EXPRESSION OF CFTR mRNA
IN NASAL EPITHELIUM
AND VAS DEFERENS

by

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A thesis submitted in conformity with
the requirements for the degree of
Master of Science

Graduate Department of Institute of Medical Science
University of Toronto

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The gene responsible for cystic fibrosis (CF), called the cystic fibrosis transmembrane conductance regulator (CFTR), encodes the cAMP-regulated chloride channel found in the apical membrane of secretory epithelial cells. It has been well established that almost all males with CF are azoospermic due to atrophy or absence of structures derived from the Wolffian duct. Interestingly, a higher than expected frequency of mutations in the CFTR gene has been identified in men with congenital absence of vas deferens and men with epididymal obstruction. In particular, these individuals have been found to have a significantly higher incidence of the 5-thymidine (5T) variant of the CFTR intron 8 polypyrimidine tract (IVS8-T tract) compared to normal or CF populations. The 5T variant results in less efficient splicing of CFTR exon 9 compared to the more common 7T and 9T variants and therefore produces less normal, full-length CFTR mRNA. The protein produced by the CFTR transcript lacking exon 9 fails to function as a cAMP-dependent chloride channel. The fact that these infertile males have no other clinical signs of classical CF suggests that the epithelia of the male reproductive tract may have the highest requirement for CFTR function or, alternatively, splicing of CFTR mRNA in the reproductive tract is less efficient than the other CF-associated organs. Nasal epithelia and segments of vas deferens were obtained from 24 healthy, previously vasectomized men who presented for vasectomy reversal. Quantitative RT-PCR was performed on these specimens, with the region of CFTR cDNA spanning exon 9 amplified. For both nasal and vasal tissues, a strong positive correlation was found between the length of the IVS8-T tract and the proportion of mRNA with exon 9 intact. In addition, within the same subject, a significantly higher level of transcripts lacking exon 9 was found in vas deferens than nasal epithelia, regardless of the IVS8-T genotype. These findings suggest that the splicing of CFTR precursor mRNA is less efficient in vasal epithelia compared to respiratory epithelia. Thus, differential splicing efficiency between the various tissues which express CFTR provides one possible explanation for the reproductive tract abnormalities observed in infertile men with CFTR gene alterations but without other manifestations of CF.
The author wishes to express sincere appreciation to Prof. Lap-Chee Tsui, Dr. Keith Jarvi, and Dr. Johanna Rommens for their assistance in the preparation of this manuscript.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>adenosine triphosphate binding cassette</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ARTs</td>
<td>assisted reproductive technologies</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAVD</td>
<td>congenital absence of vas deferens</td>
</tr>
<tr>
<td>CBAVD</td>
<td>congenital bilateral absence of vas deferens</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CF-PI</td>
<td>pancreatic-insufficient cystic fibrosis</td>
</tr>
<tr>
<td>CF-PS</td>
<td>pancreatic-sufficient cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CUAVD</td>
<td>congenital unilateral absence of vas deferens</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>IVF</td>
<td><em>in vitro</em> fertilization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IVS</td>
<td>intervening sequence (intron)</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>M</td>
<td>mole(s)/liter</td>
</tr>
<tr>
<td>MESA</td>
<td>microsurgical epididymal sperm aspiration</td>
</tr>
<tr>
<td>μg</td>
<td>microgram(s)</td>
</tr>
<tr>
<td>MIF</td>
<td>Mullerian inhibitory factor</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>μl</td>
<td>microliter(s)</td>
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<tr>
<td>ml</td>
<td>milliliter(s)</td>
</tr>
<tr>
<td>μM</td>
<td>micromole(s)/liter</td>
</tr>
<tr>
<td>mM</td>
<td>millimole(s)/liter</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram(s)</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>s</td>
<td>second(s)</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
</tbody>
</table>

vii
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSC</td>
<td>sodium citrate/sodium chloride</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate, EDTA buffer</td>
</tr>
<tr>
<td>TDF</td>
<td>testis determining factor</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxy transferase</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>chi-square</td>
</tr>
</tbody>
</table>
Mrs. Theresa Longley served as study nurse for the project and assisted in specimen collection, patient counseling, and obtaining patient consent.

Dr. Keith Jarvi from the Division of Urology, Department of Surgery, Mount Sinai Hospital, Toronto provided tissue specimens.

My contribution to this work includes the following:

1. collection of specimens
2. obtaining patient consent
3. extraction of DNA from peripheral leukocytes
4. genotyping of CFTR IVS8-T tract
5. extraction of RNA
6. quantitative RT-PCR experiments
7. determination of proportion of exon 9+ CFTR transcripts
8. design of experimental protocols
9. analysis and interpretation of data
I.A. BACKGROUND

I.A.1. HUMAN MALE INFERTILITY

Approximately fifteen percent of couples are infertile [Mosher, 1985]. An abnormality is found in only the man in 30 percent of the cases, while abnormalities detected in both partners occur in another 20 percent. Therefore, male factor infertility accounts for one half of couples with fertility problems [Mosher, 1985; Sigman and Howards, 1992]. Infection, immunologic factors, congenital anomalies, genetic defects, toxic insult, and sexual dysfunction contribute to this important medical problem. In this section, the embryology of the male gonad and reproductive tract, the physiology of male reproduction, and clinical aspects of male infertility will be reviewed.

I.A.1.a. OVERVIEW OF MALE TESTICULAR AND REPRODUCTIVE TRACT EMBRYOLOGY

I.A.1.a.i. DEVELOPMENT OF TESTES

The gonads are derived from three sources: coelomic epithelium, mesenchyme, and primordial germ cells [Moore, 1989b]. They are first recognized during the fifth week of gestation when a thickened area of coelomic epithelium develops on the medial aspect of the mesonephros. Proliferation of these epithelial cells produces a bulge on the medial side of each mesonephros called the gonadal ridge. Primary sex cords grow from the gonadal ridges into the underlying mesenchyme. The indifferent gonad now has an outer cortex and an inner medulla. The cortex will normally differentiate into an ovary while the medulla
sex chromosome constitution, the medulla normally differentiates into a testis and the cortex regresses. Primordial germ cells migrate along the dorsal mesentery of the hindgut to the gonadal ridges and become incorporated in the primary sex cords [Witschi, 1948; Peters, 1970].

The short arm of the Y chromosome (Yp) carries the sex determining gene, SRY, which encodes the testis determining factor, TDF. TDF is essential for differentiation of the gonad into a testis and its absence results in formation of the gonad into an ovary [Disteche et al., 1986; Affara et al., 1987; Page et al., 1987; Palmer et al., 1989; Sinclair et al., 1990; Berkovitz et al., 1991]. TDF permits the primary sex cords to differentiate into seminiferous tubules. The walls of the seminiferous tubules are composed of Sertoli cells, derived from the surface epithelium, and spermatogonia, derived from the primordial germ cells. The Sertoli cells produce Mullerian inhibitory factor (MIF) [Josso, 1986]. The seminiferous tubules become separated by mesenchyme that gives rise to the interstitial cells (of Leydig). These cells produce testosterone [Siiteri and Wilson, 1974].

I.A.1.a.ii. DEVELOPMENT OF MALE GENITAL DUCTS

Testosterone stimulates development of the Wolffian duct into the male reproductive tract while MIF suppresses development of the paramesonephric (Mullerian) ducts into female genital ducts [Moore, 1989b]. When the mesonephros degenerates, some mesonephric tubules near the testis persist and become efferent ductules which open into the mesonephric duct, forming the head of the epididymis. The mesonephric duct proper separates into its ureteral and reproductive divisions at approximately the seventh week of
the metanephric mesoderm. The reproductive portion of the mesonephric (Wolffian) duct forms the body and tail of the epididymis, vas deferens, seminal vesicle, and ejaculatory duct.

I.A.1.a.iii. DEVELOPMENT OF MALE EXTERNAL GENITALIA

Masculinization of the indifferent external genitalia results from androgens produced by the fetal testes. As the phallus elongates to form a penis, the urogenital folds fuse with each other along the ventral surface of the penis to form the penile urethra. Consequently, the external urethral meatus moves to the glans penis. The scrotum is formed by the fusion of the labioscrotal swellings. Feminization of the indifferent external genitalia occurs in the absence of androgenic stimulation. The final form of the external genitalia is established by the twelfth week of gestation [Moore, 1989b].

I.A.1.b. OVERVIEW OF MALE REPRODUCTIVE PHYSIOLOGY

The testis has two important functions: spermatogenesis in the seminiferous tubules and secretion of steroid hormones (androgens) by the interstitial Leydig cells. These are intimately related, as testosterone synthesis is necessary for both sperm production and development of secondary sexual characteristics. The two functions are controlled by the anterior pituitary through the secretion of gonadotropins. The anterior pituitary itself is regulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus. The hypothalamic-pituitary-testicular axis consists of a closed-loop feedback control mechanism for maintaining normal reproductive function.
The hypothalamus receives messages from the central nervous system and the testis to regulate the synthesis and secretion of GnRH, which stimulates the synthesis and release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [Schally et al., 1971]. These two hormones are synthesized in the anterior pituitary and are secreted episodically in response to the pulsatile release of GnRH. LH and FSH bind to specific receptors on the membrane of Leydig cells and Sertoli cells, respectively, to stimulate cellular metabolism.

The hypothalamic-pituitary-gonadal axis consists of a closed-loop feedback control mechanism. Testosterone, secreted by the Leydig cells in testes, inhibits LH secretion in males [Walsh et al., 1973]. It is metabolized in peripheral tissue to dihydrotestosterone (DHT) or estradiol. Secretion of FSH is inhibited by inhibin, a nonsteroidal compound produced by the Sertoli cells [Van Thiel et al., 1972].

I.A.1.b.ii. THE TESTIS

I.A.1.b.ii.a. Leydig Cells

Testosterone is secreted episodically from the Leydig cells in response to LH pulses and has a diurnal pattern, with the peak level in the early morning and the nadir in the evening [Lipsett, 1974]. The biologic effects of androgens are exerted on target organs that contain a specific androgen receptor protein in the cell cytosol. Testosterone enters target cells where it may be converted to the more potent androgen, DHT, by 5α-reductase, and either testosterone or DHT binds to a receptor protein. This bound receptor complex is translocated into the nucleus, where it binds to nuclear chromatin and results in the
mRNA then exerts its androgen action in the target cell [McClure, 1987].

The major functions of androgen in target tissue include regulation of gonadotropin secretion by the hypothalamic-pituitary axis, initiation and maintenance of spermatogenesis, differentiation of the internal and external male genital system during fetal development, and promotion of sexual maturation at puberty.

I.A.1.b.ii.b. Seminiferous Tubules

Seminiferous tubules contain germ cells and Sertoli cells. The latter are nondividing support cells found on the basement membrane of the seminiferous tubules and extend filamentous cytoplasmic ramifications toward the tubular lumen. Sertoli cells are linked by tight junctions that divide the seminiferous tubule into a basal and adluminal compartment. These tight junctions along with the myoid cells of the peritubular contractile cell layer, serve to form the blood-testis barrier [Russell, 1980]. Sertoli cells are believed to provide structural and metabolic support for the developing spermatogenic cells. They are also involved in the phagocytosis of excess cytoplasm cast off by spermatids [Wheater et al., 1987].

The germ cells are arranged in an orderly manner from the basement membrane to the lumen. Spermatogonia lie directly on the basement membrane, and found progressing centrally are primary spermatocytes, secondary spermatocytes, and spermatids [Clermont, 1963; Heller and Clermont, 1964].
Spermatogenesis is the process in which primitive stem cells either divide to renew themselves or produce daughter cells that will become spermatocytes. The most primitive undifferentiated spermatogonia are the stem cells. For stem cell renewal, the dark type A spermatogonia following mitotic division produce a fresh stock of type A cells as well as pale type A spermatogonia. The latter undergo mitotic divisions into preleptotene primary spermatocytes via type B spermatogonia [Clermont, 1972]. The primary spermatocytes undergo the first maturation division by meiosis, reducing the number of chromosomes from 46 to 23 [Kerr and deKretser, 1981]. Each primary spermatocyte gives rise to two secondary spermatocytes, and each of these divide into two spermatids. The spermatids then transform into spermatozoa, a process called spermiogenesis. This process includes nuclear condensation, acrosome formation, loss of cytoplasm, development of a tail, and arrangement of mitochondria into the middle piece of the sperm. The entire spermatogenic process requires approximately 64 days to complete [Clermont, 1972].

I.A.1.b.iii. HORMONAL CONTROL OF SPERMATOGENESIS

LH indirectly affects spermatogenesis in that it stimulates endogenous testosterone production [DiZerga and Sherins, 1981]. Sertoli cells, possessing specific high-affinity FSH receptors, are the target for FSH [Means et al., 1980; Ritzen et al., 1981]. Androgen-binding proteins produced by Sertoli cells carry androgens intracellularly [Ritzen et al., 1971; Hansson and Djoseland, 1972]. The close proximity of the Leydig cells to the seminiferous tubule and elaboration by the Sertoli cells of androgen-binding protein cause a
spermatozoa.

I.A.1.b.iv. SPERM MATURATION, STORAGE, AND TRANSPORT

While the testis is responsible for sperm production, the epididymis is involved in the maturation, storage, and transport of spermatozoa. Spermatozoa develop an increased capacity for motility [Bedford et al., 1973; Moore et al., 1983] and fertilization [Orgebin-Crist, 1969; Bedford et al., 1973; Hinrichsen and Blaquier, 1980; Moore et al., 1983; Bedford, 1988] as they migrate through the epididymis.

The epididymis consists of a single convoluted tubule, 3 to 4 metres long, and is divided into the caput (head), corpus (body), and cauda (tail) epididymis [Jenkins et al., 1978]. Sperm are transported by a hydrostatic pressure difference, ciliary propulsion, and peristaltic contraction of myoid cells along the epididymis [Johnson and Howards, 1976]. The epididymis also functions as a storage reservoir for sperm [Johnson and Varner, 1988].

Mature sperm from the cauda epididymis enter the vas deferens, which transports its contents by peristaltic motion into the ejaculatory duct [Bruschini et al., 1977]. Sperm are then propelled to the outside by emission and ejaculation. Secretions from the seminal vesicles and prostate are deposited into the posterior urethra during emission. Peristaltis of the vas deferens and contraction of the bladder neck are under sympathetic control of the autonomic nervous system [Bell and McLean, 1967; Owman and Sjoberg, 1972; Lipshultz et al., 1991]. During ejaculation, the external sphincter relaxes and the semen is propelled through the urethra by rhythmic contractions of the perineal and bulbourethral muscles, both under somatic control.
The seminal vesicles produce fructose, prostaglandins, and coagulating factors [Mann and Mann, 1981]. Seminal plasma acts as a buffer on the acidic vaginal secretions. The coagulum formed by the ejaculated semen liquefies within 20 minutes from the action of prostatic proteolytic enzymes. More fluid are contributed by the bulbourethral glands (Cowper's glands) and urethral glands (Littre's glands) through the penile urethra. The first portion of the ejaculate contains most of the spermatozoa and secretions from the bulbourethral glands and the prostate; the remaining portion consists mostly of seminal vesicle secretions and contains only a few spermatozoa [Lilja et al., 1987].

I.A.1.b.v. FERTILIZATION

Fertilization normally occurs in the fallopian tubes after ovulation. During mid-cycle of the menstrual period in the female, cervical mucus becomes more abundant, thinner, and watery, thus allowing entry of sperm into the uterus and also protecting sperm from the acidic vaginal environment [Barnes and Toot, 1986]. Spermatozoa must undergo capacitation within the female reproductive tract in order for fertilization to take place [Chang, 1951; Austin, 1951]. Capacitation is followed by the acrosome reaction. The latter is a progressive fusion between the inner and outer acrosome membrane, resulting in the release of enzymes important for oocyte penetration by the sperm [Moore, 1989a].

I.A.1.c. CLINICAL ASPECTS OF MALE INFERTILITY

Pregnancy occurs in the majority of couples within one year of natural unprotected intercourse. The term infertility applies to those couples who have not been able to conceive after this one year period. Both partners should be evaluated for factors that may
entirely to a male factor, and an additional 20% involve both males and female factors; thus, about one half of infertile unions is due to a male factor [Mosher, 1985; Sigman and Howards, 1992].

I.A.1.c.i. EVALUATION OF THE INFERTILE MALE

The work-up of a man presenting with infertility should consist of a detailed history and physical examination. The latter must include inspection and palpation of the scrotum to ensure that the testicles have properly descended into the scrotum and are normal in size and consistency, and that the epididymides and vasa deferentia are present. Abnormal or suspicious findings from the history and physical examination direct subsequent investigations, such as serum hormonal tests (e.g., TSH, LH, FSH, testosterone, prolactin) and/or radiological imaging studies (e.g., scrotal ultrasound, transrectal ultrasound). However, all men presenting with infertility should have a semen analysis.

I.A.1.c.i.a. Semen analysis

The semen analysis is the cornerstone of the infertility investigation [Sigman and Howards, 1992]. Normal parameters are shown in Table 1 [World Health Organization, 1987]. Major abnormalities in semen analysis include the following:

- asthenospermia (<50% progressively motile sperm)
- oligospermia (<20 x 10^6 sperm / mL)
- pyospermia (>1 x 10^6 white blood cell / mL)
- azoospermia (no sperm)
teratospermia (abnormal sperm morphology)

I.A.1.c.ii. ETIOLOGIC CLASSIFICATION OF MALE INFERTILITY

The causes of male infertility may be classified according to results of the semen analysis. Approximately 10% of men presenting with infertility will have azoospermia, whereas the remaining 90% will have oligospermia, asthenospermia or teratospermia [Lipshultz, 1980]. Of the patients with azoospermia, standardard laboratory and imaging studies can determine whether this abnormality is due to a pre-testicular (hypothalamic-pituitary pathology, ~10% of azoospermic men), testicular (spermatogenic deficiency including testicular failure, ~40%), or post-testicular (functional or structural obstruction to sperm outflow, ~50%). Specific causes of post-testicular azoospermia include congenital absence of vas deferens (CAVD), epididymal obstruction, and vasectomy.

I.A.1.c.iii. TREATMENT OF MALE INFERTILITY

Obviously, treatment of an individual male patient with infertility will depend on the underlying cause. Those with a pre-testicular cause can often be treated successfully with exogenous GnRH analogues or gonadotropins [Howards, 1995]. Advances in microsurgery and assisted reproductive technologies (ARTs) now make it possible for many infertile men with a testicular or post-testicular cause to father their own children. Men with previous vasectomies wishing to reverse their sterile status can undergo microsurgical vasovasostomy (re-connecting the vas deferens) or, if necessary, vasoepididymostomy (connecting the vas deferens to the epididymis). The latter procedure is also indicated in cases of acquired (usually due to infection) or idiopathic epididymal obstruction. Men with
CAVD can be effectively treated with microsurgical epididymal sperm aspiration (MESA) and routine *in vitro* fertilization (IVF) or *in vitro* fertilization-intracytoplasmic sperm injection (IVF-ICSI) [Temple-Smith et al., 1985; Silber et al., 1988; Palermo et al., 1992; Silber et al., 1995; Schlegel et al., 1995; Gil-Salom et al., 1995]. Patients with a testicular cause for their infertility such as testicular failure may also be treated with IVF-ICSI via testis-retrieved haploid sperm [Van Steirteghem et al., 1993; Vanderzwalmen et al., 1995; Fishel et al., 1995; Fishel and Thornton, 1995; Harari et al., 1995; Silber et al., 1995; Sherins et al., 1995; Tesarik et al., 1995]. However, it must be emphasized that ARTs should not be initiated until the patient and his partner have received rigorous genetic counseling on the possibility that the genetic defect responsible for his reproductive failure may be transmitted to the offspring [de Kretser, 1995; In’t Veld et al., 1995; Mak and Jarvi, 1996; Morris and Gleicher, 1996].

**I.A.2. CYSTIC FIBROSIS AND THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR**

Cystic fibrosis (CF) is the most common fatal autosomal recessive disorder in the Caucasian population, with an incidence of approximately 1 in 2,500 live births and a carrier frequency of 1 in 25 persons in populations of Northern European descent. The classical form of CF is characterized by abnormalities of electrolyte, fluid and macromolecule secretion of exocrine glands. Clinical hallmarks of CF include chronic pulmonary obstruction and infections, exocrine pancreatic insufficiency, neonatal meconium ileus, elevated sweat electrolytes, and male infertility [Welsh et al., 1995].

The gene responsible for CF, called cystic fibrosis transmembrane conductance regulator (CFTR), was identified and cloned in 1989 [Rommens et al., 1989; Riordan et
~230 kilobases (kb) of genomic DNA, and produces a mRNA transcript of 6.2 kb. The encoded protein contains 1,480 amino acids and is a member of the ATP-binding cassette (ABC) family of membrane proteins. Various studies support that CFTR is a cAMP-regulated chloride channel found in the apical membrane of secretory epithelial cells [Welsh et al., 1995].

Hundreds of mutations in the CFTR gene have been reported [Cystic Fibrosis Genetic Analysis Consortium, 1994]. These mutations may be classified as follows: class I mutations confer defective protein production; class II mutations involve defective protein processing; class III mutations involve defective channel regulation; class IV mutations involve defective channel conduction; and class V mutations lead to decreased protein synthesis [Zielenski and Tsui, 1995]. In general, class I, II or III mutations are expected to have more serious phenotypic consequences than class IV or V mutations.

I.A.3. CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR AND MESONEPHRIC DUCT ANOMALIES

Studies have shown that more than 95% of CF men have abnormalities in the structures derived from the Wolffian duct [Kaplan et al., 1968; Valman and France, 1969; Landing et al., 1969; Holsclaw et al., 1971; Taussig et al., 1972]. The body and tail of epididymis, vas deferens, seminal vesicles and ejaculatory ducts are atrophic, fibrotic, or completely absent. As a result, semen analysis typically reveals decreased volume, complete absence of spermatozoa (azoospermia), increased acidity, decreased fructose concentration, elevated levels of citric acid and acid phosphatase [Kaplan 1968]. However, spermatogenesis is present on testicular histology [Denning, 1968].
Wolffian duct abnormalities but no other manifestations of CF. These conditions may represent either a reproductive tract specific or a very mild form of CF. At present, congenital bilateral absence of the vas deferens (CBAVD), congenital unilateral absence of the vas deferens (CUAVD), and idiopathic epididymal obstruction have all been associated with CFTR gene mutations [Dumur et al., 1990; Anguiano et al., 1992; Mickle et al., 1993; Osborne et al., 1993; Patrizio et al., 1993; Culard et al., 1994; Oates and Amos, 1994; Casals et al., 1995; Chillon et al., 1995; Costes et al., 1995; Durieu et al., 1995; Jarvi et al., 1995; Mercier et al., 1995; Zielenski et al., 1995b; Schlegel et al., 1996; Donat et al., 1997].

CBAVD is found in 1-2% of men presenting with infertility [Dubin and Amelar, 1971; Greenberg et al., 1978; Jequier et al., 1985]. Prior to the identification of the CFTR gene, CBAVD was considered to be a distinct clinical and genetic entity with an autosomal recessive inheritance pattern [McKusick, 1992]. Genetic studies found that 50-82% of CBAVD men have at least one detectable CFTR gene mutation and that about 15% of these men have two detectable CFTR mutations [Dumur et al., 1990; Anguiano et al., 1992; Osborne et al., 1993; Patrizio et al., 1993; Culard et al., 1994; Oates and Amos, 1994; Casals et al., 1995; Chillon et al., 1995; Costes et al., 1995; Durieu et al., 1995; Jarvi et al., 1995; Mercier et al., 1995; Zielenski et al., 1995b; Schlegel et al., 1996; Donat et al., 1997]. CFTR gene mutations have also been reported in men with congenital unilateral absence of vas deferens (CUAVD) [Mickle et al., 1993; Schlegel et al., 1996]. Of 14 infertile patients with CUAVD tested for 20 CFTR mutations, 6 (43%) had one mutation, and, in fact, one of these patients had two brothers with CBAVD.
CFTR gene mutations was further extended by the observation that 47% of otherwise healthy men with idiopathic epididymal obstruction have a CFTR gene mutation [Jarvi et al., 1995]. These findings suggest that a broad spectrum of Wolffian duct abnormalities (absence of the vas deferens, obstruction of the epididymis, ejaculatory duct cysts, seminal vesicle cysts, etc.) may be associated with CFTR gene mutations.

Alterations in the CFTR gene identified in men with Wolffian duct abnormalities can be divided into four groups. The first group consists of mutations that have been identified in classical CF patients. These “severe” CFTR mutations associate with pancreatic insufficiency (CF-PI) and are generally class I-III mutations. Examples of such mutations reported include ΔF508, G542X, R553X, R1162X, W1282X, N1303K, 1717-1G→A, and 2184delA. The second group consists of mutations found in more benign presentations of CF. These “mild” CFTR mutations are associated with pancreatic sufficiency (CF-PS) and tend to be class IV-V mutations. Such mutations include R117H and R347P. The third group consists of novel mutations identified exclusively in some CAVD men; however, as these sequence alterations are extremely rare, it is only speculated that they contribute to the CAVD phenotype [Anguiano et al., 1992; Culard et al., 1994; Mercier et al., 1995]. The fourth group consists of CFTR gene alterations that were previously considered as benign sequence variations [Anguiano et al., 1992; Mercier et al., 1995; Zielenski et al., 1995a]. Such examples include R75Q, G576A, R668C, and 5T. In the majority of patients (~45-50%), only one mutation or sequence alteration has been identified despite exhaustive screening of all exons and immediate flanking intron sequences in the CFTR gene. For those with mutations identified in both CFTR alleles
It is reasonable to assume that otherwise healthy men with CAVD with one or no detectable CFTR mutation may harbour as yet unidentified CFTR gene mutations or variants. However, not all cases of congenital Wolffian duct anomalies are due to mutations in the CFTR gene. There is evidence that CUAVD or CBAVD men with concomitant renal aplasia or ectopia (not typical features of CF) do not result from CFTR gene mutations. Augarten et al. reported that of 47 CBAVD patients, 10 had renal malformations on ultrasonography [Augarten et al., 1994]. Of the 37 patients with normal urinary tract, 18 (49%) tested positive for CFTR mutations. Interestingly, none of the 10 individuals with renal malformations had mutant CFTR alleles or abnormal sweat tests. In a separate report, 12 of 70 men with CUAVD or CBAVD had renal anomalies on ultrasound or excretory urography, and all 12 tested negative for CFTR mutations. Of 58 patients without renal abnormalities, 46 underwent CFTR mutation analysis and 27 (59%) tested positive for mutations [Schlegel et al., 1996]. Therefore, cases with vasal agenesis and renal malformations probably do not represent a genital form of CF but rather a distinct clinical entity.

The aforementioned information provides insight into the timing for the effect of the CFTR mutation on the reproductive tract. The mesonephric duct separates into its ureteral and reproductive divisions at approximately week seven of gestation. The ureteral bud induces renal formation as it grows into the metanephric mesoderm, while the reproductive portion (Wolffian duct) forms the corpus and cauda epididymis, vas deferens, seminal vesicle, and ejaculatory duct [Moore, 1989b]. It has been proposed that in CUAVD or CBAVD not caused by a defect in the CFTR gene, anomalous changes to
and genital systems are affected [Augarten et al., 1994]. In CUAVD or CBAVD patients with CFTR mutations, changes to the Wolffian duct are believed to occur after the urinary system has separated from the reproductive system; therefore, no renal malformation results. The pathogenesis probably involves intrauterine obstruction of the Wolffian duct with inspissated secretions, which results from CFTR chloride channel dysfunction, and gives rise to a dehydrated intraluminal fluid content with secondary degeneration or obliteration of the Wolffian duct [Taussig et al., 1972]. However, a primary morphogenetic defect in development of the Wolffian duct cannot be excluded [Olson and Weaver, 1969].

Electrophysiological experiments on cultured human epididymal cells using the patch-clamp technique demonstrated the presence of a low-conductance chloride channel that was activated by addition of cAMP agonists to these cells [Pollard et al., 1991]. This channel most likely represents CFTR. Furthermore, expression studies in human fetal and adult reproductive tract by in situ hybridization showed that CFTR is expressed in the ductal epithelium by week 18 of gestation and this expression is maintained throughout post-natal life [Trezise et al., 1993; Tizzano et al., 1993]. CFTR expression is high in the head of the epididymis, low in the body and tail of the epididymis, low in the vas deferens, and undetectable in the seminal vesicles [Tizzano et al., 1994]. The decreased expression of CFTR in Wolffian derivatives suggests that these structures may be more sensitive to CFTR dysfunction than other organs.
One of the first forms of alternative splicing discovered in CFTR was the deletion of exon 9 (exon 9-) [Strong et al., 1991; Chu et al., 1991]. Exon 9 encodes the initial 21% of the first nucleotide binding fold (NBF1) of CFTR, including motif A of the Walker ATP binding consensus sequence [Walker et al., 1982]. Based on quantitative RT-PCR experiments performed on respiratory epithelia, the amount of exon 9-transcripts was found to vary between different individuals [Bremer et al., 1992; Chu et al., 1991; Chu et al., 1992]. The variability in exon 9 splicing efficiency can be explained, for the most part, by a variation in the polypyrimidine tract of the splice acceptor site in intron 8 of CFTR (IVS8-T tract) [Chu et al., 1993]. Sequencing of the splice acceptor site preceding exon 9 demonstrated that three variations of uninterrupted pyrimidines occur in the population: 5, 7, and 9 thymidines (5T, 7T, and 9T) [Chu et al., 1991]. It was also observed that a positive correlation existed between the length of IVS8-T tract and the proportion of bronchial epithelial CFTR transcripts with exon 9 intact (exon 9+) (Fig. 1) [Chu et al., 1993].

The abundance of exon 9- CFTR transcripts in respiratory epithelial cells lead two groups of investigators to determine whether it encoded a functional isoform of CFTR. Transduction of CFPAC (pancreatic adenocarcinoma cell line from a CF patient) or HeLa cells with the CFTR cDNA lacking exon 9 failed to confer cAMP-regulated chloride conductance [Delaney et al., 1993; Strong et al., 1993]. Protein analysis of transduced cells detected mutant CFTR that was immature and incompletely glycosylated in both studies, suggesting that exon 9- CFTR mRNA transcripts are translated but the protein
protein probably undergoes degradation within the endoplasmic reticulum. Expression of the CFTR missing exon 9 encoded amino acids in *Xenopus* oocytes incubated at room temperature also did not produce a cAMP-regulated chloride conductance. This finding is important as other CFTR mutants that are improperly processed at 37°C appear to fold properly and regain function at lower temperatures [Drumm et al., 1991; Denning et al., 1992]. Although some have argued that the protein made by the exon 9- transcript may have physiological function [Bremer et al., 1992], the lack of conservation of exon 9-transcripts in mice suggests otherwise [Delaney et al., 1993]. Thus, CFTR missing the region of NBF1 encoded by exon 9 does not function as a chloride channel and likely does not have any intracellular function.

I.A.5. THE 5'T VARIANT IN INTRON 8 OF THE CFTR GENE AND CONGENITAL ABSENCE OF VAS DEFERENS

Studies have shown that the frequency of the 5'T variant was significantly higher in men with CBAVD (46%), CUAVD (25%), or epididymal obstruction (29%) than the general population (5%) [Chillon et al., 1995; Costes et al., 1995; Jarvi et al., 1995; Zielenski et al., 1995b]. Indeed, the 5'T variant is the single most frequent CFTR gene sequence alteration found in CBAVD men (Table 2). The presumed role of the 5'T allele in the pathogenesis of CBAVD is supported by two separate studies showing that the frequency of the 5'T allele is lower in fathers (2.1% and 1.9%) than in mothers (5.2% and 4.7%) of CF patients [Chillon et al., 1995; Zielenski et al., 1995b]. In fact, Chillon et al. reported four fathers who had one CFTR gene with the 5'T allele and the other with a severe CFTR mutation (G542X, N1303K, 1812-1G→A, or 936delTA) [Chillon et al.,
and are phenotypically normal. Three hypotheses could explain the strong but incomplete association between the 5T allele and CBAVD. First, there could be a non-random association between 5T and CBAVD, with the allele segregating with but not being causal for the CBAVD phenotype; however, this hypothesis is probably untrue as the analysis of several intragenic DNA markers (i.e., within the CFTR gene) in the four fathers and in patients with CBAVD showed that several haplotypes are associated with the 5T allele [Chillon et al., 1995]. Second, the 5T variant may have a partially causal role, together with other CFTR gene mutations. The presence of another mutation in the same CFTR gene as the 5T allele is unlikely since all coding regions and their immediately flanking intron sequences have been screened [Chillon et al., 1995; Costes et al., 1995; Zielenski et al., 1995b]. However, this does not rule out mutations within the promoter region or introns of the CFTR gene. Third, the 5T variant could have a causal role in CBAVD, with other factors (genetic and/or environmental) accounting for these exceptional cases without CBAVD (i.e., the four fathers with the CFTR mutation/5T genotype). The association of the 5T allele with lower levels of normal, full-length CFTR mRNA supports the hypothesis that this variant generally causes CBAVD, especially when a CFTR mutation is present in the other copy of the gene. Nevertheless, the association between the 5T allele and CBAVD is not absolute. Taking into account the predicted frequency (1 in 222 men) and the reported frequency (~1 in 1,000 men) of CBAVD, it has been estimated that the penetrance of the 5T allele is ~0.6 [Zielenski et al., 1995b]. While confirmation of this estimate awaits further studies involving larger
penetrance.

I.B. HYPOTHESIS

An intriguing question pertains to the mechanism by which alterations in CFTR expression results in reproductive tract abnormalities in the absence of pathologic changes in other CF-associated organs (e.g., lung, nasal sinus, pancreas, liver). It is possible that the reproductive tract is more sensitive to CFTR dysfunction than the other tissues. Alternatively, the level of functional CFTR may vary between different tissues, with the lowest expression in the Wolffian structures. Since the 5T variant of the IVS8-T tract of the CFTR gene is associated with inefficient splicing of exon 9 and has been identified in a significant proportion of infertile men with isolated Wolffian duct anomalies, it is proposed that the splicing efficiency of CFTR exon 9 is lower in the reproductive tract than the other CF-associated organs.

I.C. PURPOSE

The purpose of my work was to compare the proportion of exon 9+ CFTR mRNA transcripts within nasal epithelia and vas deferens from the same individual, and to examine the relationship between the IVS8-T tract and the level of exon 9+ CFTR mRNA in these tissues.
II.A. PATIENTS AND SAMPLES

Twenty-four healthy men with a previous vasectomy presenting to a male infertility clinic for sterilization reversal were recruited for the study (mean age±SD=40±3 years). This study was performed with the approval of the Human Subjects Review Committee of the University of Toronto, and informed consent was obtained from each subject. None of the patients had a personal or family history of pulmonary or gastrointestinal manifestations suggestive of CF. Each patient underwent scrotal surgery (i.e., either vasovasostomy (re-connecting the vas deferens) or vasoepididymostomy (connecting the vas deferens to the epididymis)). Intraoperatively, while under general anesthesia, in each case the following were obtained: peripheral venous blood by venipuncture for analysis of the IVS8-T tract genotype, nasal epithelia by a sterile swab and a small piece of vas deferens (~5-10 mm, which would ordinarily be discarded) by excision for quantitative RT-PCR analysis. Nasal samples were placed into a 2 ml microfuge tube containing 500 μl of phosphate buffered saline and vasal samples were placed into a 2 ml microfuge tube, snap-frozen in liquid nitrogen, and stored at -70°C if not analyzed immediately.

II.B. ALLELE SPECIFIC OLIGONUCLEOTIDE ANALYSIS OF IVS8-T TRACT

Genomic DNA was isolated from peripheral blood lymphocytes according to standard protocols [Miller et al., 1988]. Exon 9 including the IVS8-T tract was amplified by the polymerase chain reaction (PCR) [Mullis et al., 1986] with primers 9i-5 (5'-TAATGGATCATGGGCCATGT -3') and 9i-3 (5'-ACAGTGTTGAATGTTGTCATGCA-3').
follows: 94°C for 2 min; denaturation at 94°C for 20 s, annealing starting at 60°C then auto-down 0.5°C/s over 20 s, and extension at 72°C for 30 s, for 20 cycles; followed by denaturation at 94°C for 20 s, annealing at 50°C for 20 s, and extension at 72°C for 30 s, for 15 cycles; followed by one final cycle of denaturation at 94°C for 20 s, annealing at 50°C for 20 s, and extension at 72°C for 7 min. The reaction mixture contained 5 µl of PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.75 mM MgCl₂, 0.001% gelatin), 5 µl of dNTP’s (2 mM each of deoxyadenine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate; Pharmacia); 50 ng of each primer; and 0.5 unit of Taq DNA polymerase (Perkin Elmer/Cetus) in a final volume of 50 µl, containing 50-100 ng of genomic DNA. All PCR amplifications were performed with the GeneAmp PCR System 9600 (Perkin Elmer/Cetus). Ten µl of PCR amplified DNA was denatured and vacuum-blotted to nylon membrane (Hybond-N+; Amersham). The following allele specific oligonucleotides were used: 5T (5’-GTGTGTTTAAACAGG-3’), 7T (5’-GTGTGTTTTTTTTAACAG-3’), and 9T (5’-TGTGTTTTTTTTTTAACAG-3’). Each oligonucleotide was labeled with ³²P by terminal transferase (Pharmacia), and hybridization was performed overnight at 42°C and the washings were done once with 3xSSC/0.1%SDS at room temperature for 20 min, and twice with 0.2xSSC at 36°C for 20 min. Amplified DNA from three individuals previously shown to have the 5T/7T, 7T/7T, or 9T/9T genotype were used to control for oligonucleotide specificity [Jarvi et al., 1995].
For reconstruction studies, a segment of exon 9+ cDNA was obtained from a plasmid containing full-length CFTR cDNA [Rommens et al., 1991] following two rounds of nested PCR. First round PCR was performed with plasmid wild-type CFTR cDNA, 5’ primer X5-5 (5’-GCTGTCAGCCGTGTTCTAG-3’, in exon 5) and 3’ primer 13i-3sA (5’-TGTCGAAAAGATCCACATCC-3’, in exon 13) for 35 cycles (94°C, 20 s; 60°C, 20 s; 72°C, 30 s) (Fig. 3A). The reaction mixture contained 5 μl of PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.75 mM MgCl2, 0.001% gelatin), 5 μl of dNTP’s (2 mM of each of the four nucleotides triphosphate; Pharmacia); 200 ng of each primer; and 0.5 unit of Taq DNA polymerase (Perkin Elmer/Cetus) in a final volume of 50 μl, containing 400 ng of plasmid wild-type CFTR cDNA. Nested PCR was performed on 1/5 of the reaction product from the first round under identical PCR conditions, except that 5’ primer 7i-5s (5’-TTCAATAGCTCAGCCTTC-3’, in exon 7) and 3’ primer X12-3 (5’-GTTAAAACATCTAGGTATCC-3’, in exon 12) were used. The PCR products were size fractionated on 1% agarose gel in 1 X TAE (40 mM Tris-acetate; 1 mM EDTA, pH 8.0). The exon 9+ cDNA fragments were extracted from the agarose gel and purified according to the manufacturer’s protocol (QIAquick Gel Extraction Kit/QIAGEN). Exon 9- cDNA fragments were isolated from commercially-obtained human lung total RNA (Clontech) after conversion to cDNA and amplification by two rounds of nested PCR as described above, except the exon 9- fragments were extracted from the agarose gel and purified (Fig. 3B).
Nasal epithelial cells were collected by centrifugation and lysed in 800 µl of TRIzol Reagent (Gibco/BRL). Vas deferens samples were homogenized with a rotor-stator homogenizer (PRO 200 Handheld Homogenizer, PRO Scientific Inc.) in 800 µl of TRIzol Reagent. Total RNA was extracted from the lysates according to the manufacturer’s instructions (Gibco/BRL). First strand cDNA was synthesized from total RNA using Superscript II RNase H\(^{-}\) reverse transcriptase (RT) and random hexanucleotide primers. The reaction (21 µl) contained up to 11 µl of total RNA, 2 µl 10xPCR buffer (100 mM Tris-HCl (pH 8.4), 500 mM KCl), 2 µl MgCl\(_2\) (25mM), 1 µl 10 mM dNTP mix, 2 µl DTT (0.1 M), 1 µl random hexamers (50ng/µL), 1 µl Superscript II RT (200 units/µl), and 1 µl \(E.\) coli RNase H (2 units/µl), with all components obtained commercially from Gibco/BRL.

To ensure that total RNA was successfully extracted and synthesized into cDNA, mRNA of the human housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Tokunaga et al., 1987] from each sample was amplified using the PCR conditions described below. Primers used in the reaction were 5’primer GAPDH5B (5’-GGTCGGAGTCAACGGATTTGGTCG-3’) and 3’ primer GAPDH3B (5’-CCTCCGACGCCTGCTTCACCAC-3’). The amplified products (788 bp) were size fractionated by agarose gel (1.5%) electrophoresis, transferred to Hybond N+ nylon membrane (Amersham) by the method of Southern [Southern, 1975] and evaluated with a \(^{32}\)P-labeled human GAPDH cDNA probe (GAPDH 5A; 5’-CCAATATGATTCCACCCATG-3’) internal to the amplified region. The resultant
After being adjusted with GAPDH mRNA transcript level for each individual sample, transcripts of the CFTR gene were amplified by PCR. The oligonucleotide primers included 5' primer X8-5 (5'-ACGACTACAGAAGTAGTGATGGAG-3', in exon 8) and 3' primer C16D (5'-GTGGCCATGCTTTGATGACGCTTC-3', in exon 10) (Fig. 5A), and amplification was performed as follows: 94°C for 2 min; denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s, for 30 cycles; followed by one final cycle of denaturation at 94°C for 20 s, annealing at 50°C for 20 s, and extension at 72°C for 7 min. The PCR products were blotted to nylon membrane and hybridized with a CFTR cDNA oligonucleotide probe derived from exon 10, which would anneal to both exon 9+ and exon 9- fragments (C16B; 5'-GTTTTTCCTGGATTATGCCTGGCAC-3') (Fig. 5B). The expected size of the exon 9+ and exon 9- products were 416 bp and 233 bp, respectively. To ensure that the 416 bp and 233 bp fragments respectively represented exon 9+ and exon 9-, two oligonucleotide probes were designed: 9i-5s (5'-ACAGGGATTTGGGAATTATTG-3'), a sequence within exon 9, and ex8/10 (5'-TGGGAGGAGACTTCACTT-3'), a sequence which spans the 3' end of exon 8 and the 5' end of exon 10 (Fig. 5B).

The absence of contaminants in RT-PCR assays was regularly assessed by controls that did not contain any cDNA template, starting RNA, or RT enzyme.
Nasal epithelial and vas deferens total RNA were extracted, converted to cDNA, amplified by PCR with CFTR primers, and subjected to Southern analysis using a \(^{32}\text{P}\)-labeled exon 10 probe (C16B) as described in Section II.D. The resulting autoradiograph was analyzed by scanning densitometry, and the proportion of exon 9+ transcripts, as a percentage of total CFTR transcripts, was derived by using the total densitometric units of both transcripts as 100%.

As standards for quantification of exon 9+ and exon 9- mRNA species, the isolated exon 9+ and exon 9- cDNA fragments (see Section II.C.) were serially mixed in varying, known quantities and subjected to the same PCR conditions as for the nasal and vas deferens samples as outlined in Section II.D. Quantification of exon 9+ and exon 9- was then determined as described above. The results were plotted and graphed. All experimental proportions of exon 9+ and exon 9- CFTR transcripts were accordingly adjusted, based on this graph.

II.G. ANALYSIS OF DATA

Differences in IVS8-T allele frequencies between study subjects and the general population were compared by the \(\chi^2\) statistic or Fisher’s exact test. The mean proportions of CFTR exon 9+ mRNA transcripts associated with the different IVS8-T genotypes were compared using analysis of variance (ANOVA). Post-hoc pairwise comparisons between the different means were evaluated by the Bonferroni test. The correlation between the proportion of exon 9+ transcripts and length of the IVS8-T tract was analyzed by the Spearman rank correlation. Intra-individual nasal and vas deferens levels of exon 9+
comparisons and those less than 0.05 were considered to indicate statistical significance.

All statistical analyses were performed using *SPSS for Windows 1994*.
III.A. IVS8-T TRACT GENOTYPE

Leukocyte genomic DNA was isolated and amplified by PCR using CFTR primers which encompass the IVS8-T tract and exon 9 region (Fig. 2A). Subsequent ethidium bromide staining and gel electrophoresis confirmed successful amplification (Fig. 2B). Slot blot analysis using allele specific oligonucleotides for the 5T, 7T, and 9T variants revealed that, of the 24 patients, 8 were 7T/9T heterozygotes, 14 were 7T/7T homozygotes, and the remaining 2 were 5T/7T heterozygotes (Fig. 2C). As shown in Table 3, the frequencies of the 9T, 7T, and 5T alleles were not significantly different between individuals in the present study and the general population [Kiesewetter et al., 1993; Cuppens et al., 1994; Dork et al., 1994; Chillon et al., 1995] (9T: 16.7% vs. 11.5%, p=0.28; 7T: 79.2% vs. 83.3%, p=0.46; 5T: 4.2% vs. 5.2%, p=0.75).

III.B. CONVERSION OF EXPERIMENTAL TO ACTUAL PROPORTIONS OF EXON 9+ CFTR mRNA

To establish accurate measurement of the proportion of exon 9+ CFTR transcripts, it was first necessary to establish a standard curve using known concentrations of isolated fragments of exon 9+ and exon 9- transcripts. A plasmid containing full-length CFTR cDNA served as the source of exon 9+ transcripts [Rommens et al., 1991]. After two rounds of nested PCR using primers as shown in Fig. 3A, the exon 9+ fragments were extracted from agarose gel and purified. Exon 9- CFTR transcripts were similarly obtained, except lung total RNA served as the source (Fig. 3B). Analysis by electrophoresis and ethidium bromide staining revealed that a greater concentration of
was then serially diluted and mixed with a constant quantity of the latter, and analyzed by Southern transfer and scanning densitometry. This demonstrated that the concentration of the isolated exon 9+ species was higher than that of exon 9- (Fig. 3D). Therefore, the former was diluted to obtain equivalent concentrations of exon 9+ and exon 9- transcripts. We then verified our calibration and the purity of the isolated exon 9+ and exon 9- fragments by subjecting them to Southern analysis and hybridization with both specific and common radiolabeled oligonucleotide probes (Fig. 3E). Hybridization with the probe common to both transcripts (C16B) and subsequent densitometric analysis confirmed that we had accurately obtained equivalent concentrations of the exon 9+ and exon 9- fragments (Fig. 3F). Furthermore, hybridization with probes unique for exon 9+ or exon 9- mRNA, validated the purity of the exon 9+ and exon 9- isolates, as the exon 9+ specific probe (9i-5s) and the exon 9- specific probe (ex8/10) annealed only to the exon 9+ and exon 9- cDNA fragments, respectively (Fig. 3F).

Next, the exon 9+ and exon 9- fragments were serially mixed in known proportions and subjected to the identical PCR conditions and quantification protocol as for the nasal and vas deferens specimens (Fig. 3G-I). The experimental proportions of exon 9+ and exon 9- transcripts were derived from densitometric scanning, taking the sum of densitometric units for the exon 9+ and exon 9- bands as 100%. The corresponding actual and experimental proportions of exon 9+ transcripts were then recorded graphically (Fig. 3J). Consequently, all experimental proportions of exon 9+ transcripts from our tissue samples were adjusted according to this graph.
Total RNA was extracted from nasal and vas deferens specimens, converted to cDNA, amplified initially with primers for the transcript of the ubiquitously expressed GAPDH gene, and analyzed by Southern hybridization. Quantification of the autoradiographic bands by scanning densitometry showed differences in the yield of RNA extraction and cDNA synthesis (Fig. 4). This information was then employed to estimate the volume of cDNA to be used for PCR amplification with CFTR primers. For example, Fig. 4 illustrates that cDNA obtained from the vas of patient 5 was ~3.4 times less than that of patient 1, hence 3.4 times the amount of cDNA from patient 5 was used for the CFTR transcript analysis. These steps minimized inter-sample variability in RNA extraction and cDNA synthesis, thus allowing comparison of the proportion of exon 9+ CFTR transcripts within and between subjects.

Evaluation of CFTR mRNA transcripts from nasal epithelial and vas deferens cells in the region encompassing exons 8 to 10 after conversion to cDNA and PCR amplification revealed two different fragments. The difference in size between these two bands (416 bp and 233 bp) corresponded to the size of exon 9 (183 bp) (Fig. 5A). Southern analysis with an exon 10 specific probe (C16B) showed that both fragments contained exon 10 sequences (Fig. 5B; Fig. 5C, lane 1). However, Southern analysis with the exon 9+ specific probe (9i-5s) annealed to the larger fragment only (Fig. 5B; Fig. 5C, lane 2), while the exon 9- specific probe (ex8/10) detected only the smaller transcript (Fig. 5B; Fig. 5C, lane 3). These results confirm that the larger transcript represented the normal, full-length transcript with exon 9 intact, and that the smaller transcript represented the in-frame exon 9 deleted transcript.
III.D. QUANTIFICATION OF EXON 9+ CFTR mRNA

All proportions of exon 9+ transcripts reported hereafter refer to the corrected, actual proportions, based on Fig. 3J. The proportion of exon 9+ nasal transcripts were 93±2%, 82±3%, and 74±1% (mean±SD) for the 7T/9T, 7T/7T, and 5T/7T genotypes, respectively, while in the vas deferens samples, the proportion of exon 9+ transcripts were 88±3%, 76±3%, and 64±4% for the 7T/9T, 7T/7T, and 5T/7T groups, respectively (Fig. 6 & 7; Table 4).

III.E. RELATIONSHIP BETWEEN LEVEL OF EXON 9+ CFTR mRNA AND THE IVS8-T TRACT

For both the nasal and vas deferens samples, significant differences were noted in the mean exon 9+ levels for the different IVS8-T genotypes (p<0.0001, ANOVA; p<0.05, Bonferroni). In addition, a strong positive correlation was found between the length of the IVS8-T tract and the proportion of exon 9+ transcripts in nasal epithelia (r=0.88, p<0.001, Spearman) and in vas deferens (r=0.87, p<0.001, Spearman) (Fig. 7).

III.F. COMPARISON OF THE PROPORTION OF EXON 9+ CFTR mRNA IN NASAL EPITHELIUM TO VAS DEFERENS

The mean levels of nasal and vasal exon 9+ transcripts were 85±7% and 79±8% (mean±SD), respectively. When evaluating the proportions of exon 9+ transcripts for these two different CFTR expressing tissues from the same subject as paired samples, a significantly higher proportion of exon 9+ transcripts was found within the nasal epithelium compared to the vas deferens (p<0.001, paired t-test) (Fig. 8).
IV. DISCUSSION

IV.A. SPlicing Efficiency of CFTR Exon 9 Is Related to the IVS8-T Tract Length

In the present study, we found a relationship between the IVS8-T genotype and the proportion of normal, full-length CFTR mRNA transcripts (exon 9+) in nasal epithelia and vas deferens. It appears that the longer the IVS8-T tract, the greater the proportion of normal CFTR transcripts in nasal epithelia and vas deferens cells (Fig. 7; Table 4). This finding is consistent with that reported by Chu et al., who also demonstrated a positive correlation between the proportion of exon 9+ transcripts in bronchial epithelial cells and the length of the IVS8-T tract [Chu et al., 1993]. It is likely that this relationship holds true for other CFTR-expressing tissues as well.

IV.B. Higher Proportion of Normal CFTR mRNA in Nasal Epithelium Than Vas Deferens

Our study also showed that there is a greater proportion of the normal, full-length CFTR message in nasal epithelia than in vas deferens from the same subject, regardless of the IVS8-T genotype (p<0.001, paired t-test) (Fig. 8). In other words, the precise excision of intron 8 with in-frame joining of exon 8 and exon 9 of CFTR mRNA may be less efficient in epithelia of the reproductive tract compared to those of the respiratory tract. Two recent reports corroborate our findings. Teng et al. found that, for the same IVS8-T tract genotype, the proportion of exon 9+ transcripts was lower in a series of vas deferens samples obtained from vasectomized men than in a series of nasal biopsies obtained from different men and women with chronic nasal obstruction or sinusitis [Teng et al., 1997]. Rave-Harel et al. documented that three men with CBAVD had an increased level of exon
Taken together, these and our observations support the hypothesis that splicing efficiency varies between the different tissues affected in CF.

IV.C. THE CFTR IVS8-T TRACT MODIFIES PHENOTYPE BY VARIABLE SPLICING OF EXON 9 IN DIFFERENT TISSUES

The discovery of differential splicing efficiency between the various tissues which express CFTR provides important insights into the relationship between levels of normal CFTR and phenotypic variation. For instance, the R117H mutation is associated with pancreatic-sufficient CF (CF-PS) [Kristidis et al., 1992]. CF-PS patients with this mutation have pulmonary dysfunction but do not have pancreatic exocrine insufficiency and their sweat chloride measurements are only modestly elevated [Hamosh et al., 1994]. Not surprisingly, the mild R117H mutation has been identified in otherwise healthy males with CBAVD. However, further genetic analysis uncovered that individuals heterozygous for the R117H mutation on a 5T background (i.e., R117H and 5T on the same chromosome) and a “severe” CFTR mutation (e.g., ΔF508, G551D) developed lung disease characteristic of CF, whereas the R117H mutation found in CBAVD men is associated exclusively with the more efficient splice acceptor 7T (i.e., R117H and 7T on the same chromosome) [Kiesewetter et al., 1993]. It is also important to note that the R117H mutation gives rise to a partially functional CFTR protein [Sheppard et al., 1993]. Therefore, the R117H/5T allele results in a low enough level of partially functioning CFTR in the lung and an even lower level in the reproductive tract such that both organs are affected. In contrast, the R117H/7T allele, although producing a sufficient level of partially functional CFTR in the lung to prevent disease, the lower level in the genital
results strongly suggest that the specific IVS8-T tract background on which a CFTR mutation resides can modulate disease severity in a tissue-specific manner.

IV.D. PROPORTION OF NORMAL CFTR mRNA PRODUCED BY THE IVS8-T ALLELES

The frequencies of the 9T, 7T, and 5T alleles of the IVS8-T tract of the CFTR gene in our study sample are similar to those in the general population [Kiesewetter et al., 1993; Cuppens et al., 1994; Dork et al., 1994; Chillon et al., 1995] (Table 3). This finding is not unexpected as both groups consist of normal, healthy subjects. The fact that our study lacked subjects with the 9T/9T, 5T/9T, or 5T/5T genotype is likely a consequence of the relatively small sample size. Despite the absence of these groups, based on our subjects with the 7T/9T, 7T/7T and 5T/7T genotypes (Table 4) and the assumption that each of the two CFTR alleles contributes equally to the total amount of CFTR transcripts, it is inferred that the 7T allele produces ~41% exon 9+ transcripts (i.e., ~41% of the total amount of CFTR transcripts is exon 9+) in nasal epithelium and ~38% exon 9+ transcripts in vasal epithelium. It follows, then, that the 5T allele produces ~32% and ~26.5%, and the 9T allele produces ~49.5% and ~49%, exon 9+ CFTR mRNA in nasal epithelial and vas deferens cells, respectively (Table 5). Therefore, although the present study does not consist of any individuals with the 9T/9T, 5T/9T, or 5T/5T genotypes, it can be deduced that they would have 99% and 98%, 82% and 76%, and 64% and 53%, exon 9+ CFTR transcripts in nasal epithelia and vas deferens, respectively (Table 6).
phenotype and the amount of normal CFTR message (Fig. 9). A phenotypically normal, male CF carrier typically has the 7T variant on one chromosome and a severe CFTR gene mutation (e.g., ΔF508) on the other. The latter will result in absent or non-functional CFTR protein while the 7T variant may give rise to ~41% normal CFTR in respiratory tract and ~38% normal CFTR in reproductive tract, enough to sustain a normal phenotype in these tissues. On the other hand, a typical CBAVD patient may harbor the 5T variant on one chromosome and a severe CFTR mutation on the other. He may produce ~32% normal CFTR in the lung, which is adequate to sustain normal pulmonary function, but ~26% in the reproductive tract, an insufficient level to confer a normal genital duct phenotype. However, the occurrence of fertile males with the severe CFTR mutation/5T genotype, such as fathers of some CF patients [Chillon et al., 1995], suggests that other genetic factors (e.g., expression of alternative chloride channels) and/or environmental influences can ameliorate the unfavorable effects of certain CFTR gene sequence alterations. Alternatively, since a range of exon 9+ mRNA level does exist for the same IVS8-T genotype (Fig. 7; Table 4), these healthy, fertile men with a severe CFTR mutation and the 5T allele may produce a level of normal CFTR from the 5T chromosome that exceeds a minimal essential threshold. Furthermore, CBAVD men with the severe CFTR mutation/5T genotype may harbor additional mutations not detectable by our current DNA mutation screening methods (e.g., mutations within the promoter region or introns of the CFTR gene).

Various studies have reported different mean values for the proportion of exon 9+ transcripts produced from the various IVS8-T tract genotypes. For example, with respect
to the 7177T genotype, Chu et al. reported a mean proportion of exon 9+ nasal epithelial transcripts to be 75% [Chu et al., 1993] while Teng et al. documented 86% [Teng et al., 1997]. Although these estimates are not significantly different from ours (82%), the slight discrepancy may be partly explained by the different methods used in the quantitative RT-PCR analysis, such as the number of cycles and nested rounds of PCR employed, which pairs of primers were used in the PCR reaction, whether oligodeoxythymidine or random primers were utilized in the first strand cDNA synthesis, etc. In addition, these studies employed a differential RT-PCR which may lead to preferential amplification of the smaller exon 9- cDNA [Walsh et al., 1992]. Recently, Rave-Harel et al. designed nondifferential RT-PCR reactions in which both exon 9+ and exon 9- cDNA products were of the same size [Rave-Harel et al., 1997]. However, this necessitated the use of different primers and oligonucleotide probes. The former may result in different amplification efficiencies while the latter will require deprobing and rehybridization procedures which may lead to inadequate stripping of the previous probe and/or some loss of membrane-bound PCR products. In our approach, we minimize the number of PCR cycles and introduce a standardization curve which should more accurately reflect the actual proportion of exon 9+ transcripts (Fig. 3). The methodology involved is simple and may be applied broadly to other similar quantitative RT-PCR analyses.

**IV.E. MOLECULAR MECHANISM OF DIFFERENTIAL SPlicing EFFICIENCY OF CFTR EXON 9**

Several consensus sequences are found within introns of higher organisms that are important for the efficient splicing of nuclear pre-mRNA. These include the 5'-GU splice donor, 3'-AG splice acceptor, branch-point A at ~20 to 50 bases from the 3' splice site,
consensus has eleven consecutive nucleotides consisting of thymidine and/or cytosine [Krainer and Maniatis, 1988]. Extensive polypyrimidine tracts can make these sites more competitive as splice acceptor sites [Helfman and Ricci, 1989; Smith and Nadal-Ginard, 1989], while deletions in the polypyrimidine tract has been shown to inhibit the 5' cleavage reaction [Frendeway and Keller, 1985; Reed and Maniatis, 1986; Ruskin and Green, 1985], binding of the splicing factors U2AF and U2snRNP [Ruskin et al, 1988], and assembly of the splicing complex [Frendeway and Keller, 1985]. Furthermore, the observation that identical pre-mRNA transcripts are processed into alternatively spliced forms in a cell-type specific manner strongly suggests that the splicing environment of different cells varies for these transcripts [Breibart and Nadal-Ginard, 1987; Wieczorek et al., 1988]. This tissue-specific difference in the splicing environment may be due to the presence of specific alternative splicing factors or variations in the activities or levels of constitutive splicing factors. Thus, the short polypyrimidine tract in CFTR intron 8, especially with 5T, likely makes it underutilized as a splice acceptor site, resulting in reduced efficiency of exon 9 splicing. This effect is presumably more pronounced in the reproductive tract than in the other CF-associated organs.

IV.F. SUMMARY

In summary, our study confirms the hypothesis that splicing efficiency of CFTR pre-mRNA varies between the different organs affected in CF. Specifically, depending on the IVS8-T tract background, the splicing efficiency of CFTR exon 9 may be poor in the genital tract but adequate in other tissues, thus explaining some cases of the CAVD or
However, the existence of fertile males with the 5T genotype [Chillon et al., 1995] imply that other genetic factors (e.g., expression of alternative chloride channels) and environmental influences contribute to the overall phenotype. Studies such as ours may have important future implications for CF gene therapy, which will involve delivery of the appropriate level of normal CFTR into affected cells. Moreover, the study of the molecular pathogenesis underlying CF-associated male infertility is especially relevant given that the barrier to conception for men with CF, CAVD, or epididymal obstruction has been overcome by advances made in the assisted reproductive technologies, thus raising the concern of passing deleterious genetic traits onto subsequent generations [Mak and Jarvi, 1996].
Alternative splicing variants other than exon 9- have been detected in CFTR transcripts by RT-PCR [Bremer et al., 1992; Delaney et al., 1993], including ones which involve the 5’ end, portions of TM1, portions of NBD1, or the 3’ end of the CFTR gene. To date, these variants have not been demonstrated to result in functional CFTR isoforms. These patterns of alternative RNA splicing have been identified for the CFTR gene in a variety of tissues, including lung, pancreas, intestine, and testis; however, similar experiments have not been conducted in Wolffian-derived tissues. Since the latter structures are highly sensitive to aberrant splicing as our study suggests, it would be reasonable to assume that alternatively spliced transcripts may occur with greater frequency in the reproductive tract. Therefore, we hypothesize that aberrantly spliced transcripts other than exon 9-, previously discovered or novel, occur in the vas deferens and/or epididymis.

The tissue-specific difference in the splicing environment between the various organs which express CFTR may be due to the presence of specific alternative splicing factors. The identification and characterization of alternative splicing factors can be approached by developing in vitro splicing systems able to accurately reproduce cell-specific splicing patterns. This should be possible either by using nuclear extracts from the different cell types, or by complementing HeLa nuclear extracts [Smith et al., 1989; Hodges and Bernstein, 1994]. Other strategies for the isolation of factors include co-injection of nuclei or nuclear extracts into Xenopus oocytes, along with the experimental transcript [Kelley and Perry, 1986; Kedes and Steitz, 1988]. This system would also
vitro. With these approaches, alternative splicing factors which act on CFTR may be identified and their mode of action subsequently characterized. The elucidation of alternative splicing mechanisms could play an important role in development of novel therapeutic strategies for CF. For example, it may be possible to design pharmacologic agents which upregulate the level and/or activity of constitutive splicing factors, or which downregulate that of alternative splicing factors as means of augmenting levels of the normal CFTR gene product.

Our findings may have a more immediate application to our study population in terms of providing prognostic information. Although too early in the post-operative period for us to address this issue critically, it would be interesting to see whether a correlation exists (after controlling for variables such as patient age, interval between vasectomy and vasectomy reversal, and CF carrier status) between the length of the IVS8-T tract and the presence of sperm in the ejaculate post-vasectomy reversal. Indeed, it appears that azoospermic men with idiopathic epididymal obstruction (who have a significantly higher frequency of the 5T variant (29%) compared to the general population (5%) [Jarvi et al., 1995]) have a lower rate of surgical success (defined as presence of sperm in the ejaculate post-vasoepididymostomy) compared to men with acquired epididymal or vasal obstruction [Jarvi, K., unpublished data]. Although the difficulty of surgery and the actual site of anastomosis are paramount in prognosticating post-operative success, inadequate CFTR chloride function in the former group may contribute to re-stenosis of the excurrent genital ducts.


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<tr>
<td>Percentage motility:</td>
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<td>Percentage normal morphology:</td>
<td>$&gt;50%$</td>
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<td>White blood cell count:</td>
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</tr>
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<td>CFTR genotype</td>
<td>No. of CBAVD men (%)</td>
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<td>---------------</td>
<td>----------------------</td>
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</tr>
<tr>
<td>- / -</td>
<td>40 (18.4)</td>
</tr>
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</table>

*aCalculated from a total of 217 CBAVD men [Chillon et al., 1995; Costes et al., 1995; Zielenski et al., 1995b]. -b"-" indicates no CFTR mutations detected.*
<table>
<thead>
<tr>
<th>Group</th>
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</tr>
<tr>
<td>no. with allele / no. studied (%)</td>
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<td></td>
</tr>
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<td>38/48</td>
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<tr>
<td>General population (498 chromosomes)</td>
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<td>415/498</td>
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aData pooled from Kiesewetter et al., 1993, Cuppens et al., 1994, Dork et al., 1994, and Chillon et al., 1995.

b$\chi^2 = 1.1, p = 0.28$ for comparison with the 9T allele frequency in the general population.

c$\chi^2 = 0.5, p = 0.46$ for comparison with the 7T allele frequency in the general population.

d$p = 0.75$, Fisher's exact test, for comparison with the 5T allele frequency in the general population.
<table>
<thead>
<tr>
<th>Subject number</th>
<th>IVS8-T genotype</th>
<th>Nasal exon 9+ (%)</th>
<th>Vas deferens exon 9+ (%)</th>
<th>Mean± SD nasal exon 9+ (%)</th>
<th>Mean±SD vas deferens exon 9+ (%)</th>
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<td>7T/9T</td>
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*Values represent corrected proportions of exon 9+ transcripts, according to Fig. 3J.*
T tract alleles

<table>
<thead>
<tr>
<th>IVS8-T allele</th>
<th>Proportion of nasal exon 9+ (%)</th>
<th>Proportion of vasal exon 9+ (%)</th>
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Values represent corrected proportions of exon 9+ transcripts, according to Fig. 3J.
T tract genotypes<sup>a</sup>

<table>
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<th>IVS8-T genotype</th>
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<th>Proportion of vasal exon 9+ (%)</th>
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<tr>
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<sup>a</sup>Values represent corrected proportions of exon 9+ transcripts, according to Fig. 3J.
The intron 8 polypyrimidine tract of the CFTR gene and alternative splicing of CFTR exon 9. Three alleles have been identified in the polypyrimidine tract within the splice acceptor site of intron 8 of the CFTR gene: one with 9 thymidines, one with 7 thymidines, and one with 5 thymidines (9T, 7T, 5T) [Chu et al., 1991]. All three alleles are associated with two transcripts, one that is normally spliced with exon 9 intact (exon 9+) and one that is aberrantly spliced, with skipping of exon 9 but in-frame joining of exons 8 to 10 (exon 9-) [Chu et al., 1993]. Transfection studies demonstrated that the protein made from the exon 9- transcript is incapable of functioning as a cAMP-regulated chloride channel [Strong et al., 1993; Delaney et al., 1993]. In addition, quantitative RT-PCR analysis performed on bronchial epithelial cells showed that individuals with the longer T-tract length had a higher proportion of exon 9+ transcripts [Chu et al., 1993]. Therefore, CBAVD men with the 5T variant likely produce lower levels of normal CFTR mRNA due to a less efficient splicing machinery.
Figure 1.
Figure 2. Detection of IVS8-T genotype by oligonucleotide hybridization.

A) Leukocyte genomic DNA from study subjects were extracted and the region encompassing the intron 8 polypyrimidine tract was amplified by PCR with primers indicated in the schematic to generate a predicted product of 560 bp. 

B) Amplification was evaluated by ethidium bromide staining and gel electrophoresis. PCR product was generated from all subjects (numbered 1 to 24) (M=100 bp marker; Neg=no DNA template). 

C) An aliquot of amplified DNA was denatured and vacuum-blotted to nylon membrane and hybridized with radiolabeled oligonucleotides which specifically recognize 5T, 7T, or 9T alleles. Autoradiography revealed that subjects 1, 9, and 23 possess the 7T/9T, 7T/7T, and 5T/7T genotypes, respectively.
Figure 2A.

Figure 2B.
<table>
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**probes**

| Virus genotype | TT/9T | TT/TT | 5T/TT |

**IVS8-T genotype**

**Figure 2C.**
type (WT) CFTR cDNA (exon 9+) were subjected to two rounds of nested PCR with primers as shown. Amplified products were assessed by ethidium bromide staining and agarose gel electrophoresis (Neg control=no DNA). Second round PCR fragments (834 bp) were extracted from the gel and purified. B) Fragments of CFTR exon 9- transcripts (651 bp) were obtained in a similar manner from human lung total RNA. C) Same volumes of the isolated exon 9+ and exon 9- fragments were loaded onto separate lanes and size fractionated by agarose gel electrophoresis for comparison. Note that a higher yield was obtained for the exon 9+ fragments, as reflected by their greater intensity under ultraviolet light. D) To obtain equivalent concentrations of exon 9+ and exon 9- transcripts, the exon 9+ fragments were serially diluted and mixed with a constant volume of the exon 9- fragments, and evaluated by Southern analysis. Hybridization with an exon 10 specific probe (C16B) produced bands of the two fragments, and quantification by optical density (OD) measurements demonstrated that the concentration of the isolated exon 9+ fragments was ~1.82 times that of exon 9- fragments. Thus, the exon 9+ fragments were diluted 1.82 times, making them equivalent in concentration to the exon 9- fragments. E) In order to verify calculations and the purity of the exon 9+ and exon 9- isolates, Southern analysis and hybridization with a common probe (C16B), exon 9+ specific probe (9i-5s), and exon 9- specific probe (ex8/10) were used. F) Hybridization with C16B and OD measurements confirmed that concentrations of exon 9+ (OD=1.482) and exon 9- (OD=1.478) fragments were equivalent. Hybridization with 9i-5s and ex8/10 validated the purity of the isolates, as shown by their annealing to only the exon 9+ and exon 9- fragments, respectively. G-I) The exon 9+ and exon 9- fragments were serially
subjected to the same PCR conditions and quantification procedure as for the nasal and vas deferens specimens. The experimental proportions of exon 9+ and exon 9- transcripts were evaluated by scanning densitometry, with the sum of OD measurements for exon 9+ and exon 9- taken as 100%. J) Graph of actual versus experimental proportions of CFTR exon 9+ transcripts. All experimental proportions of nasal and vas deferens exon 9+ mRNA were adjusted according to this graph.
Figure 3C.

Figure 3D.

Exon 9+  5.5 μL  OD 1.4955
Exon 9-  10.0 μL  OD 1.4915
Exon 9+ ~1.82 x Exon 9-
**Figure 3E.**

<table>
<thead>
<tr>
<th>Exon 9+ fragments</th>
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<tr>
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<td>651 bp</td>
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<tr>
<td>9i-5s</td>
<td>ex8/10</td>
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<td>C16B</td>
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**Figure 3F.**

- Exon 9+ OD 1.482
- Exon 9- OD 1.478
Figure 3G.

Figure 3H.
Figure 3I.

Figure 3J.
Figure 4. Evaluation of RNA extraction and cDNA synthesis. Total RNA was extracted from nasal and vas deferens specimens, converted to cDNA, and PCR amplified for a region of the GAPDH transcript. The products were analyzed by gel electrophoresis, Southern blotted, and hybridized with a $^{32}$P-labeled GAPDH probe specific for the amplified region. The resultant autoradiographic bands were quantified by densitometric scanning. Differences in the yield of RNA extraction and cDNA synthesis were adjusted for, prior to amplification with CFTR primers. For example, the concentration of cDNA obtained from the vas of subject 5 was ~3.4 times less than that from subject 1, hence 3.4 times the amount of cDNA from subject 5 was used for CFTR mRNA analysis.
Figure 4.
Figure 5. Evaluation of CFTR mRNA. A) Total RNA was extracted from nasal and vas deferens samples, converted to cDNA, and PCR amplified using primers from exons 8 and 10 (X8-5 and C16D, respectively), producing two different sized fragments which corresponded to the exon 9+ (416 bp) and exon 9- (233 bp) transcripts. B) The CFTR cDNA products were then separated by agarose gel electrophoresis and transferred to nylon membrane by Southern blotting, followed by hybridization with $^{32}$P-labeled probes specific for the exon 9+ or exon 9- fragments (9i-5s and ex8/10, respectively) and one common to both (C16B). C) C16B, a sequence within exon 10, identified two species (lane 1). The exon 9+ specific probe (9i-5s) detected only the larger transcript, corresponding to CFTR mRNA with exon 9 intact (lane 2), while the exon 9- specific probe (ex8/10) detected only the smaller transcript (lane 3) which represented the in-frame deleted exon 9 message.
Figure 5A.

Exon 9+ mRNA

Exon 9- mRNA

Figure 5B.

Figure 5C.
Figure 6. Quantification of nasal and vas deferens CFTR mRNA. The density of cDNA fragments on the autoradiographs was measured by scanning densitometry. The proportions of nasal and vas deferens exon 9+ mRNA, as a percentage of total CFTR transcripts, were determined by using the total densitometric units of both transcripts as 100%.
Figure 6.
Figure 7. Relationship between CFTR IVS8-T genotype and proportion of exon 9+ transcripts in nasal epithelia (♦) or vas deferens (●). Each specimen is represented by a separate symbol. Horizontal bars indicate mean values.
Figure 7.
Figure 8. Comparison of the proportion of CFTR exon 9+ transcripts within nasal epithelia and vas deferens from the same subject. Intra-individual values are connected by straight lines.
Figure 8.
Figure 9. Influence of CFTR genotype on phenotype by the proportion of normal CFTR mRNA. A typical patient with CBAVD may have a severe CFTR gene mutation (e.g., ΔF508) in one CFTR allele and the less efficiently splicing 5T variant in the other. He may produce a sufficient proportion of normal (exon 9+) CFTR mRNA (and therefore protein) from the 5T allele in the lung (32% of total CFTR transcripts) to confer a normal phenotype but not so in the reproductive tract (26%), thus giving him the isolated absence of vas deferens phenotype without any pulmonary manifestations. In contrast, a typical CF carrier may have a severe CFTR mutation in one copy of the CFTR gene but the more efficiently splicing 7T variant in the other chromosome. Although he will produce less of the normal CFTR mRNA in the reproductive tract (38%) compared to the lung (41%) from the 7T allele, both proportions exceed the threshold for normalcy, thus giving him a completely normal phenotype. The threshold level for normal/abnormal phenotype depicted in the figure is arbitrary and may vary between different tissues.
Figure 9.