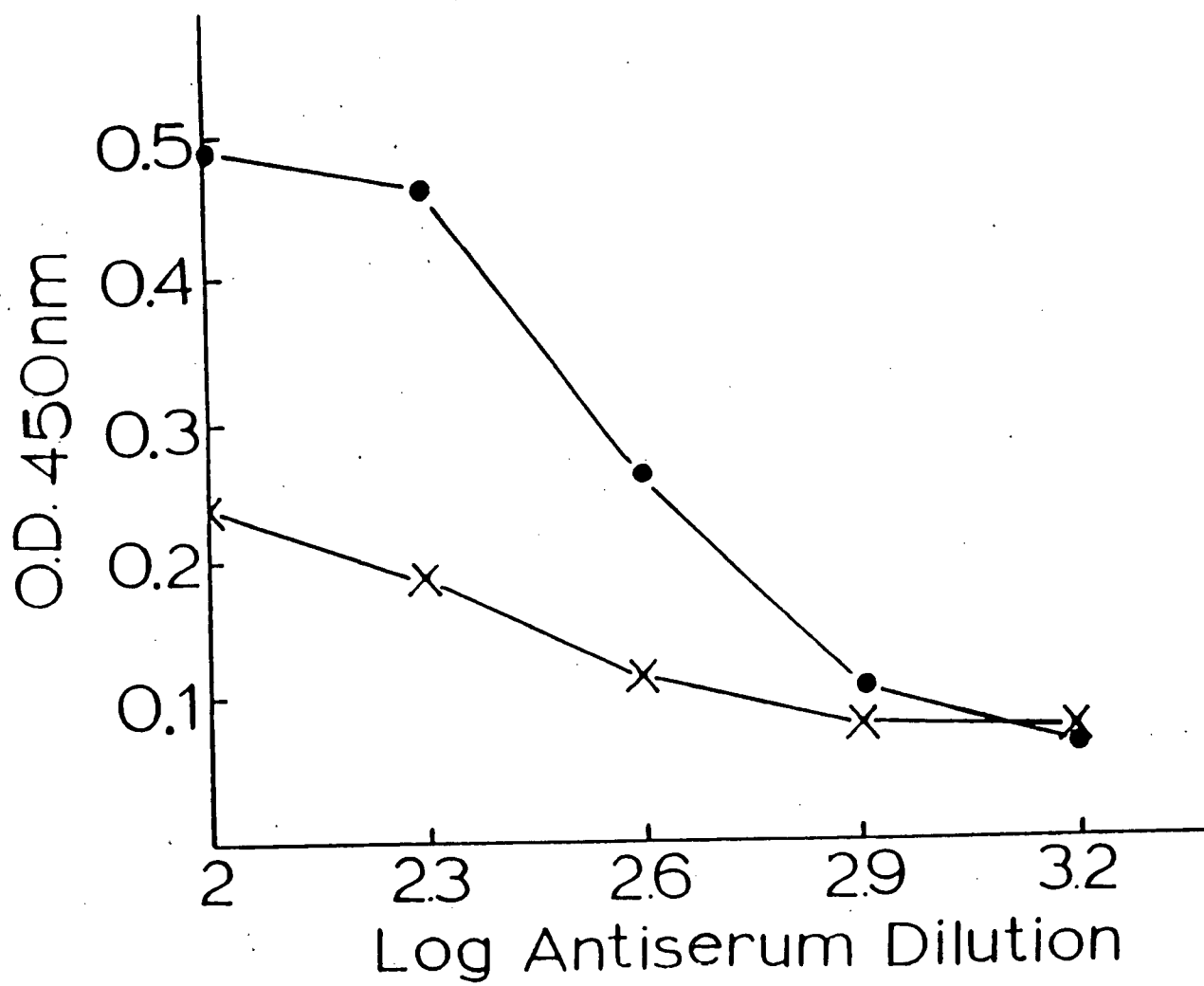


Figure 21. ELISA showing the binding between mouse antisera against MSA-63 fusion protein and mouse sperm homogenate coated on microwells. Absorbance at 450 nm was plotted as a function of antiserum dilution on a log scale, where (●) denotes the mouse antiserum against fusion MSA-63 protein and (X) is that against *E. coli* host Y1089. (The data were published in Liu et al., 1990)



By using the mouse in vitro fertilization experiment, the mouse antisera against recombinant fusion protein could significantly inhibit the in vitro fertilization of mouse oocyte at a serum dilution of 1:10 (fertilization rate of 37.7 % vs. 74.1 % for the control, $P < 0.001$). Supplemental details are presented in Table 9.

Table 9. Inhibitory effect of mouse antisera raised against MSA-63 fusion protein on in vitro fertilization of mouse oocytes (Liu et al., 1990).

Immunogen	Number of assay	Ova examined (No.)	Ova fertilized (No.)	Fertilization rate (%)	P ^C value
Homogenate of ^a host Y1089	2	31	23	74.1	
Recombinant ^b MSA-63 fusion protein	2	61	23	37.7	<0.001

^aThe titre of antiserum to MSA-63 fusion protein is 1:500 as determined by the indirect immunofluorescent assay.

^bRecombinant MSA-63 fusion protein was partially purified from homogenate of bacteria lysogens by Sephacryl S-300 gel filtration chromatography.

^cCompared with the control group.

Part IV. DEVELOPMENTAL STUDIES OF MSA-63 ANTIGEN

A. CONSERVED NATURE OF SA-63 GENE AND ITS LOCATION ON HUMAN CHROMOSOME

To examine the diversity of SA-63 gene among different mammalian species, genomic DNA isolated from dog, rabbit, rat, guinea pig, human, and mouse of both sexes were shown to hybridize with cDNA probe of MSA-63. Results of this Southern blot analysis are shown in Figure 22. This analysis revealed the conserved nature of SA-63 gene among different mammalian species. Moreover, the genomic DNA from either sex (Figure 22, lanes 5-8) gave essentially the same pattern and intensity of hybridization through this analysis.

The location of SA-63 gene on human chromosome was also determined by using the blot of human-hamster somatic cell hybrid panel (from BIOS Co.). By using the MSA-63 cDNA insert with the size of 1.2 Kb as a probe, the hybridization signal was detected on lanes 16 and 17 in Figure 23. Preliminary results of this analysis seemed to suggest that the SA-63 gene is located on human chromosome 11 according to the instruction manual from BIOS Co.

Figure 22. Southern blot analysis of SA-63 gene from different mammalian species. A: The Bgl II digested genomic DNA on 0.8 % agarose gel. B: The pattern of hybridization with ^{32}P -labeled MSA-63 cDNA probe. DNA specimens are from: lane 1, dog; lane 2, rabbit; lane 3, rat; lane 4, guinea pig; lane 5, male mouse; lane 6, female mouse; lane 7, male human; lane 8, female human. Mixture of Lambda DNA digested by Hind III and ϕX174 RF DNA digested by Hae III in Kb are shown on the left as size markers. (Work performed in Dr. Chris Lau's lab at University of California, San Francisco) (The data were published in Liu et al., 1990)

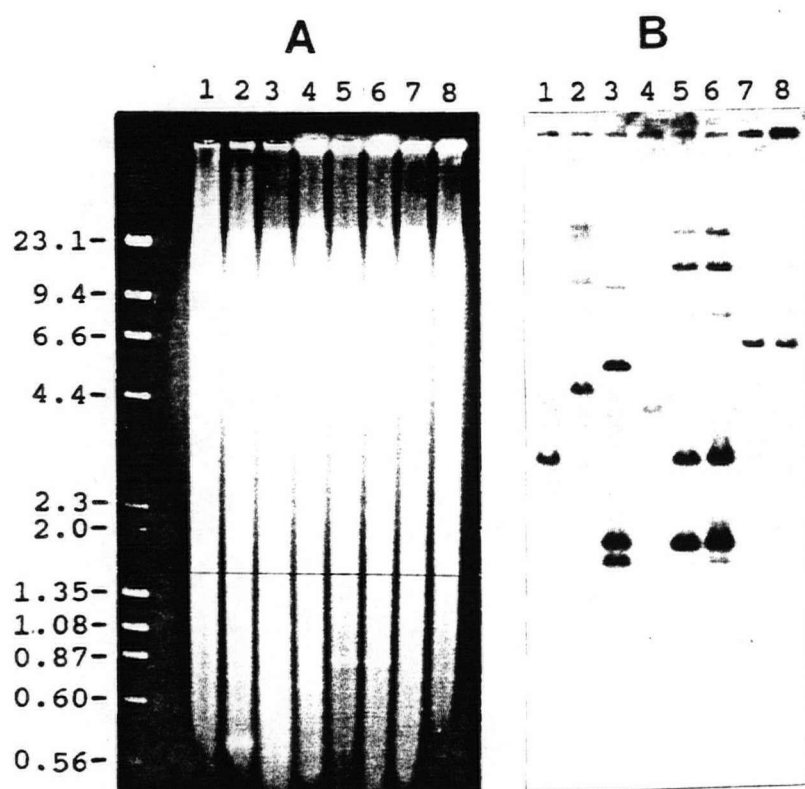
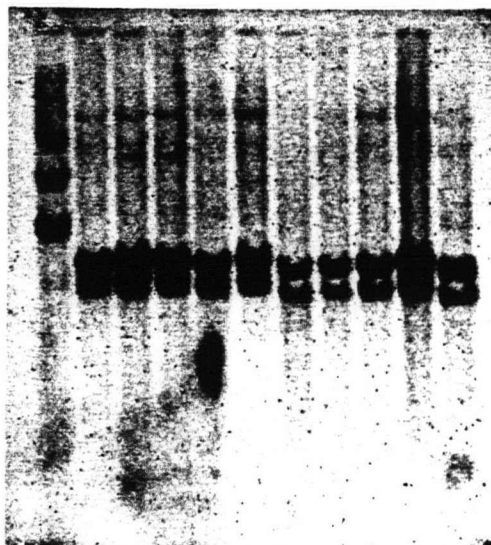
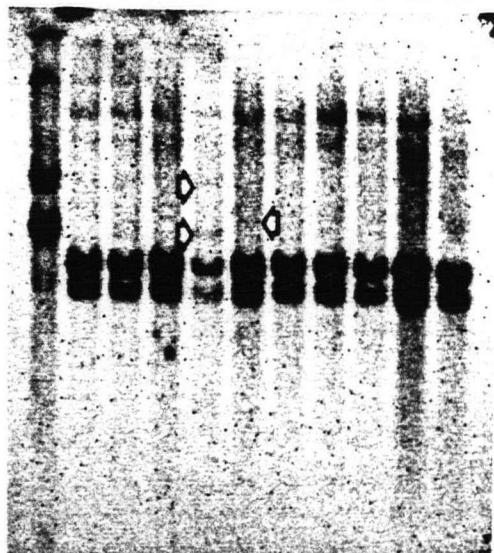


Figure 23. Southern blot analysis of SA-63 gene by using a blot of EcoR I digested human-hamster somatic cell hybrid panel. The ^{32}P labeled-MSA-63 cDNA clone was used as hybridization probe. Lanes 1 and 12 are the control of human DNA and lanes 11 and 22 are the control of hamster DNA. The human specific positive hybridization signals are indicated by arrows. (The blot was purchased from BIOS Co., New Haven, CT)

1 2 3 4 5 6 7 8 9 10 11



12 13 14 15 16 17 18 19 20 21 22



B. TISSUE-SPECIFIC EXPRESSION OF MSA-63 GENE

By using the cDNA insert of clone 12 as a probe, the expression of MSA-63 gene was examined in different mouse tissues by Northern blot analysis. Results of this analysis indicated that this cDNA probe could hybridize to a 1.5 Kb mRNA in adult mouse testis. However, no hybridization of this cDNA probe was observed with fetal testis, ovary or any other mouse somatic tissues (i.e. brain) from either sex. Results of this analysis are shown in Figure 24.

C. EXPRESSION OF MSA-63 GENE IN DEVELOPING MOUSE TESTES

RNA extracted from mouse testes of different ages (5 days to 35 days old) were separated by agarose gel electrophoresis. Northern blot hybridization with ^{32}P -labeled cDNA probe of MSA-63 gene was performed. mRNA expression of MSA-63 was first detected on day 20 after birth in the developing mouse testis and reached a maximum level on day 35 after birth. The hybridization analysis is presented in Figure 25.

The expression of MSA-63 protein in developing mouse testis

Figure 24. Northern blot analysis of MSA-63 gene expression in different mouse tissues. The filter was hybridized with ^{32}P -labeled MSA-63 cDNA clone 12 and washed under high stringent conditions. The band was visualized by autoradiography. Denatured PM2 DNA digested by Hind III are used as a size marker in Kb and illustrated on the left. RNA samples of mouse tissues are: lane 1, male brain; lane 2, female brain; lane 3, fetal male testis; lane 4, fetal female ovary and lane 5, adult testis, respectively. (Work carried out in Dr. Chris Lau's lab at University of California, San Francisco) (The data were published in Liu et al., 1990)

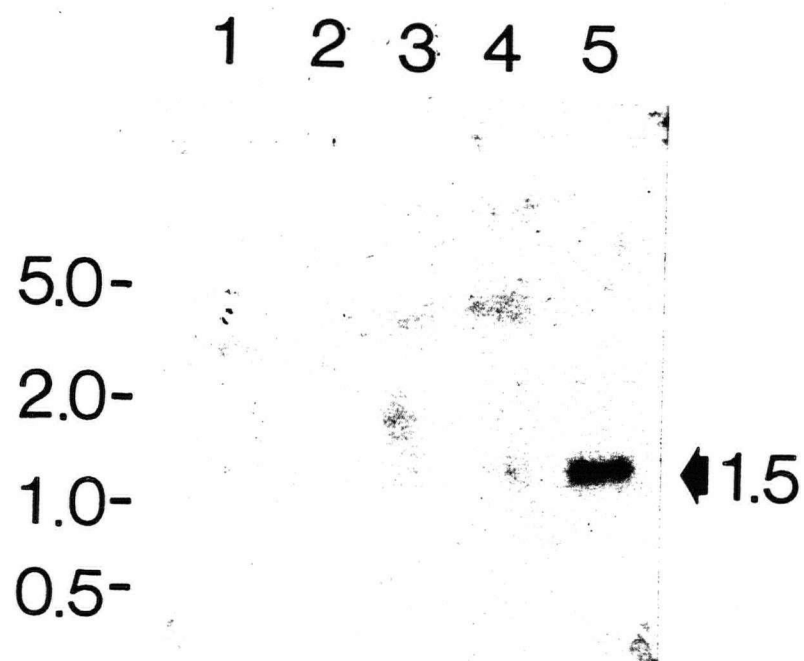
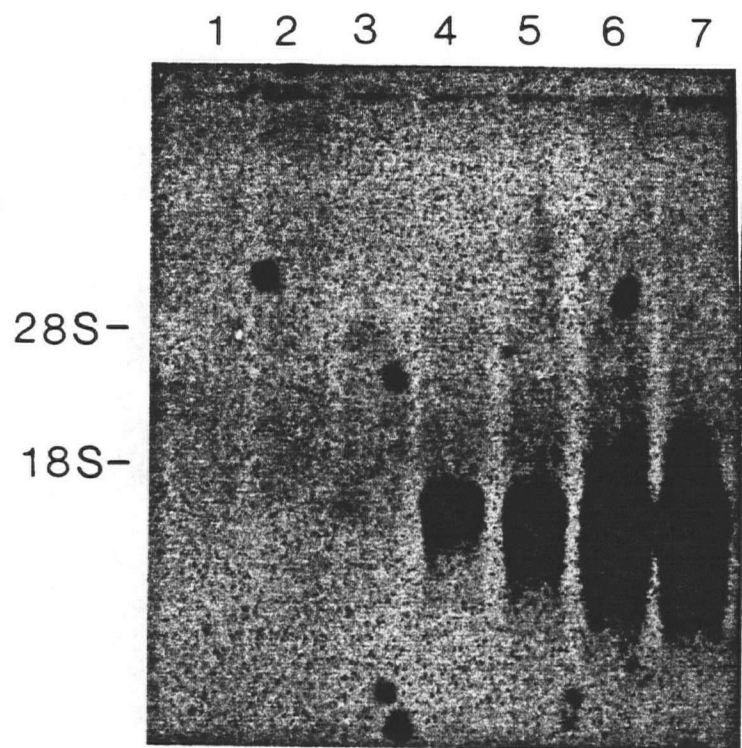


Figure 25. Northern analysis of RNA obtained from mouse testes at various ages of development. Lane 1 to 7 contains 20 ug of total RNA each from testes at age of 5, 10, 15, 20, 25, 30, and 35 days old mice, respectively. The filter was hybridized with ^{32}P -labeled MSA-63 cDNA clone 12 and washed under high stringent conditions. The size markers, 28S and 18S ribosomal RNA are shown on the left.



was also studied. Rabbit antisera against MSA-63 served as the detecting probe in a dot blot enzyme immunoassay for following the specific expression of MSA-63 protein in testis from mice of different ages. As shown in Figure 26, the MSA-63 antigen in developing mouse testis was not detected in mouse testis until day 20 after the birth.

D. IMMUNOHISTOCHEMICAL STUDIES OF MSA-63 ANTIGEN IN MOUSE TESTIS

An immunohistochemical method was used to analyze the expression of MSA-63 protein in developing mouse testis. By using indirect immunofluorescent assay on the frozen testis sections, it could be clearly demonstrated that MSA-63 was not detected until day 25 after birth. MSA-63 protein was mainly localized on the acrosomal region of the elongated spermatids. Results of this assay are presented in Figure 27.

Figure 26. Expression of MSA-63 antigen in developing mouse testes. Dot 1 to 8 contains 0.5 mg each supernatant of mouse testes homogenate at the ages of day 5, 10, 15, 20, 25, 30, 35, and 60 respectively. Dot 9 and 10 contain 0.5 mg of mouse liver homogenate supernatant and 12.5 ug BSA, respectively. Immunoblot assay is performed with (A) rabbit anti-MSA63 sera; (B) rabbit anti-LDHX sera (C) normal rabbit serum. Column (D) was stained by amido black for protein quantitative control. Details of this assay procedure are given in the section of Materials and Methods.

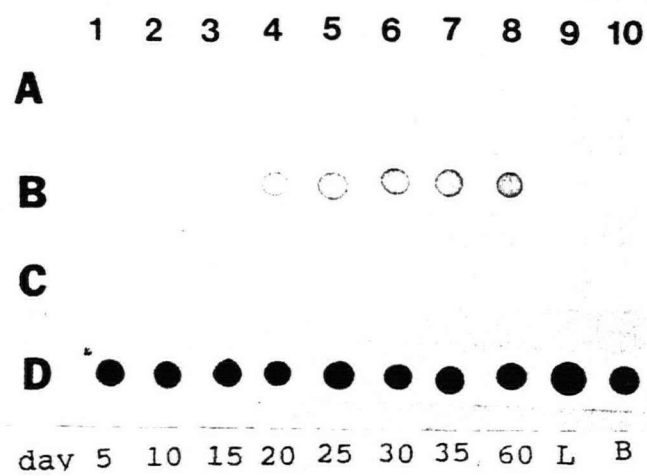
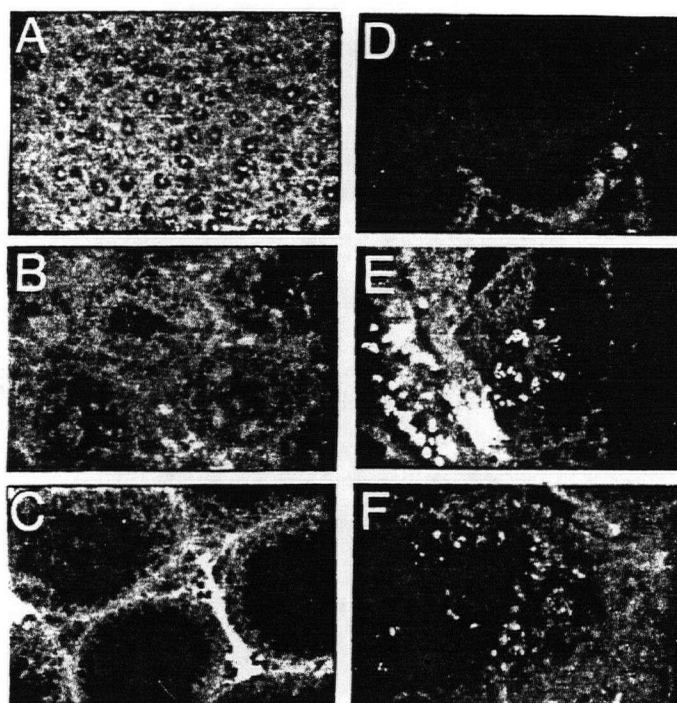


Figure 27. Immunohistochemical analysis of MSA-63 in mouse testes at various stage of development. The testicular tissue sections from A to F are at age of day 5, 10, 15, 20, 25, and 30 respectively (X 400).



IV. DISCUSSION

PART I. CHARACTERIZATION OF HS-63 REACTIVE SPERM ANTIGENS AND EVALUATION OF THEIR ROLES DURING FERTILIZATION PROCESSES

The process of mammalian fertilization is a continuous and progressive event. Spermatozoa must undergo a sequence of programmed events including capacitation, acrosome reaction and sperm-oocyte fusion in order to complete the overall fertilization process (Fraser and Ahuja, 1988; Wassarman, 1987a,b). To study such a complicated system, it is essential to identify those sperm proteins which are involved in the crucial steps of the fertilization process. Numerous efforts by others have been made to identify those crucial sperm components mainly by using monoclonal sperm antibodies as the initial tools for the identification (Naz, et al., 1984a; O'Rand et al., 1984; Gerton et al., 1988; Saling, 1986; Primakoff et al., 1988a).

In our laboratory, HS-63 monoclonal antibody was initially found to react with the acrosome of human and mouse sperm (Figure 8) and inhibit mouse in vitro fertilization (Lee et al. 1986; Menge et al. 1987; Anderson et al., 1987; Table 2, 3, 4). It was

therefore hypothesized that the corresponding sperm antigen (SA-63) in the acrosome may be involved in fertilization process.

HS-63 was shown to react with the acrosome of live capacitated sperm, but not with the fresh uncapacitated ones (Figure 3). Sperm capacitation is a unique process required for mammalian fertilization. Certain multifaceted changes of sperm surface membrane and intracellular components are required during the capacitation process (Fraser, 1983, 1984). Redistribution of intramembranous particles and intracellular calcium content in the head and midpiece regions of spermatozoa might be associated with sperm capacitation (Koehler and Gaddum-Rose, 1975; Bearer and Friend, 1982). Following the treatment of uncapacitated sperm with ethanol, high salt (0.5 M KCl) or detergent such as Triton X-100, SA-63 may be exposed to the sperm surface and become available for antibody binding. Therefore, it is reasonable to assume that following capacitation, the SA-63 becomes exposed as the result of a sperm surface membrane change. This observation seemed to suggest that SA-63 may be initially localized beneath the plasma membrane of the sperm acrosome, and becomes exposed following the capacitation process.

The sperm acrosome reaction involves the fusion of plasma and outer acrosomal membranes and the release of hydrolytic enzymes, which is similar to the process of exocytosis (Russell et al., 1979; Wassarman, 1987a). Some of the sperm acrosomal proteins reside in the plasma and outer acrosomal membrane were found to shed during the acrosome reaction (Morton, 1975; Meizel and Mukerji, 1975). However, proteins associated with the inner acrosomal membrane will remain on the sperm surface after completion of the acrosome reaction (i.e. acrosin) (Dunbar et al., 1976). In view of the fact that HS-63 could no longer react with the acrosome-reacted sperm, it can be suggested that SA-63 may lie in between the inner acrosomal membrane and plasma or outer acrosomal membrane.

In summary, our experiments suggest that SA-63 can be a useful marker for studying sperm capacitation and the acrosome reaction. However, the exact location of SA-63 antigen in mammalian sperm remains to be determined by electron microscopy.

The inhibitory effect of HS-63 on in vitro fertilization of mouse oocytes was found to be dose-dependent (Table 2). When the concentration of HS-63 was diluted to 0.04 mg/ml, there was no

significant inhibition of fertilization in vitro. Results of our experiments also revealed that HS-63 had little effect on sperm motility and sperm-zona binding in the mouse system. However, Wolf and his coworkers (Archibong et al., 1991) observed that HS-63 could significantly inhibit human sperm-zona binding in a typical human hemizona assay system. The discrepancy could be due to the different affinity between HS-63 binding to human sperm and that to mouse sperm.

In the human sperm penetration assay, HS-63 was found to inhibit not only the penetration rate but also the mean number of human sperm attached to zona-free hamster ova (Table 3). In a given sperm penetration assay, it is known that the resulting fertilization rate is associated with many physiological factors such as the degree of sperm acrosome reaction, sperm motility, avidity of binding to the egg surface and efficacy of sperm-egg fusion. (Fukuda et al., 1989; van Kooij et al., 1986a,b; Aitken et al., 1983; Yang et al., 1988). The binding of HS-63 to capacitated human sperm may block some of these factors so that the sperm penetration rate is reduced.

It has been reported that human sperm bound to zona-free

hamster ova were found to be acrosome-reactive (Singer et al., 1985). The number of bound human sperm on the surface of zona-free hamster ova may be related to the degree of sperm acrosome reaction (Singer et al., 1985). It is possible that the binding of HS-63 to capacitated sperm could impede the rate of sperm acrosome reaction, so that the number of sperm attached to zona-free hamster ova was reduced significantly.

The ability of acrosome-reactive sperm to fertilize eggs is not well known and could be species specific (Longo, 1987). In the case of mouse, only acrosome-intact sperm show the fertilizing capacity. Acrosome-reacted mouse sperm can no longer bind to the zona pellucida of the ova (Saling et al., 1979; Storey et al., 1984; Wassarman, 1987a). However, in humans and guinea pigs, experimental evidence suggested that the acrosome-reacted sperm might retain their ability to fertilize the egg (Wolf, 1986; Yanagimachi, 1984). In view of the fact that HS-63 only reacts with the acrosome-intact capacitated sperm, one would expect that HS-63 can only inhibit the initial fertilization steps prior to sperm penetration into zona pellucida and sperm-egg membrane fusion.

In order to further explore the possible inhibitory mechanism of HS-63 on fertilization, several in vitro assays were performed including its effect on sperm-zona binding and zona-induced acrosome reaction. Our preliminary experimental evidence showed that although HS-63 has little inhibitory effect on mouse sperm-zona binding, it does significantly inhibit zona-induced sperm acrosome reaction. Judging from this observation, it can be assumed that the inhibitory effect of HS-63 on fertilization could result from its interference with the steps of zona-induced acrosome reaction prior to sperm penetration into zona pellucida.

In view of the fact that the mechanism of zona-induced acrosome reaction is currently unknown (Roldan and Harrison, 1990), the exact stage at which HS-63 interferes with the acrosome reaction remains to be elucidated.

According to previous studies using chlortetracycline (CTC) fluorescent assay, the process of acrosome reaction can be divided into three stages (Saling and Storey, 1979; Storey et al., 1984). They are (1) F phase - sperm with intact acrosome, (2) S phase - intermediate stage of sperm acrosome reaction, and (3) AR phase - acrosome reacted sperm, respectively. This assay

was also successfully used to study the inhibition of sperm antibody to the acrosome reaction (Saling et al., 1986). M42 monoclonal antisperm antibody was found to inhibit zona-induced acrosome reaction at F phase and had no effect on ionophore-induced acrosome reaction (Leyton et al., 1989). It was suggested that M42 could affect calcium influx during the acrosome reaction and might not be involved in membrane fusion (Lyeton et al., 1989). In view of this report, CTC staining assay may be used to evaluate the effect of HS-63 on sperm acrosome reaction in the future.

From the in vivo mating studies with passive immunization of female mice with HS-63, the antibody titre of HS-63 in mouse circulating sera could reach to 1:200,000 by using indirect immunofluorescent assay. However, the local antibody titre of HS-63 in oviduct fluid was estimated to be 1:2,500. Judging from the antibody dose-dependent effect on the fertilization of mouse oocytes in vitro (Table 2), the local antibody titre in oviduct fluid might be too low to cause any inhibition on in vivo fertilization.

In vivo mating experiments via passive immunization of

monoclonal antibodies have been previously reported by Saling and coworkers (Saling et al., 1986). Two monoclonal antibodies, M29 (IgM subclass) and M42 (IgG1 subclass) that reacted separately with mouse sperm equatorial segment and acrosome were clearly demonstrated to inhibit mouse fertilization in vivo following passive immunization in female mice. It was observed that increasing the dose of these two antibodies led to a significant reduction in fertilization level. Since sperm antigens recognized by HS-63 and M29 or M42 are apparently different, the differential effectiveness between HS-63 and M29/M42 could be explained.

The stability of the antigenic determinant recognized by HS-63 was studied via treatments of sperm with detergents, proteolytic enzymes, glycosidases, or carbohydrate modifying chemicals. Through such extensive analyses, it was concluded that HS-63 reacts with a peptide determinant of SA-63 which is still immunoactive in the presence of nonionic detergent. Therefore, the recombinant fusion protein carrying part of SA-63 could still be recognized by HS-63 (Figure 19). Meanwhile, through the molecular cloning of MSA-63 cDNA, the antigenic determinants were tentatively assigned from the amino acid sequence of the deduced

protein (Figure 16 and 17). However, hydrophilicity is only one of the predictors for antigenic determinant.

Part II. PURIFICATION AND CHARACTERIZATION OF MSA-63

By means of indirect immunofluorescent assay, it was clearly established that MSA-63 antigen in mouse testis exists as a membrane-associated soluble protein. Thus, when the conventional soluble protein purification procedure was employed for purification of MSA-63 antigen, the purified MSA-63 precipitated out during storage. This is perhaps due to hydrophobic aggregation of MSA-63 protein in the absence of detergent (Hjelmeland and Chrambach, 1984). Also, this observation further suggested that MSA-63 antigen is a membrane-associated soluble protein and exists as aggregates in the native state (Figure 8). In view of this problem, Triton X-100 was introduced in the second purification procedure. In the presence of 0.1 % Triton X-100, no precipitation was found in the solution containing purified MSA-63 solution.

As shown in Table 5, the detergent extraction method resulted in a much higher protein recovery of MSA-63 as compared to that of the soluble extraction method. It was also noticed that in the presence of Triton X-100, the estimated immunoactivity of purified MSA-63 is somewhat lower.

After 2D-gel electrophoresis, purified MSA-63 displayed a number of protein spots (Figure 6A and 6B). Some of the protein spots in group 1, 2, and 4 were identified to be sperm actin fragments through amino acid sequence analysis of the isolated peptides. The major actin spots on a 2D-gel were found to have molecular weights of 50 Kd (group 1) and 45 Kd (group 2) and pIs around 5. Presumably, they are major actin fragments in mouse sperm (Flaherty et al., 1986). Rabbit antisera against protein spots in group 1 exhibited high cross-reactivity to α -actin. However, those raised against protein spots in group 2 showed little cross-reactivity to α -actin. Through the analysis of amino acid sequence homology of peptides isolated from protein spots in group 1, 2, and 4, it was concluded that they are either actin-related proteins or their degradation products.

By using Western blot analysis, antisera raised against protein spots in group 3 (MSA-63) could cross-react with all the protein spots on a 2D-gel. However, antisera against protein spots of group 1 and 2 (actins) could not react with MSA-63 in group 3. It is possible that protein spots in group 3 which were used to raise anti-MSA-63 sera may be contaminated with actin-related

protein fragments.

Through gel filtration analysis, it was clearly demonstrated that MSA-63 exists as aggregates in the native form with a molecular size ranging from 200 to 300 KD (Figure 8). The aggregated form of MSA-63 could result from either self association or complex formation with actin-related proteins. It is intriguing to find that actin-related proteins or fragments are tightly associated with MSA-63 protein.

The interaction between MSA-63 and actin (Sigma A2522) has also been investigated (Figure 9). It is assumed that changes of molecular distribution of α -actin may be due to the interaction between MSA-63 and actin or degradation caused by MSA-63.

The presence of actin in mammalian spermatozoa has been reported (Camatnini et al., 1986; 1987; Casale et al., 1988; Flaherty et al., 1983, 1986; Olson and Winfery, 1985). However, the locations of actin in sperm vary with different mammalian species (Flaherty, 1986). The precise functional role of actin in sperm is currently unknown (Vogl, 1989).

In mouse sperm, F-actin was found on the surface of

postacrosomal region (Clarke and Yanagimachi, 1978), midpiece and anterior principal piece (Flaherty, 1986). Results of our study may suggest that, actins are associated with MSA-63 protein in the acrosomal region and may be involved in sperm acrosome reaction.

The proteolytic activity in the purified MSA-63 preparation was studied. When the purified MSA-63 was incubated at 37°C for 5 days and two weeks at neutral pH, the protein spots in group 3 revealed no significant changes. However, groups 1 and 2 protein spots were found to disappear when analyzed on a 2D-gel (Figure 15C). On the other hand, new protein spots with molecular sizes ranging from 10 to 20 Kd appeared on 2D-gel after long incubation. The origin and the nature of the proteolytic activity in the purified MSA-63 preparation remain to be elucidated.

MSA-63 protein (group 3 on a 2D-gel) showed serial of protein spots with similar pI (about 4.2) but different molecular sizes ranging from 24 to 45 Kd. According to the amino acid sequence deduced from full length cDNA insert, the molecular weight of MSA-63 should be 27,948 dalton. The treatment of O- and N-glycosidase or reducing reagent failed to change the

microheterogenicity of MSA-63 protein on 2D-gel (Figure 10).

Hence, one can assume that the presence of carbohydrate moiety and disulfide linkage may not be the cause of such microheterogenicity in MSA-63.

It is therefore proposed the molecular size heterogeneity of MSA-63 protein may result from the cross-linking or covalent aggregations of MSA-63 or its association with other molecules such as actins. Further tryptic analysis of MSA-63 protein (group 3) should enable us to solve this question.

Part III. CLONING AND CHARACTERIZATION OF MSA-63 cDNA

The cDNA encoding MSA-63 protein was successfully cloned by using immunoscreening of a mouse testis cDNA library constructed in the lambda gt 11 expression vector (Young and Davis, 1983).

Generally most, cDNA inserts isolated from the immuno-detectable cDNA clones are not full length (Synder et al., 1987). Polyclonal antisera have the advantage of recognizing multiple epitopes of a given protein. A monoclonal antibody can only recognize a single epitope and is therefore of limited use for immunoscreening. Thus, polyclonal antisera are usually recommended as probes for immunoscreening (Huynh et al., 1985).

Using this technique, the cDNA clones of MSA-63 antigen were isolated from a mouse testis cDNA library by immunoscreening using rabbit antiserum against MSA-63. Through immunoscreening of 6×10^5 bacteriophages from mouse testis cDNA library, more than 60 positive clones were identified (Figure 12).

The most difficult part of cDNA isolation by immunoscreening is to determine whether the immunoreactive clones are derived

from the gene of interest. Southern blot analysis was usually employed to determine if the inserts of the positive cDNA clones can cross-hybridize with each other. Following hybridization, only those with a high degree of homology showed positive signals under highly stringent wash conditions. As a result, four positive cDNA clones were isolated (see Figure 13). From the restriction mapping analysis, they were shown to be derived from a single structural gene.

Experiments were performed to determine if the isolated cDNA clones express MSA-63 protein. The recombinant fusion protein prepared from the positive cDNA clones could be recognized by anti-MSA-63 sera in the Western blot assay (Figure 18). Cell lysates of lysogen which produced the recombinant fusion protein were able to inhibit HS-63 binding to sperm (see Figure 19). Therefore, it can be suggested that HS-63 reactive epitope exists in the recombinant fusion protein produced from E coli. Results of this experiment further confirmed that the HS-63 specific epitope is a peptide and not the carbohydrate moiety.

The PC\GENE computer program was used to analyze the hydrophobic/hydrophilic nature of cDNA deduced amino acid

sequence of MSA-63 protein. A hydrophobic amino terminus was identified in the deduced amino acid sequence, possibly being an eukaryotic secretory signal (von Heijne, 1986). Therefore, MSA-63 protein may be transported across the membrane as a secretory protein after its synthesis. Due to the lack of membrane-spanning segment in the entire amino acid sequence, MSA-63 is considered as a peripheral protein, but not a membrane integral protein (Klein et al., 1985). This prediction is in agreement with our earlier observation that MSA-63 is a membrane-associated soluble protein. Other cytoskeletal protein components such as actins may be required to anchor MSA-63.

The size of mRNA identified from mouse testis was close to 1.5 Kb (Figure 24). However, the isolated cDNA clones had a size of only 1.2 Kb (Figure 16). It is possible that the poly A tail of the mRNA may be more than 200 bp. Further study by using the method of mRNA primer extension may be required to identify the size of full length MSA-63 cDNA.

Usually, the polyadenylation in eukaryotic mRNA is required to stabilize the mRNA for long term expression (Burton et al., 1983; Belasco et al., 1985). However, an mRNA consensus

degradation sequence (AUUUA) was found in the 3' noncoding region. The presence of this sequence seems to promote mRNA decay (Shaw and Kaman, 1986; Caput et al., 1986). Transiently-expressed mRNAs, such as those for oncogenes, lymphokines and interferons, also carry such a segment in their 3' noncoding region (Wilson and Treisman, 1988; Jones and Cole, 1988). Therefore, the expression of MSA-63 gene may also be regulated by this segment during spermatogenesis.

The computer search from the data base of the PC\GENE program (release 6.25, July 10, 1990) revealed that there was no significant homology of MSA-63 with any other known nucleotides and proteins. However, 60 to 70 % homology was observed between MSA-63 cDNA sequence and that of a human sperm acrosomal antigen, SP-10 (Wright et al., 1990). In contrast, SP-10 antigen was not conserved in all species. Sperm from several mammalian species including rabbit, bull, rat, guinea pig, and cat do not express any antigen which is immunologically or structurally related to SP-10 antigen (Herr et al., 1990a,b). MHS-10, the monoclonal antibody by which SP-10 antigen was identified, was shown previously to inhibit fertilization by the WHO workshop (Anderson et al., 1987). The structural and functional relationship between

MSA-63 and SP-10 antigen in sperm acrosome remain to be explored in the near future.

Part IV. DEVELOPMENTAL STUDY OF MSA-63 ANTIGEN

A previous study showed that HS-63 monoclonal antibody is species conserved and sperm specific (Anderson et al., 1987). At the molecular level, by Southern blot analysis using MSA-63 cDNA as a probe, the SA-63 gene was found to be conserved among different species in both sexes (Figure 22). In human, the location of the SA-63 gene was suggested to be on chromosome 11 (Figure 23). Therefore, the SA-63 gene is localized on an autosome and not on the sex chromosome. Results of these molecular analyses are consistent with those of immunological analysis by the WHO workshop regarding the broad species cross-reactivity of HS-63 monoclonal antibody (Anderson et al, 1987).

Results of the Northern blot analysis showed that the mRNA of MSA-63 was only expressed in adult testis but not in fetal testis or any other somatic tissues (Figure 24). In the developing mouse testis, spermatogenesis begins on day 5 postnatally, when the primitive type A spermatogonia begin to proliferate (Bellve' et al., 1977). On day 9, type B spermatogonia pass through a mitotic division to yield preleptotene spermatocytes. By day 10, leptotene spermatocytes appear and quickly differentiate to form

zygotene spermatocytes. By day 14, pachytene spermatocytes are formed. Around day 18 of development, the first meiotic division occurs to form secondary spermatocytes (Bellve', 1979). On day 20, a significant number of pachytene spermatocytes in mouse testis are ready to enter the round spermatid stage (Hecht et al., 1985). The transcription of MSA-63 was first detected in mouse testis on day 20 after birth and the maximum expression was found on day 35 (Figure 25). Therefore, the nature of gene expression of MSA-63 appears to be postmeiotic.

At the protein level, the immunoactivity of MSA-63 antigen was first detected in testis of 20 day old mice (Figure 26). This observation suggests that transcription and translation of MSA-63 gene are initiated simultaneously in the developing mouse testis.

Sperm specific genes are valuable for the study of the regulation of gene expression during spermatogenesis (Kleene et al., 1984; Hecht et al., 1985; Waters et al., 1985; Villasante et al., 1986; Mutter and Wolgemith, 1987). Lactate dehydrogenase-X (LDH-X), a sperm specific isozyme, is expressed in the haploid germ cell (Hintz and Goldberg, 1977; Wheat et al., 1977;

Meistrich et al., 1977; Wieben, 1981; Tanaka and Fujimoto, 1986). Transcription and translation of the LDH-X gene are initiated at the pachytene stage of germ cell differentiation (Jen et al., 1990), the stage of which is considered premeiotic.

Protamine, a testis-specific histone, is found to be expressed in the diploid cell during meiotic prophase (Hecht et al., 1985). However, the message of protamine is stored without translation until the stage of haploid spermatids.

Phosphoglycerate kinase-2 (PGK-2) is a sperm specific isozyme which is involved in the glycolytic metabolism of spermatozoa (Gold et al., 1983). The postmeiotic expression of PGK-2 is also subject to strong translational control. As well as protamine, the mRNA of PGK-2 is stored for many days before being translated in the round spermatid stages of spermatogenesis (Gold et al., 1983).

Several proto-oncogenes are also found to be expressed during spermatogenesis (i.e. c-mos, c-abl and N-ras). The expression of these proto-oncogenes may be associated with the control of sperm proliferation and differentiation in the testis (Willison and

The timing of MSA-63 expression is around the onset of spermiogenesis. At this moment, morphological changes in round spermatids occur concomitantly with alterations in the complement of constituent proteins (Boitani et al., 1980; Millette and Moulding, 1981; Stern et al., 1983). Meanwhile, acrosome formation is initiated immediately after the completion of meiosis and intimately involves the Golgi apparatus in early spermatids (Clermont and Tang, 1985). From our study on the gene expression of MSA-63, it can be concluded that MSA-63 is produced at the initial stages of acrosome formation.

Factors that regulate the MSA-63 gene expression in spermatogenesis are unknown. In testis, sperm cells are in intimate contact with Sertoli cells during all phases of spermatogenesis, which includes meiotic proliferation, meiotic division, and differentiation (Russell, 1980). It is believed that Sertoli cells may play an important regulatory role in spermatogenesis (Tindall et al., 1985). Hence, the expression of MSA-63 antigen might be regulated directly or indirectly by the Sertoli cells. This hypothesis remains to be confirmed.

V. CONCLUSION AND FUTURE RESEARCH DIRECTION

During the last two decades, numerous biotechniques have been developed for applications in biomedical research. They have greatly revolutionized the approaches by which scientists solve the important problems in reproductive biology.

In this study, hybridoma, protein chemistry, and recombinant DNA technology represent the most important tools in dealing with problems related to structural and functional roles of specific sperm antigens during the fertilization. Without adequate applications of these techniques, it would have been impossible to obtain the information presented in this thesis.

Systematically, with the application of hybridoma technology, numerous monoclonal antibodies to sperm were generated in our laboratory. Based on their respective sperm specificity and inhibitory effects on sperm functions and fertilization, HS-63 monoclonal antibody was among those selected. By means of immunoaffinity chromatography using this antibody as the ligand, specific mouse sperm antigen (MSA-63) recognized by HS-63 was purified and characterized biochemically and immunologically.

Furthermore, through the immunoscreening of a mouse testis cDNA library with antisera against MSA-63 as the probe, cDNA clones expressing this sperm protein were isolated. The molecular cloning work enabled us to determine mRNA and protein structures. The isolated specific cDNA fragments were also used as probes to study the expression of this sperm antigen during spermatogenesis. Moreover, mass-production of specific sperm antigens can also be achieved via applications of recombinant DNA technology for the development of a sperm antigen based immunocontraceptive vaccine.

Although much information has been obtained regarding the mRNA and protein structure of this sperm specific acrosomal antigen (SA-63), more studies need to be carried out for the full understanding of its structure and function.

In order to determine the organizational and structural features of the genes encoding the SA-63 protein, the entire SA-63 gene will be cloned and sequenced. Its exons, introns and regulatory elements can be investigated. Furthermore, the regulation of the SA-63 gene expression can be evaluated by transfection studies by introducing the entire gene or putative

regulatory regions into tissue culture cells or a transgenic animal system so that specific expression of SA-63 gene can be studied in response to transacting regulatory factors such as growth factors and hormones.

Moreover, the availability of HS-63 monoclonal antibody and a the specific cDNA probe for SA-63 will enable us to study the subcellular location of protein and mRNA in various differentiated spermatogenic cells in the testis and in mature spermatozoa during epididymal transit. Particular attention will be paid to identify how SA-63 is associated with cytoskeletal proteins such as actins.

In view of the protein chemistry, it would be interesting to determine how the protein expressed by the SA-63 gene can exhibit such a broad molecular size distribution. The molecular mechanisms of such a wide-spread microheterogeneity of SA-63 protein and its structural and functional implications are an important subject for further investigations.

Although the results of our preliminary studies revealed that MSA-63 is ineffective as an immunocontraceptive vaccine in

mouse, the contraceptive efficacy of SA-63 in primate species remains to be explored. Because of significant anatomical and physiological differences in the reproductive systems between rodents and primates, one would expect differences in contraceptive efficacy in these two mammalian species. According to recent studies by Wolf and his coworkers in Oregon Regional Primate Center (Archibong et al., 1991), a strong inhibition of HS-63 and rabbit anti-MSA-63 sera on sperm-zona binding was found in a human hemi-zona assay system. The result of this study suggests that SA-63 may be a suitable candidate for immunocontraceptive vaccine in humans. Active immunizations with purified SA-63 from rhesus monkey testes in rhesus monkeys are already being underway at the Oregon Regional Primate Center. The resulting contraceptive efficacy will be assessed by in vitro fertilization and in vivo mating experiments in primates.

Finally, it is important to mention that the strategy developed in this study could serve as a model to study several other sperm antigens and potential sperm vaccine candidates in the future.

References:

- Ahuja, K.K. (1985) Carbohydrate determinants involved in mammalian fertilization. *Am. J. Anat.* 174, 207
- Aitken, R.J., Wang, Y-F, Liu, J., Best, F. and Richardson, D.W. (1983) The influence of medium composition, osmolarity and albumin content on the acrosome reaction and fertilizing capacity of human spermatozoa: development of an improved zona-free hamster egg penetration test. *Int. J. Androl.* 6, 180
- Anderson, D.J. and Alexander, N.J. (1983) A new look at antifertility vaccines. *Fertil. Steril.* 40, 557
- Anderson, D.J., Johnson, P.M., Alexander, N.J., Jones, W.R. and Griffin, P.D. (1987) Monoclonal antibodies to human trophoblast and sperm antigens: Report of two WHO-sponsored workshops. *J. Reprod. Immunol.* 10, 231
- Archibong, A.E., Vijayaraghavan, S., Liu, M.S., Lee, C.Y.G. and Wolf, D.P. (1991) Characterization of the acrosomal antigen (PSA-63) in primate sperm function. *Biol. Reprod.* (submitted)
- Austin, C.R. (1951) Observations on the penetration of sperm into the mammalian egg. *Austral. J. Sci. Res. (B)* 4, 581
- Austin, C.R. and Short, R.V. Eds. (1982) *Germ Cells and Fertilization* (2nd Ed.) (Cambridge University, Cambridge)
- Babcock, D.F., Singh, J.P. and Lardy, H.A. (1979) Alternation of membrane permeability to calcium ions during maturation of bovine spermatozoa. *Dev. Biol.* 69, 85
- Barros, C., Bedford, J.M., Franklin, L.E. and Austin, C.R. (1967) Membrane vesiculation as a feature of the mammalian acrosome reaction. *J. Cell Biol.* 34, C1-C5
- Bavister, B. D. (1982) Evidence for a role of postovulatory cumulus components in supporting fertilizing ability of hamster spermatozoa. *J. Androl.* 3, 365
- Bearer, E.L. and Friend, D.S. (1982) Modifications of anion-lipid domains preceding membrane fusion in guinea pig sperm. *J. Cell Biol.* 92, 604
- Bedford, J.M. (1967) Effect of duct ligation on the fertilizing ability of mammalian spermatozoa from different regions of the rabbit epididymis. *J. Exp. Zool.* 166, 271
- Bedford, J.M. and Rifkin, J.M. (1979) An evolutionary view of the male reproductive tract and sperm maturation in a monotreme mammal-the echidna, *Tachyglossus aculeatus*. *Am. J. Anat.* 156, 207
- Belasco, J.G., Beatty, J.T., Adams, C.W., von Gabain, A. and Cohen, S.N. (1985) Differential expression of photosynthesis gene

in *R. capsulata* results from segmental differences in stability within the polycistronic *rxr A* transcript. *Cell* 40, 171

Bellev', A.R. (1979) The molecular biology of mammalian spermatogenesis. In: Finn, C.A. (ed.) *Oxford Reviews of Reproductive Biology*. Oxford University Clarendon Press, pp. 159

Bellev', A.R., Cavicchia, J.C., Millette, C.F., O'Brien, D.A. Bhatnagar, Y.M. Dym, M. (1977) Spermatogenic cells of the prepuberal mouse: isolation and morphological characterization. *J. Cell Biol.* 74, 68

Biggers, J.D., Whitten, W.K. and Whittingham, D.G. (1971) The culture of mouse embryos in vitro. In: Denial, J. C. (ed.) *Mammalian Embryology*. Freeman and Company, San Francisco. pp. 86

Bleil, J. and Wassarman, P.M. (1980a) Structure and function of the zona pellucida: Identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev. Biol.* 76, 185

Bleil, J. and Wassarman, P. (1980b) Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse zona pellucida possessing receptor activity for sperm. *Cell* 20, 873

Bleil, J. and Wassarman, P. (1983) Sperm-egg interactions in the mouse: Sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* 95, 317

Bleil, J.D. and Wassarman, P.M. (1986) Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *J. Cell Biol.* 102, 1363

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

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248

Burton, Z.F., Gross, C.A., Watanabe, K.K. and Burgers, R.R. (1983) The operon that encodes the sigma subunit of RNA polymerase also encodes ribosomal protein S21 and DNA primase in *E. Coli* K12. *Cell* 32, 335

Camatini, M., Anelli, G. and Casale, A. (1986) Immunocytochemical localization of calmodulin in intact and acrosome-reacted boar sperm. *Eur. J. Cell Biol.* 41, 89

Camatini, M. and Casale, A. (1987) Actin and calmodulin coexist in the equatorial segment of ejaculated boar sperm. *Gamete Res.* 17, 97

Casale, A., Camatini, M., Skalli, O. and Gabbiani, G. (1988) Characterization of actin isoforms in ejaculated boar

spermatozoa. Gamete Res. 20, 133

Caput, D., Beutler, B., Hartog, K., Thayer, R., Bown-Shimer, S. and Cerami, A. (1986) Identification of a common nucleotide sequence in the 3'- untranslated region of mRNA molecules specifying inflammatory mediators. Pro. Natl. Acad. Sci. USA 83, 1670

Chang, M.C. (1951) The fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168, 697

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

Clark, G.N. and Yanagimachi, R. (1979) Actin in mammalian sperm heads. J. Exp. Zool 205, 125

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

Clowin, L.H. and Clowin, A.L. (1967) in: Fertilization, vol. 1. Academic Press, New York. pp.295

Cross, N.L., Morales, P., Overstreet, J.W., and Hanson, F.W. (1986) Two simple methods for detecting acrosome-reacted human sperm. Gamete Res. 15, 213

Dan, J.C. (1967) Acrosome reaction and lysins. In: Fertilization, vol. 1. Academic Press, New York. pp. 237

De Jonge, C.J., Mack, S.R., and Zaneveld, L.J.D. (1989) Inhibition of the human sperm acrosome reaction by proteinase inhibitors. Gamete Res. 23, 387

Distel, R.J., Kleene, K.C. and Hecht, N.B. (1984) In: Hormone action and testicular function. Ann N.Y. Acad Sci 438, 525

Dunbar, B.S., Munoz, M.G., Cordle, C.T. and Metz, C.B. (1976) Inhibition of fertilization in vitro by the treatment of rabbit spermatozoa with univalent isoantibodies to rabbit sperm hyaluronidase. J. Reprod. Fert. 47, 381

Engvall, E. and Perlmann, P. (1971) Enzyme-linked immunosorbent assay (ELISA): Quantitative assay of immunoglobulin G. Immunochemistry, 8, 871

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

Fawcett, D.W. and Bedford, J.M. Eds. (1979) The Spermatozoa (Urban and Schwarzenberg, Baltimore)

- Feuchter, F.A., Vernon, R.B. and Eddy, E.M. (1981) Analysis of the sperm surface with monoclonal antibodies: topographically restricted antigens appearing in the epididymis. *Biol. Reprod.* 24, 1099
- Flaherty, S.P., Breed, W.G. and Sarafis, V. (1983) Localization of actin in the sperm head of the plains mouse, *Pseudomys australis*. *J. Exp. Zool.* 225, 497
- Flaherty, S.P., Winfrey, V.P. and Olson, G.E. (1986) Localization of actin in mammalian spermatozoa: A comparison of eight species. *Anat. Rec.* 216, 504
- Florman, H. M., and Storey, B.T. (1982) Mouse gamete interactions: The zona pellucida is the site of the acrosome reaction leading to fertilization in vitro. *Dev. Biol.* 91, 121
- Fraser, L.R. (1983) Mouse sperm assessed by kinetics and morphology of fertilization in vitro. *J. Reprod. Fertil.* 69, 419
- Fraser, L.R. (1984) Mouse sperm capacitation in vitro involves loss of a surface-associated inhibitory component. *J. Reprod. Fertil.* 72, 373
- Fraser, L.R. and Ahuja, K.K. (1988) Metabolic and surface events in fertilization. *Gamete Res.* 20, 491
- Fujimoto, H. and Erickson, R.P. (1982) Functional assay for mRNA detect many new messages after male meiosis in mice. *Biochem. Biophys. Res. Commun.* 108, 1369
- Fukuda, M., Cross, N.L., Cummings-Paulson, L. and Yee, B. (1989) Correlation of acrosomal status and sperm performance in the sperm penetration assay. *Fertil. Steril.* 52, 836
- Gaunt, S. J. (1982) A 28K-Dalton cell surface autoantigen of a monoclonal antibody. *Dev. Biol.* 89, 92
- Gerton, G.L., O'Brien, D.A. and Eddy, E.M. (1988) Antigens recognized by monoclonal antibody to mouse acrosomal components differ in guinea pig spermatogenic cell and sperm. *Biol. Reprod.* 39, 431
- Gold, B., Fujimoto, H., Kramer, 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
- Goldberg, E. (1973) Infertility in female rabbits immunized with Lactate Dehydrogenase X. *Science* 181, 458
- Goldberg, E. (1975) Effects of immunization with LDH-X on fertility. *Endocrinology (suppl.)*, 78, 202

- Goldberg, E., Wheat, T. E., Powell, J. E. and Stevens, V. C. (1981) Reduction of fertility in female baboons immunized with Lactate Dehydrogenase C4. *Fertil. Steril.* 35, 214
- Greenwood, F. C., and Hunter, W. N. (1963) The preparation of I-131-labeled human growth hormone of high specific radioactivity. *Biochem. J.*, 89, 114
- Hamilton, D.W. (1977) The epididymis. In: *Frontiers in Reproduction and Fertility Control*. MIT, Cambridge, MA. pp.411
- Harboe, N. and Ingild, A. (1973) in: *A Manual of Quantitative Immuno-electrophoresis*. Axelson, N.H. et al. (eds) Universitetsforlaget, Oslo. pp. 161
- Hart, G.W., Brew, K. Grant, G.A., Bradshaw, R.A. and Lennarz, W.J. (1979) Primary structural requirements for the enzymatic formation of the N-glycosidic bond in glycoproteins studies with natural and synthetic peptides. *J. Biol. Chem.* 254, 9747
- Hartmann, J.F. (Ed.) (1983) *Mechanism and Control of Animal Fertilization*. Academic Press, New York.
- Hattori, M. and Sakaki, Y. (1986) Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem.* 152, 232
- Hecht, N.B., Bower, P.A., Kleene, K.C. and Distel, R.J. (1985) Size changes of protamine 1 mRNA provide a molecular marker to monitor spermatogenesis in wild-type and mutant mice. *Differentiation* 29, 189
- Hecht, N.B., Kleene, K.C., Distel, R.J. and Silver, L.M. (1984) The differential expression of the actins and tubulins during spermatogenesis in the mouse. *Exp. Cell Res.* 153, 275
- Heide, K. and Schwick, H.G. (1973) Chemistry and significance of the carbohydrate moieties of human serum glycoproteins. *Angew Chem.* 12, 721
- Herr, J.C., Flickinger, C.J., Homyk, M., Klotz, K. and John, E. (1990a) Biochemical and morphological characterization of the intra-acrosomal antigen SP-10 from human sperm. *Biol. Reprod.* 42, 181
- Herr, J.C., Wright, R.M., John, E., Foster, J. and Flickinger, C.J. (1990b) Identification of human acrosomal antigen SP-10 in primates and pigs. *Biol. Reprod.* 42, 377
- Hintz, M. and Goldberg, E. (1977) Immunohistochemical location of LDH-X during spermatogenesis in mouse testes. *Dev. Biol.* 57, 375
- Hjelmeland, L.M. and Chrambach, A. (1984) Solubilization of functional membrane proteins. *Methods Enzymol.* 104, 305
- Holden, C.A., Hyne, R.V., Sathananthan, A.H. and Trounson, A.O.

(1990) Assessment of the human sperm acrosome reaction using Concanavalin A lectin. *Mol. Reprod. Dev.* 25, 247

Hoskins, D.D., Brandt, H. and Acott, T.S. (1978) Initiation of sperm motility in the mammalian epididymis. *Fed. Proc.* 37, 2534

Huynh, T.V., Young, R.A. and Davis, R.W. (1985) Constructing and Screening cDNA libraries in lambda gt 10 and lambda gt 11. in: *DNA cloning volume 1*. Glover, D.M. (ed.) IRL Press, Oxford. pp 49

Jackson, J. J., Hagopian, A., Carol, D. J., Limjuco, G. A. and Eyler, E. H. (1975). Experimental allergic aspermatogenic orchitis. *J. Biol. Chem.* 250, 6141

Jamil, K. and White, I.G. (1981) Induction of acrosomal reaction in sperm with ionophore A23187 and calcium. *Arch. Androl.* 7, 283

Jen, J., Deschepper, C.F., Shackleford, G.M., Lee, C.Y.G. and Lau, Y.-F.C. (1990) Stage-specific expression of the lactate dehydrogenase-X gene in adult and developing mouse testes. *Mol. Reprod. Dev.* 25, 14

Jones, T.R. and Cole, M.D. (1988) Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3' untranslated sequences. *Mol. Cell. Biol.* 7, 4513

Khandjian, E.W. (1987) Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. *Biotechnology Vol.* 5, 165.

Kille, J.W. and Goldberg, E. (1979) Female reproductive tract immunoglobulin responses to a purified sperm-specific antigen (LDH-C₄). *Biol. Reprod.* 20, 863

Kille, J.W. and Goldberg, E. (1980) Inhibition of oviductal sperm transport in rabbits immunized against sperm specific Lactate Dehydrogenase (LDH-C₄). *J. Reprod. Immunol.* 2, 15

Kleene, K.C., Distel, R.J. and Hecht, N.B. (1984) Translational regulation and deadenylation of a protamine mRNA during spermatogenesis in the mouse. *Dev. Biol.* 105, 71

Klein, P., Kanehisa, M. and Delisi, C. (1985) The detection and classification of membrane-spanning protein. *Biochim. Biophys. Acta* 815, 468

Koehler, J.K. and Graddum-Rosse, P. (1975) Media induced alterations of the membrane associated particles of the guinea pig sperm tail. *J. Ultrastruct. Res.* 51, 106

Kozak, M. (1984) Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* 12, 857

- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680
- Lakoski, K.A., Williams, C. and Saling, P. (1989) Proteins of the acrosomal region in mouse sperm: Immunological probes reveal post-testicular modifications. *Gamete Res.* 23, 21
- Lakoski, K.A., Carron, C.P., Cabot, C.L. and Saling, P. (1988) Epididymal maturation and the acrosome reaction in mouse sperm: response to zona pellucida develops coincident with modification of M42 antigen. *Biol. Reprod.* 38, 221
- Lanz, T. von. and Neuhauser, G. (1964) Morphometrische analyse des menschlichen nebenhodens. *Z. Anat. Entwicklungsgesch* 124, 126
- Leblond, C.P. and Clermont, Y. (1952) Spermiogenesis of the rat, mouse, hamster and guinea pig as revealed by the 'periodic acid-fuchsin sulfurous acid technique.' *Am. J. Anat.* 90, 167
- Lee, C.Y.G., Wong, E., Richter, D.E. and Menge A.C. (1984a) Monoclonal antibodies to human sperm antigens II. *J. Reprod. Immunol.* 6, 227
- Lee, C.Y.G., Wong, E. and Teh, C.Z. (1984b) Analysis of mouse sperm isoantigen using specific monoclonal antibodies. *Am. J. Reprod. Immunol.* 6, 27
- Lee, C.Y.G., Wong, E. and Menge, A. C. (1984c) Monoclonal antibodies to rabbits sperm autoantigen. *Fertil. Steril.* 41, 131
- Lee, C.Y.G., Wong, E., Teh, C.Z. and Nishizawa, Y. (1985) Generation of mouse oocyte monoclonal antibodies: their effects and those of antisperm monoclonal antibodies on in vitro fertilization. *J. Reprod. Immunol.* 7, 3
- Lee, C.Y.G. and Wong, E. (1986a) Developmental studies of sperm surface antigens using specific monoclonal antibodies. *J. Reprod. Immunol.* 9, 275
- Lee, C.Y.G., Wong, E. and Zhang, J.H. (1986b) Inhibitory effects of monoclonal sperm antibodies on the fertilization of mouse oocyte in vitro and in vivo. *J. Reprod. Immunol.* 9, 261
- Lee, C.Y.G., Zhang, J.H., Wong, E., Chow, S.N., Yang, Y., Sun, P. and Leung, H.Y. (1987) Sex difference of antifertility effect by passive immunized monoclonal sperm antibodies. *Am. J. Reprod. Immunol. Microbiol.* 13, 9
- Lenz, R. W., Ax, R. L., Grimek, H. J. and First, N. L. (1982) Proteoglycan from bovine follicular fluid enhances an acrosome reaction in bovine spermatozoa. *Biochem. Biophys. Res. Commun.* 106, 1092
- Leyton, L. Robinson, A. and Saling, P. (1989) Relationship

between the M42 antigen of mouse sperm and the acrosome reaction induced by ZP3. *Dev. Biol.* 132, 174

Liu, M.S., Yang, Y., Pan, Y., Liu, H.W., Menge, A.C. and Lee, C.Y.G. (1989) Purification of an acrosomal antigen recognized by a monoclonal antibody and antifertility effects of isoimmune sera. *Int. J. Androl.* 12, 451

Liu, M.S., Chan, K., Lau, Y.F. and Lee, C.Y.G. (1990) Molecular cloning of an acrosomal sperm antigen gene and the production of its recombinant protein for immunocontraceptive vaccine. *Mol. Reprod. Dev.* 25, 302

Longo, F.J. (1987) *Fertilization*. Chapman and Hall Press, London.

Mack, S.R., Bhattacharyya, A.K., Joyce, C., Van der Ven, H.H., Zaneveld, L.J.D. (1983) Acrosomal enzymes of human spermatozoa before and after in vitro capacitation. *Biol. Reprod.* 28, 1032

Maniatis, T., Fritsch, E.T. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York.

Mann, T. (1964) *The Biochemistry of semen and of the Male Reproductive Tract*. Wiley, New York.

Mathur, S., Goust, J. M., Williamson, H. O. and Fudenberg, H. H. (1981) Crossreactivity of sperm and T lymphocyte antigens. *Am. J. Reprod. Immunol. Microbiol.* 1, 113

McMaster, R., Yanagimachi, R., and Lopata, A. Penetration of human eggs by human spermatozoa in vitro. (1978) *Biol. Reprod.* 19, 212

Meistrich, M.L., Trostle, P.K., Frapart, M. and Erickson, R.P. (1977) Biosynthesis and localization of lactate dehydrogenase X in pachytene spermatocytes and spermatids of mouse testes. *Dev. Biol.* 60, 428

Meizel, S. and Mukerji, S.K. (1975) Proacrosin from rabbit epididymal spermatozoa: Partial purification and initial biochemical characterization. *Biol. Reprod.* 13, 83

Menge, A. C., Peegal, H. and Riolo, M. L. (1979). Sperm fractions responsible for immunologic induction of pre- and post-fertilization infertility in rabbits. *Biol. Reprod.*, 20, 931

Menge, A.C., Shoultz, G.K., Kelsey, D.E. and Lee, C.Y.G. (1987) Characterization of monoclonal antibodies against human sperm antigens by immunoassays including sperm function assays and epitope evaluation. *Am. J. Reprod. Immunol. Microbiol.* 13, 108

Millette, C.F. and Moulding, C.T. (1981) Cell surface marker proteins during mouse spermatogenesis. Two-dimensional electrophoretic analysis. *J. Cell Sci.* 48, 367

- Moore, H.D.M. and Bedford, J.M. (1983) The interaction of mammalian gametes in the female. In: Mechanism and Control of Animal Fertilization. Academic Press, New York. pp.453
- Moore, H.D.M. and Hartman, T.D. (1984) Localization by monoclonal antibodies of various surface antigens of hamster spermatozoa and the effect of antibody on fertilization in vitro. J. Reprod. Fertil. 70, 175
- Morton, D.B. (1975) Acrosomal Enzymes: Immunological localization of acrosin and hyaluronidase in ram spermatozoa. J. Reprod. Fertil. 36, 235
- Mutter, G.L. and Wolgemuth, D.J. (1987) Distinct developmental patterns of c-mos proto-oncogene expression in female and male mouse germ cells. Proc. Natl. Acad. Sci. USA 84, 5301
- Myles, D.G. Koppel, D.E., Cowan, A.E., Phelps, B.M. and Primakoff, P. (1987) Rearrangement of sperm surface antigens prior to fertilization. Ann. N.Y. Acad. Sci. 513, 262
- Nakane, P. K. and Kawaoi, A. (1974) Peroxidase-labeled antibody. A new method of conjugation. J. Histochem. Cytochem. 22, 1084-1091
- Naz, R.K., Alexander, N.J., Isahakia, M. and Hamilton, M.S. (1984a) Monoclonal antibody to a human germ cell glycoprotein that inhibits fertilization. Science, 225, 342
- Naz, R.K., Rosenblum, B.B. and Menge, A.C. (1984b) Characterization of a membrane antigen from rabbit testis and sperm isolated by using monoclonal antibodies and effect of its antiserum on fertility. Proc. Natl. Acad. Sci. (USA) 81, 857
- Naz, R.K., Philips, T.M. and Rosenblum, B.B. (1986) Characterization of the fertilization antigen (FA-1) for the development of a contraceptive vaccine. Proc. Natl. Acad. Sci. (USA) 83, 5713
- O'Brien, D.A., Gerton, G.L. and Eddy, E.M. (1988) Acrosomal constituents identified with a monoclonal antibody are modified during late spermatogenesis in the mouse. Biol. Reprod. 38, 955
- O'Farrell, P. Z., Goodman, H. M., and O'Farrell, P.H. (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12, 1133
- Olson, G.E. and Winfrey, V.P. (1985) Substructure of a cytoskeletal complex associated with the hamster sperm acrosome. J. Ultrastruct. Res. 92, 167
- O'Rand, M.G., Irons, G.P. and Porter, J.P. (1984) Monoclonal antibodies to rabbit sperm autoantigens I: Inhibition of in vitro fertilization and location on the egg. Biol. Reprod. 30, 721

- Perreault, S., Zaneveld, L.J.D. and Rogers, B.J. (1979) Inhibition of fertilization of hamster by sodium aurothiomalate, a hyaluronidase inhibitor. *J. Reprod. Fertil.* 60, 461
- Phillips, D.M. (1972) Substructure of mammalian sperm acrosome. *J. Ultrastruct. Res.* 38, 591
- Pless, D.D. and Lennarz, W.J. (1977) Enzymatic conversion of proteins to glycoproteins. *Proc. Natl. Acad. Sci. USA* 74, 134
- Primakoff, P. and Myles, D.G. (1983) A map of the guinea pig sperm surface constructed with monoclonal antibodies. *Dev. Biol.* 98, 417
- Primakoff, P., Cowan, A., Hyatt, H., Tredick-Kline, J. and Myles, D.G. (1988a) Purification of the guinea pig sperm PH-20 antigen and detection of a site-specific endoproteolytic activity in sperm preparations that cleaves PH-20 in two disulfide-linked fragments. *Biol. Reprod.* 38, 921
- Primakoff, P., Laphrop, W., Woolman, L., Cowan, A. and Myles, D.G. (1988b) Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20. *Nature*, 335, 543
- Reyes, R., Carranco, A., Hernandez, O., Rosado, A., Merchant, H., and Delgado, N. M. (1984) Glycosaminoglycan sulfate as acrosomal reaction-inducing factor of follicular fluid. *Arch. Androl.* 12, 203
- Roldan, E.R.S. and Harrison, R.A.P. (1990) Molecular Mechanisms leading to exocytosis during the sperm acrosome reaction. in: *Fertilization in Mammals*. Bavister, B.D. et al., (eds) Serono Symposia Press, Massachusetts. pp. 179
- Russell, L., Peterson, R.N. and Freund, M. (1979) Direct evidence for formation of hybrid vesicles by fusion of plasma and outer acrosomal membranes during the acrosome reaction in boar spermatozoa. *J. Exp. Zool.* 208, 41
- Russell, L.D. (1980) Sertoli-germ cell interactions: A review. *Gamete Res.* 3, 179
- Russell, L.D., Weber, J.E. and Vogl, A.W. (1986) Characterization of fragments within the subacrosomal space of rat spermatids during spermiogenesis. *Tissue Cell* 18, 887
- Saling, P.M., and Storey, B.T. (1979) Mouse gamete interactions during fertilization in vitro. Chlorotetracycline as fluorescent probe for the mouse sperm acrosome reaction. *J. Cell Biol.* 83, 544
- Saling, P.M., Raines, L.M. and O'Rand, M.G. (1983) Monoclonal antibody against mouse sperm blocks a specific event in the fertilization process. *J. Exp. Zool.* 227, 481

- Saling, P. M., Morton, P. C. and Waibel, R. (1986) Contraceptive effect of two anti-sperm monoclonal antibodies, administered singly and in combination, in the mouse. In: Talwer, G. P. (ed.) Contraception and Promotion of Fertility. Plenum Publishing Co. New Jersey. pp.191
- Schachuer, M., Wortham, R. A., Carter, L. D. and Chafe, J. K. (1975) NS-4 (Nervous system antigen 4): A cell surface antigen of developing and adult mouse brain and sperm. Dev. Biol. 44, 313
- Schaffner, W. and Weissmann, C. (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal. Biochem. 56, 502
- Shaw, G. and Kamen, R. (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46, 659
- Siiter, J.E., Dandekar, P. and Meizel, S. (1988) Human sperm acrosome reaction-initiating activity associated with the human cumulus oophorus and mural granulosa cells. J. Exp. Zool. 246, 71
- Silber, S.J. (1980) Vasoepididymostomy to the head of the epididymis: recovery of normal spermatozoa motility. Fertil. Steril. 34, 149
- Singer, S.L., Lambert, H., Overstreet, J.W., Hanson, F.W. and Yanagimachi, R. (1985) The kinetics of human sperm binding to the human zona pellucida and zona-free hamster oocyte in vitro. Gamete Res. 12, 29
- Snyder, M., Elledge, S., Sweetser, D., Young, R.A., and Davis, R.W. (1987) Lambda gt11: Gene isolation with antibody probes and other applications. Meth. Emzyl. 154, 107
- Soupart, P., and Strong, P.A. (1974) Ultrastructural observations on human oocytes fertilization in vitro. Fertil. Steril. 25, 11
- Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503
- Stern, L., Kleene, K.C., Gold, B., Hecht, N.B. (1983) Changes in populations of mRNA during spermatogenesis. Exp. Cell Res. 143, 247
- Steube, K., Gross, V. and Heinrich, P.C. (1985) Deglycosylation of α_1 -proteinase inhibitor by endo-B-N-acetylglucosaminidase F. Biochem. 24, 5587
- Storey, B.T., Lee, M.A., Muller, C. et al. (1984) Binding of mouse spermatozoa to the zona pellucida of mouse eggs in cumulus: Evidence that the acrosomes remain substantially intact. Biol. Reprod. 31, 1119

- Talbot, P., and Chacon, R. (1980) A new procedure for rapidly scoring acrosome reactions of human sperm. *Gamete Res.* 3, 211
- Talbot, P., and Chacon, R. (1981) A triple-stain technique for evaluating normal acrosome reactions of human sperm. *J. Exp. Zool.* 215, 201
- Talwar, G.P. (1986) Immunological approaches to contraception: the need, basic premise, and overview. in: *Immunological Approaches to Contraception and Promotion of Fertility*. Talwar, G.P. (ed.) Plenum Press, New York. pp. 17
- Tanaka, S. and Fujimoto, H. (1986) A postmeiotically expressed clone encodes lactate dehydrogenase isozyme X. *Biochem. Biophys. Res. Commun.* 136, 760
- Tesarik, J., Travnik, P., Mecova, L. and Pilka, L. (1985) Binding of antisperm isoantibodies from a woman at risk for immunological infertility to intraacrosomal components of donor spermatozoa. *Fertil. Steril.* 44, 138
- Teuscher, C., Wild, G. C. and Tung, K. S. K. (1982). Immunochemical analysis of guinea pig sperm autoantigens. *Biol. Reprod.* 26, 218.
- Teuscher, C., Wild, G. C. and Tung, K. S. K. (1983). Acrosomal autoantigens of guinea pig sperm. *J. Immunol.* 130, 317.
- Thomas, P. S. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. (USA)* 77, 5201.
- Tindall, D.J., Rowley, D.R., Muethy, L., Lipshultz, L.I. and Chang, C.H. (1985) Structure and Biochemistry of the Sertoli cell. *Int. Rev. Cyto.* 94, 127
- Towbin, H., Srachelin, T., Gordon, J.A. (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. (USA)* 76, 4350
- Tung, K.S.K. (1980). Immunological Aspects of Infertility and Fertility Regulation. In Dhindsa, D.S. and Schumacher, G.F.B. (eds.) Elsevier Press, North Holland. pp. 33
- van Kooij, R.J., Balerna, M., Roatti, A., Campana, A. (1986a) Oocyte penetration and acrosome reactions of human spermatozoa. I: Influence of incubation time and medium composition. *Andrologia* 18, 152
- van Kooij, R.J., Balerna, M., Roatti, A., Campana, A. (1986b) Oocyte penetration and acrosome reactions of human spermatozoa. II: Correlation with other seminal parameters. *Andrologia* 18, 503

- Varmus, H.E., Quintrell, N., and Ortiz, S. (1981) Retroviruses as mutagens : insertion and excision of a nontransforming provirus after expression of a resident transforming provirus. *Cell* 25, 23.
- Villasante, A., Wang, D., Dobner, P., Dolpph, P., Lewis, S.A. and Cowan, N.J. (1986) Six mouse alpha-tubulin mRNAs encode five distinct isoforms: Testis-specific expression of two sister genes. *Mol. Cell Biol.* 6, 2409
- Vogl, A.W. (1989) Distribution and function of organized concentrations of actin filaments in mammalian spermatogenic cells and sertoli cells. *Int. Rev. Cyto.* 119, 1
- von Heijne, G. (1986) A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14, 4683
- Wassarman, PM (1987a) The biology and chemistry of fertilization. *Science* 235, 553
- Wassarman, PM (1987b) Early events in mammalian fertilization. *Ann. Rev. Cell Biol.* 3, 109
- Waters, S.H., Distel, R.J. Hecht, N.B. (1985) Mouse testes contain two size classes of actin mRNA that are differentially expressed during spermatogenesis. *Mol. Cell Biol.* 5, 1649
- Welch, J.E. and O'Rand, M.G. (1985) Identification and distribution of actin in spermatogenic cells and spermatozoa of the rabbit. *Dev. Biol.* 109, 411
- Wheat, T.E., Hintz, M., Goldberg, E. and Margoliash, E. (1977) Analysis of stage-specific multiple forms of lactate dehydrogenase and of cytochrome c during spermatogenesis in the mouse. *Differentiation* 9, 37
- Wieben, E.D. (1981) Regulation of the synthesis of lactate dehydrogenase-X during spermatogenesis in the mouse. *J. Cell Biol.* 88, 492
- Willison, K. and Ashworth, A. (1987) Mammalian spermatogenic gene expression. *Trends Genet.* 3, 351
- Wilson, T. and Treisman, R. (1988) Removal of poly (A) and consequent facilitated by 3' AU-rich sequences. *Nature* 336, 396
- Wolf, D.P., Sokoloski, J.E., Dandekar, P. and Bechtol, K.B. (1983) Characterization of human sperm surface antigens with monoclonal antibodies. *Biol. Reprod.* 29, 713
- Wolf, D.P., Boldt, J., Byrd, W., Bechtol, K.B. (1985) Acrosomal status evaluation in human ejaculated sperm with monoclonal antibodies. *Biol. Reprod.* 32, 1157
- Wolf, D.P. (1986) Research frontiers in human in vitro

fertilization. Adv. Exp. Med. Biol. 207, 429

Wright, R.M., John, E., Klotz, K., Flickinger, C.J. and Herr, J. C. (1990) Cloning and sequencing of cDNAs coding for the human intra-acrosomal antigen SP-10. Biol. Reprod. 42, 693

Yanagimachi, R., Yanagimachi, H. and Rogers, B.J. (1976) The use of zona-free animal ova as a test system for the assessment of the fertilizing capacity of human spermatozoa. Biol. Reprod. 15, 471

Yanagimachi, R. (1981) Mechanisms of fertilization in mammals. in: Fertilization and Embryonic Development In Vitro. Plenum, New York. pp. 81

Yanagimachi, R. (1984) Fertilization in mammals. Tokai J. Exp. Clin. Med. 9, 81

Yang, Y-S, Rojas, F.J. and Stone, S.C. (1988) Acrosome reaction of human spermatozoa in zona-free hamster egg penetration test. Fertil. Steril. 50, 954.

Young, R. A., and Davis, R. W. (1983) Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. (USA) 80, 1194.

APPENDIX

1. The formula of solutions in ELISA are summarized as follows:

- (1). Blocking buffer
 - Tris-HCl (pH 8.0) 0.1 M
 - BSA 0.25 %
 - Sucrose 2 %
 - Thimerosal 0.1 %
- (2). Substrate solution
 - Citrate-phosphate (pH 5.0) 0.1 M
 - 3,3',5,5' tetramethyl benzidine 0.03 %
 - H₂O₂ 0.02 %

2. The formula of stock solutions mentioned in SDS-PAGE are summarized as follows:

- (1). Stacking gel acrylamide solution
 - acrylamide 28.38 gm
 - bisacrylamide 1.62 gm
 - dH₂O to 100 ml
- (2). Stacking Tris-HCl solution
 - Tris base 6.06 gm
 - SDS 0.4 gm
 - pH to 6.8 with HCl
 - dH₂O to 100 ml
- (3). Running gel acrylamide solution
 - acrylamide 30.0 gm
 - bisacrylamide 0.8 gm
 - dH₂O to 100 ml
- (4). Running Tris-HCl solution
 - Tris base 18.17 gm
 - SDS 0.40 gm
 - pH to 8.8 with HCl
 - dH₂O to 100 ml
- (5). Electrophoresis buffer (10 X)
 - Tris base 30 gm
 - glycine 144 gm
 - SDS 10 gm
- (6). Sample buffer (2 X)
 - 1 M Tris-HCl, pH 6.8 1.25 ml
 - 10 % SDS 4 ml
 - glycerol 1 ml
 - bromophenol blue 20 mg
 - B-mercaptoethanol 0.4 ml
 - Bring volume to 10 ml with dH₂O

3. The formula of stock solutions mentioned in 2D-gel electrophoresis above are summarized as follows:

- (1). Ampholine mixture
pH range 3.5-10:4-6:5-8 = 1:1:3
- (2). Gel mixture (5 ml)

urea	2.5 gm
30 % acrylamide	0.7 ml
(for upper gel of SDS-PAGE)	
glycerol	0.25 ml
dH ₂ O	1.25 ml
ampholine mixture	0.25 ml
10 % ammonium persulfate	10 ul
TEMED	10 ul
- (3). Sample buffer

urea	0.57 gm
20 % NP-40	0.1 ml
ampholine mixture	0.05 ml
1 M DTT	0.025 ml
dH ₂ O to 1 ml	
- (4). SDS sample buffer

1 M Tris-HCl pH 6.8	1.8 ml
10 % SDS	6.0 ml
2-mercaptoethanol	1.5 ml
10 % glycerol	3.0 ml
dH ₂ O to 30 ml	

4. The formula of stock solutions mentioned in dot blot assay are listed as follows:

- (1). TBS
50 mM Tris-HCl (pH 7.9)
150 mM NaCl
- (2). TBST
TBS plus 0.05 % Tween-20
- (3). Substrate solution
30 mg (W/V) urea peroxide in 50 ml 0.1 M Tris-HCl, pH 8.0
30 mg (W/V) 4-Chloro-1-naphthol in 10 ml methanol
mix well before use

5. The formula of stock solutions mentioned in Western blot are summarized as follows:

- (1). Blocking solution
3 % BSA and 0.1 % TMS in TBST solution

- (2). Transfer buffer
 25 mM Tris base
 192 mM glycine
 After Tris and glycine are dissolved, add 20 % (V/V) methanol
 pH to 8.3 with HCl
- (3). Amido black staining and destaining solution
- a. staining solution:
 0.1 % (W/V) amido black in 45 % methanol, 45 % H₂O and 10 % acetic acid
- b. destaining solution:
 staining solution without amido black
6. The formula of stock solutions mentioned in mouse in vitro fertilization are listed as follows:
- (1). BWW medium (Bigger et al., 1971)
- | | | |
|-------------------------------------|--------|----|
| NaCl | 0.554 | gm |
| KCl | 0.0356 | gm |
| CaCl ₂ | 0.0189 | gm |
| KH ₂ PO ₄ | 0.0162 | gm |
| MgSO ₄ 7H ₂ O | 0.0294 | gm |
| Dextrose | 0.1 | gm |
| pyruvic acid (Na salt) | 0.003 | gm |
| NaHCO ₃ | 0.2106 | gm |
| BSA | 0.35 | gm |
| DL-lactic acid | 0.37 | ml |
| 1 M Hepes, pH 7.4 | 2 | ml |
| 0.5 % phenol red | 0.05 | ml |
| Pen-Strep | 1 | ml |
| ddH ₂ O to | 100 | ml |
- Filtrate the medium by using 0.22 um filter
- (2). IVF staining solution
 0.25 % Lacmoid in 45 % acetic acid
- (3). IVF destaining solution
 0.25 % glycerol in 20 % acetic acid
7. The stock solution required for tissue DNA extraction are listed as follows:
- (1). TE buffer
 Tris-HCl pH 8.0 10 mM
 EDTA 1 mM
- (2). TNE buffer
 TE buffer plus 0.1 M NaCl

- (3). "Phenol" means phenol equilibrated with buffer and containing 0.1 % hydroxyquinoline and 0.2 % 2-mercaptoethanol.
- (4). "Chloroform" means a 24:1 (v/v) mixture of chloroform and isoamyl alcohol.

8. The stock solution used for bacteriophage DNA extraction are listed as follows:

- (1). LB medium
- | | |
|----------------------------|------------|
| Bacto-tryptone | 10 gm |
| Bacto-yeast extract | 5 gm |
| NaCl | 10 gm |
| dH ₂ O | to 1 liter |
| Adjust pH to 7.5 with NaOH | |
- (2). SM buffer
- | | |
|--------------------------------------|--------|
| NaCl | 5.8 gm |
| MgSO ₄ 7 H ₂ O | 2 gm |
| 1 M Tris-HCl (pH 7.5) | 50 ml |
| 2 % gelatin | 5 ml |
- (3). TE buffer
- | | |
|-------------------------|--|
| 10 mM Tris-HCl (pH 7.5) | |
| 1 mM EDTA (pH 8.0) | |

All the solutions were sterilized by autoclaving prior to use.

9. The stock solution used in extraction of plasmid DNA are listed as follows:

- (1). Solution I
- | | |
|-------------------------|--|
| 50 mM glucose | |
| 25 mM Tris-HCl (pH 8.0) | |
| 10 mM EDTA | |
- (2). Solution II
- | | |
|------------|--|
| 0.2 M NaOH | |
| 1 % SDS | |
- (3). Solution III
- | | |
|--------------------------------|---------|
| 5 M potassium acetate (pH 4.8) | 60 ml |
| Acetic acid | 11.5 ml |
| dH ₂ O | 28.5 ml |

10. The compositions of solutions used for RNA extraction are listed as follows:

- (1). Solution D
- | | |
|-------------------------|-----|
| Guanidinium thiocyanate | 4 M |
|-------------------------|-----|

Sodium citrate, pH 7	25 mM
Sarcosyl	0.5 %
2-mercaptoethanol	0.1 M

11. The formula of solutions mentioned in nick translation are summarized as follows:

- (1). dNTP mixture

500 uM dATP	2 ul
500 uM dGTP	2 ul
500 uM dTTP	2 ul
- (2). 10 X nick translation buffer

0.5 M	Tris-HCl pH 7.2
0.1 M	MgSO ₄
1 mM	DTT
500 ug/ml	BSA
- (3). STE buffer

10 mM	Tris-HCl pH 8.0
0.1 M	NaCl
1 mM	EDTA

12. The stock solutions used in Southern blot hybridization are listed as follows:

- (1). 20 X SSC

NaCl	175.3 gm
Sodium citrate	88.2 gm
dH ₂ O to 1000 ml	
adjust pH to 7.0	
- (2). Hybridization buffer

Na ₂ HPO ₄ pH 7.2	0.25 M
SDS	7.0 %
dextran sulfate	7.5 %

13. The solutions used in immunoscreening are listed as follows:

- (1). Avidin-biotinylated horseradish peroxidase complex

avidin solution	40 ul
biotinylated horseradish peroxidase	40 ul
in 10 ml TBST buffer	

This mixture is incubated at room temperature for 30 minutes before use.

- (2). Peroxidase substrate solution

4-chloro-1-naphthol (3 mg/ml) in methanol	2 ml
TBS plus 0.01 M imidazole	10 ml
30 % hydrogen peroxide	5 ul