Characterization of
Human Prostate-Specific Transglutaminase

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Gedrukt door Febodruk BV, Enschede

ISBN 90-9013327-5

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Karakterisering van
Humaan Prostaat-Specifiek Transglutaminase

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr. P.W.C Akkermans M.A.
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 8 december 1999 om 11.45 uur

door

Hendrikus Jan Dubbink

geboren te Utrecht

Promotiecommissie

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Dit proefschrift werd bewerkt binnen de Afdeling Urologie (Experimentele Oncologie) van de Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam. De uitgave van dit proefschrift is mede mogelijk gemaakt dankzij een financiële bijdrage van Yamanouchi Nederland B.V. en de Stichting Urologisch Wetenschappelijk Onderzoek (SUWO), Rotterdam.

'Our little systems have their day
They have their day and cease to be
They are but broken lights of Thee
And Thou o Lord art more than they.'

Alfred Tennyson

Voor Roely

Voor mijn moeder

Ter nagedachtenis aan mijn vader

Contents

Abbreviations

AP1	adaptor primer 1
ARE	androgen response element
BSA	bovine serum albumin
BPH	benign prostatic hyperplasia
CE	cornified cell envelope
CGAP	Cancer Genome Anatomy Project
DP1	dorsal protein 1
ECM	extracellular matrix
EMSA	electrophoretic mobility shift assay
ERSPC	European Randomized Study for Prostate Cancer
ESTs	expressed sequence tags
FCS	fetal calf serum
FXIII	plasma factor XIII
GPI	glycosylphosphatidylinositol
hTG_P	human prostate-specific TGase
IL-2	interleukin-2
kb	kilo base pairs
kDa	kilo Dalton
LTBP	latent TGF-β-binding protein (LTBP)
LI	autosomal recessive lamellar ichthyosis
PAP	prostatic acid phosphatase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	poly(ethylene glycol)
PIN	prostatic intraepithelial neoplasia
PLC-δ1	phospholipase C-δ1
PPIase	peptidyl-prolyl *cis-trans* isomerase
PPIP	cyclophilin A pseudogene
PSA	prostate-specific antigen
PSP94	prostatic secretory protein94 or β-microseminoprotein
RA	retinoic acid
5'-RACE	5'-rapid amplification of cDNA ends
rTG_P	rat prostate TGase
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecyl sulfate
TGase	transglutaminase
TG_C	tissue type TGase
TG_E	epidermal TGase
TGF-β	transforming growth factor-β
TG_K	keratinocyte TGase
TGM1,2,3,4,5	genes encoding TG_K, TG_C, TG_E, hTG_P, and TG_X
TUR	transurethral resection
UTR	untranslated region

Chapter 1

Introduction

1 General Introduction

During the last few years, research efforts on the prostate have increased considerably. This was stimulated by the fact that prostatic diseases, such as prostate cancer and benign hyperplasia of the prostate (BPH), have become a major medical problem, particularly since prostate diseases occur most frequently in elderly men, and life expectancy of the male population has increased over the past several years. Consequently, prostate research has mostly focussed on issues associated with these diseases, such as factors involved in their etiology, and the identification of new markers for early prostate cancer detection, prostate cancer staging, monitoring of prostate cancer treatment, and development of new therapeutic applications. Recent research also shows an increasing interest in regulatory regions of prostate marker genes which are potentially useful for gene therapy by directing expression of cytotoxic compounds to prostate cancer cells. In contrast to the considerable progress that was made in these research areas, knowledge of (normal) prostate physiology has increased to a more limited extent.

The present study was undertaken to investigate the occurrence and possible function of human prostate-specific transglutaminase (hTG_P), an enzyme produced by the human prostate. hTG_P is one out of seven distinct human transglutaminases (TGase) that are presently known. With one exception, all TGases share a common enzymatic function, leading to the post-translational modification of proteins by means of cross-linking. The specific function of each TGase is defined by its tissue location, differential regulation, substrate specificity, and other distinct functional (e.g. enzymatic) characteristics present within each enzyme. Although the rodent prostate TGase has already been described in 1896 by Gamus and Gley (see Aumuller and Seitz 1990), and extensively characterized during later years, only minimal research data up to now address the human analogue. The physiological function of hTG_P has not yet been elucidated.

The first part of Chapter 1 describes in more detail the physiology and pathology of the prostate and the usefulness of prostate markers in diagnosis and monitoring of prostate cancer. The second part of this chapter focusses on general characteristics of TGases and presents more specific data about the individual TGase family members.

2 Prostate physiology and prostate-specific proteins

2.1 Human prostate histology and anatomy

The prostate is a male accessory sex gland situated around the urethra just below the urinary bladder. It is an exocrine gland composed of branching secretory tubules and acini lined with secretory epithelium. The prostate excretory ducts originate from, and secrete their products into, the urethra (Cunha et al. 1987, Aumuller and Seitz 1990).

Several cell types can be distinghuished within the prostate. The epithelial cell compartment contains basal, luminal, and neuroendocrine cells. Basal epithelial cells are flattened cells with little cytoplasm localized parallel to the underlying basement membrane (McNeal 1988). Immunohistochemically, these cells can be selectively stained with antibodies against basal-cell specific cytokeratins such as 34ß-E12 which stains keratins 5, 10 and 11 (Allsbrook and Pfeifer 1997, Bostwick and Devaraj 1997). The basal epithelial cell layer consists of undifferentiated cells. There is some evidence that the basal cell compartment harbors the pluripotent stem cell population of the prostate that have the potency to differentiate into luminal secretory and neuroendocrine cells (McNeal 1988, Bonkhoff and Remberger 1996, Bostwick and Devaraj 1997, Xue et al. 1998). The basal cells have the capacity to divide and represent a considerable part of the proliferative compartment of the prostate (Bonkhoff and Remberger 1996).
The luminal epithelial cells are the fully differentiated secretory cells of the prostate. These cells have a cuboidal to high columnar appearance and contain numerous secretory vacuoles (McNeal 1988, Aumuller and Seitz 1990). In contrast to basal cells, luminal epithelial cells express typical prostatic secretory proteins like prostate-specific antigen (PSA) and prostatic acid phosphatase [PAP, (Cleary et al. 1983, McNeal 1988, Allsbrook and Pfeifer 1997)]. Proposed mechanisms of secretion by prostate epithelial cells are merocrine and apocrine secretion (Aumuller and Seitz 1990, Aumuller et al. 1997a, 1997b). The merocrine pathway uses N-terminal signal peptides present in particular secretory proteins, that direct the proteins to storage granules which in turn release their contents into the glandular lumen by fusion with the plasma membrane. Apocrine secretion represents the regulated release from the apical membrane of membrane-bound blebs containing secretory proteins. Evidence for secretion in the apocrine fashion has been obtained for dorsal protein I (DP1) or rat prostate TGase (Seitz et al. 1990, Steinhoff et al. 1994).
Neuroendocrine or endocrine-paracrine cells represent only a small part of the epithelial cell population and are randomly scattered through the prostate (Di Sant'Agnese 1992, Noordzij et al. 1995). Ultrastructurally, neuroendocrine cells can be recognized by the presence of dense secretory granules. The cells frequently display long dendritic processes extending under or between adjacent epithelial cells. Neuroendocrine cells express and secrete a wide variety of products including chromogranins A and B and many peptide hormones with potential growth modulating properties e.g. seretonin, bombesin, somatostatin, parathyroid hormone-related protein and calcitonin. Of these, chromogranin A and serotonin are most commonly expressed by prostatic neuroendocrine cells. The other peptides are produced by subsets of neuroendocrine cells. The role of prostatic neuroendocrine cells is so far unclear, but an autocrine or paracrine influence on growth and secretory activity of prostate cells has been suggested (Di Sant'Agnese 1997, 1998).
The epithelial cell layer is separated from the surrounding stromal component by a basement membrane. Important components of prostate stroma are smooth muscle cells, fibroblasts, nerve terminals, blood and lymph vessels, all of which are embedded in a loose collagenous extracellular matrix (Aumuller 1983, Cunha et al. 1987). Stroma-epithelial interactions play an essential role in prostate growth and development and in determining the final organ size in the adult (Cunha et al. 1987, Chung et al. 1991). Mesenchyme

influences gene expression and secretory cytodifferentiation of the luminal epithelial cells of the prostate (Cunha et al. 1992, Hayashi et al. 1993, Kinbara et al. 1996). The smooth muscle cells, present in thin sheaths around the acini or as broad strands separating larger parts of the gland, make the prostate well suited for the rapid expulsion of secretory fluid during ejaculation (Aumuller 1983, McNeal 1988).
McNeal has proposed that the human prostate can be subdivided in three major glandular regions: the peripheral zone, the central zone and the transition zone (McNeal 1988, 1997). These zones are distinguished by specific architectural and stromal features and their position relative to the urethra. Whereas the typical prostate epithelial cell markers PSA and PAP are expressed throughout the entire prostate, the expression of other proteins (pepsinogen II, tissue plasminogen, lactoferrin) is differentially regulated in the separate zones. Pathologically, this zonal subdivision is important because the majority of the prostate carcinomas arise in the peripheral zone, whereas the transition zone is the place of origin of BPH (McNeal 1992).

2.2 Prostate-specific proteins and their regulation by androgens

The human prostate is characterized by the expression of several tissue-specific proteins. By definition, their expression should be strictly confined to the prostate. However, as a consequence of the use of sensitive detection methods such as RT-PCR and extension of the number of tissues investigated, a previously established prostate-specific mRNA and/or protein is later often detected in other organs as well. This is also true for most of the proteins discussed below. It can be anticipated that more prostate-specific proteins will be identified in the near future (Vasmatzis et al. 1998).
Prostate-specific proteins can be categorized into secretory and non-secretory proteins. In Table 1 some properties of the discussed proteins are summarized. Most of the presently identified prostate-specific proteins are secretory proteins expressed by the luminal epithelial cells. These proteins are secreted into the prostatic ducts and ultimately become part of semen during ejaculation. To this group belong the structurally related kallikreins PSA and hK2 (for recent review, see Rittenhouse et al. 1998), PAP (Lilja and Abrahamsson 1988, Solin et al. 1990), prostatic secretory protein94 [β-microseminoprotein, PSP94 (Lilja and Abrahamsson 1988)], and human prostate-specific TGase [hTG_P (Dubbink et al. 1999b)]. Recently, a third prostate-specific kallikrein, called prostase, has been discovered (Nelson et al. 1999). Putative functions of hK2, PSA and hTG_P in semen physiology will be discussed in more detail below. The physiological functions of PSP94 and PAP are unclear as yet and their discussion is beyond the scope of this chapter. The non-secretory proteins include prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), and Nkx3.1. PSMA is a transmembrane glycoprotein (Israeli et al. 1994, Liu et al. 1997) that possesses hydrolase activity (Carter et al. 1996) and might function as a receptor mediating the internalization of a ligand (Liu et al. 1998). Immunohistochemically, PSMA has been demonstrated in luminal epithelial cells of normal and benign prostates, but usually not in basal cells (Wright et al. 1995, Bostwick et al. 1998). By *in situ* hybridization, however, PSMA expression could only be observed in the basal epithelial

Table 1. Prostate-specific proteins [a]

Protein	Epithelial Localization	Property	Androgen [b] regulation	Mass kDa
hK2	luminal	secretory	+	31
hTG_P	luminal	secretory	+	77
Nkx3.1	luminal	non-secretory	+	38
PAP	luminal	secretory	+/-	100 [c]
Prostase	luminal	secretory	+	33
PSA	luminal	secretory	+	33
PSCA	basal	non-secretory	nd	24
PSMA	luminal	non-secretory	-	100
PSP94	luminal	secretory	ai	16

[a] For references, see text

[b] +, up-regulated; -, down-regulated; +/-, both up- and down-regulation have been observed

[c] Consists of two disulfide-linked 50-kDa subunits

ai, androgen independent; nd, not determined yet.

cell layer (Kawakami and Nakayama 1997). Although PSMA is not a typical secretory protein it can readily be detected in seminal plasma, perhaps due to membrane shedding during the secretory process (Aumuller and Seitz 1990, Troyer et al. 1995). PSCA is a glycosylphosphatidylinositol (GPI)-anchored cell surface antigen of the basal epithelial cells, and the biological function of this antigen is so far unknown (Reiter et al. 1998). Overexpression of PSCA by cDNA transfection shows that the protein can be secreted (Reiter et al. 1998). It is conceivable, however, that PSCA is usually not secreted into prostatic fluid, because the protein is expressed by the basal epithelial cells. Nkx3.1 is a homeodomain-containing protein and probably represents a transcription factor. During development of the prostate in mice, Nkx3.1 has an intriguing, male-specific, expression pattern that suggests a regulatory role in the earliest stages of prostate formation: its expression (shown by *in situ* hybridization) coincides with the regions where prostatic buds will arise from the urogenital sinus epithelium. Also postnatal Nkx3.1 mRNA expression appeared to be indicative for ductal outgrowth and morphogenesis of the prostate. Nkx3.1 expression increases during sexual maturation, and in adulthood it may regulate the expression of specific secretory proteins (Sciavolino et al. 1997, Bhatia-Gaur et al. 1999). In contrast to PSA, hK2, PAP, and PSP94, which are expressed by most luminal epithelial cells (Lilja and Abrahamsson 1988, Young et al. 1992, 1996), hTG_P and PSMA expression is heterogeneous among the cells of the normal prostate (Wright et al. 1995, Bostwick et al. 1998, Dubbink et al. 1999b).

The expression of many of these prostate-specific proteins is under androgen control. Androgens act through interaction with the nuclear androgen receptor (for review, see Trapman and Cleutjens 1998). The complex which is formed by this interaction can directly

regulate transcription by binding to androgen response elements (AREs) present in the regulatory regions of target genes. The secretory proteins PSA, hK2, prostase, and hTG_P, as well as Nkx3.1 have been shown to be up-regulated by androgens (Riegman et al. 1991a, Young et al. 1992, Banas et al. 1994, Dubbink et al. 1996, He et al. 1997, Prescott et al. 1998, Nelson et al. 1999), whereas PSMA is down-regulated (Israeli et al. 1994). Contradictory results were reported on androgen regulation of PAP (Henttu et al. 1992, Banas et al. 1994). In case of hK2 and PSA several functional AREs have been demonstrated in the corresponding promoters (Riegman et al. 1991b, Murtha et al. 1993, Schuur et al. 1996, Cleutjens et al. 1997b, Yu et al. 1999). For the gene promoters of other prostate-specific proteins the presence of functional AREs remain to be elucidated. The proximal 1.5-2.1 kb of the PAP and hTG_P promoter regions do not respond to androgen and thus seem not to contain functional AREs (Shan et al. 1997, Dubbink et al. 1999a). It is possible that expression of these genes is more indirectly influenced by androgens, e.g. by stabilization of their transcripts or by regulation of other factors that can direct their transcription. In this regard, it would be of interest to know whether Nkx3.1, as prostate-restricted transcription factor, directly or indirectly is involved in the transcriptional regulation of genes encoding secretory proteins of the prostate.

2.3 Prostate-specific proteins as tumor markers

Prostate cancer is the most frequently diagnosed malignancy and the second highest cause of cancer death in men in Western countries (Parker et al. 1996). The specific causes of prostate cancer are poorly understood. Prostate cancer incidence increases as a function of age, family history of prostate cancer and, due to environmental factors (possibly diet), living in a westernized society (Carter and Coffey 1990, Giovannucci 1995, Key 1995). As long as prostate cancers remain confined to the prostate, surgical removal of the prostate by radical prostatectomy can cure the patient (Zincke et al. 1994). Once metastasized, however, prostate cancer becomes a fatal disease for which no curative treatment is presently available (Isaacs 1997).

Prostate-specific proteins might be useful for the early detection of organ-confined disease, for staging of prostate cancer and for monitoring of the patient's response towards a treatment schedule. At present, only PSA has been found suitable in a clinical setting. PSA meets many of the criteria for an ideal tumor marker (Oesterling 1991, Montie and Meyers 1997). In healthy man, PSA is almost completely confined to the prostate due to natural barriers which prevent leakage into the blood circulation and ensure that it is secreted into the prostatic ducts. Physical trauma or diseases like prostatitis, BPH and prostate cancer can disrupt these barriers causing PSA to be released into the circulation (Rittenhouse et al. 1998). Circulating levels of PSA rise and decline with tumor size increase and decrease, respectively, and can therefore be used for detection and monitoring the effects of prostate cancer treatment. Determination of PSA serum levels is complicated by the fact that multiple molecular forms exist, i.e., free and complexed with protease inhibitors. There is evidence that the relative amount of free and complexed PSA can be used as a prognostic indicator (for reviews, see Vessella and Lange 1997, Rittenhouse et al. 1998). Although

PSA expression reduces after malignant transformation, many tumor cells continue to express PSA (Abrahamsson et al. 1988, Gallee et al. 1990). During prostate tumor growth, cells may be shedded into the bloodstream. Measurement of PSA mRNA levels by RT-PCR in the peripheral blood cell population is therefore potentially useful in detecting circulating prostate tumor cells and may indicate metastatic cancer (Olsson et al. 1997, Verkaik et al. 1997).

Currently, there is a search for new prostate tumor markers complementary to or even better than PSA. Of particular interest would be to have markers that are only expressed after malignant transformation of the prostate occurs and can reliably be detected at the time the tumor achieves clinical importance (Oesterling 1991). Thus far such an ideal tumor marker has not been found, but also markers up-regulated after malignant transformation could be of importance. In this respect hK2 and PSMA might be of interest. Like PSA, hK2 leaks into the circulation and its serum concentration is elevated (Finlay et al. 1998) and may be present at levels comparable to PSA in patients with prostate cancer (Rittenhouse et al. 1998). Contrary to PSA, hK2 expression is found to be the highest in poorly-differentiated prostate adenocarcinoma when compared to benign and well-differentiated tissue and thus appears to be more tumor-associated than PSA (Rittenhouse et al. 1998). PSMA expression is enhanced with increasing tumor grade both due to increased expression and an increased number of positive cells (Wright et al. 1995, Kawakami and Nakayama 1997, Bostwick et al. 1998). Serum PSMA levels of prostate cancer patients are elevated (Murphy et al. 1998). In addition, like PSA, PSMA can often be demonstrated immunohistochemically in metastatic cancers (Wright et al. 1995). Research is ongoing to determine if hematogenous spread of prostate cancer cells can be measured with high sensitivity and specificity by means of RT-PCR of *hK2* and *PSMA* transcripts (Young et al. 1996, Olsson et al. 1997, Verkaik et al. 1997, Gala et al. 1998, Rittenhouse et al. 1998). It can be expected that the clinical value of these markers will soon be determined and that other prostate markers that become up-regulated after malignant transformation will be discovered in the near future (Israeli et al. 1997, Vasmatzis et al. 1998, Nelson et al. 1999).

2.4 Processes in the male genital tract

During the semen ejaculatory process, products of the prostate become part of the ejaculate (Figure 1). To understand the prostate function, it is important to know more of semen generation and composition.

After at least two months of spermatogenesis in the testis, the still infertile and immotile flagellar spermatozoa undergo further maturation in the epididymis into fertile sperm cells (Mann and Lutwak-Mann 1981, Kirchhoff et al. 1997). Sperm cells leaving the epididymis are motile, though this capacity is not used until after ejaculation (Mann and Lutwak-Mann 1981). During the ejaculatory process sperm cells encounter secretions from the male accessory glands which are located along the reproductive tract, including seminal vesicles, ampullae, prostate and bulbourethral or Cowper's glands, and become surrounded by the secretory products from these organs. The ultimate ejaculate consists for over 95% of

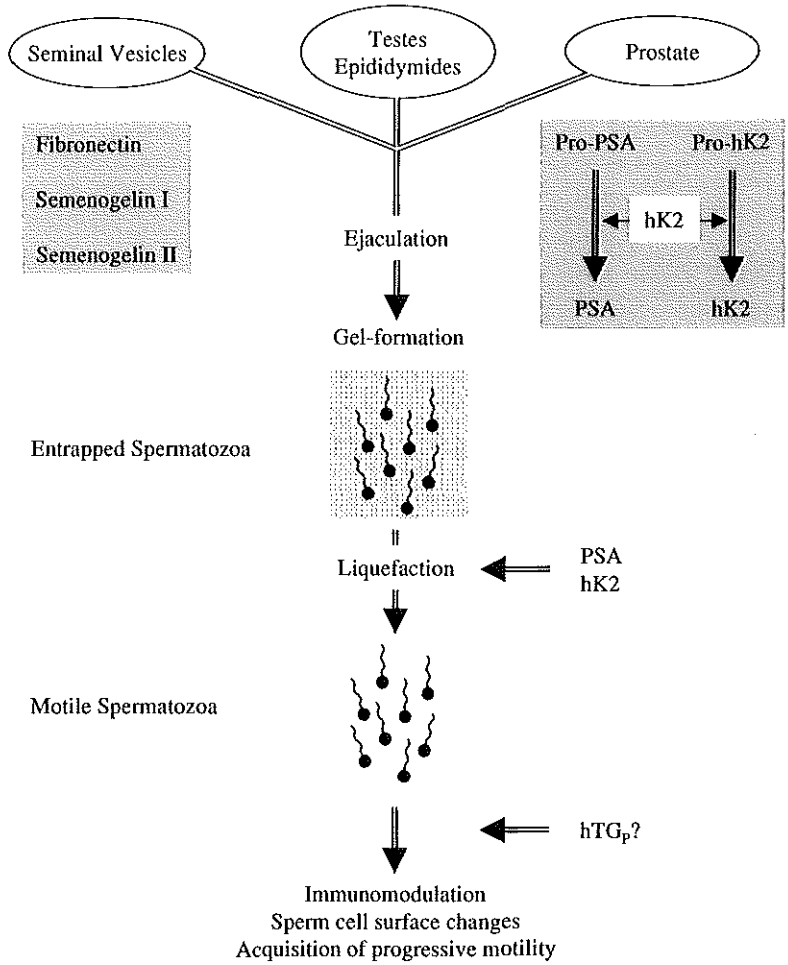

Figure 1. Major processes and proteins in semen. See text for details.

secretions derived from the accessory glands. The contributions of the prostate and the seminal vesicles to an ejaculated semen volume of 2-6 ml are approximately 30% and 60%, respectively. Immediately upon ejaculation, semen rapidly turns into a gel or coagulum in which most spermatozoa become entrapped (Zaneveld et al. 1974, Mann and Lutwak-Mann 1981). Subsequent liquefaction of the coagulum in 5 to 20 minutes releases the trapped immotile spermatozoa, which then gain progressive motility. Full fertilizing competence of the spermatozoa is acquired in the female reproductive tract after a process called capacitation. During this process further modifications occur at the sperm plasma membrane (Fraser 1995, Aitken 1997, Cooper and Yeung 1997).

2.5 Secretory products of the prostate in semen physiology

The precise biological importance of many of the seminal plasma constituents, including those contributed by the prostate, for fertilization and other aspects of sperm function, including interaction of spermatozoa with the immune system, is largely unclear. In general, it is suggested that secretions of the accessory glands optimize the fertilization process. Potential functions and biochemistry of selected prostatic products will be discussed in relation to physiological functions which can be attributed to semen as an entity (Figure 1).

2.5.1 Semen coagulation and liquefaction

Seminal gel formation

The main source of the gel-forming proteins is the secretory epithelium of the seminal vesicles, as has become clear from biochemical studies with split (partitioned) ejaculates (Tauber et al. 1980) from patients with a congenital absence or functional deficiency of these glands (Amelar 1962, Lilja et al. 1987) and from immunohistochemical studies (Lilja et al. 1989, Aumuller et al. 1990, 1997b). The predominant structural proteins of the seminal gel are now identified as fibronectin, semenogelin I and semenogelin II, which are high molecular mass proteins of 200-250 kDa, 52 kDa and dependent on its glycosylation status 71 or 76 kDa, respectively (Lilja et al. 1987, 1989, 1990, Malm et al. 1996). Despite the presence of disulphide linkages in the coagulum (Chaistitvanich and Boonsaeng 1983, Lilja and Laurell 1985), gel formation essentially results from non-covalent interactions of seminal vesicles proteins, because interference with non-covalent bonds, but not cleavage of the disulphide bonds, solubilizes the clot (Lilja and Laurell 1984, 1985).

Seminal gel proteolysis

Lysis of the gel is achieved by cleavage of the gel-forming proteins by proteolytic enzymes mainly derived from the prostate, generating low molecular mass protein products (Tauber et al. 1976, 1980, Lilja 1985). Gel proteolysis is important since poor semen liquefaction is often associated with subfertility (Zaneveld et al. 1974, Mann and Lutwak-Mann 1981). Although other prostatic proteases may participate in the liquefaction process, e.g metalloproteases, caseinolytic and gelatinolytic activities (see Wilson 1995), at present most is known about the involvement of the kallikreins hK2 and prostate-specific antigen (PSA or hK3, recently extensively reviewed by Dube and Tremblay 1997, Rittenhouse et al. 1998). PSA is one of the major protein constituents of seminal plasma and is present at concentrations ranging from 0.5 mg/ml to 5.5 mg/ml (Wilson 1995, Rittenhouse et al. 1998). Its contribution to and importance for gel hydrolysis is well established (Lilja 1985, Lilja et al. 1987). For hK2, this is more difficult to prove, because the seminal plasma concentration of hK2 is much lower, in the order of 2-12 μg/ml, and in contrast to PSA its activity disappears soon after ejaculation, because of proteolyic cleavage and complexation with seminal vesicle secreted protein C inhibitor [PCI (Deperthes et al. 1996, Dube and Tremblay 1997)]. PSA and hK2 are proposed to fulfill synergistic or complementary roles

in dissolving the coagulum, because they have different substrate specificities (Deperthes et al. 1996). Apart from the fact that PSA has chymotrypsin-like protease activity and hK2 trypsin-like activity (Mikolajczyk et al. 1998), determining their specific cleavage sites within protein substrates, both enzymes may show differential degradation potentials toward the same substrate. Semenogelin I and semenogelin II are hydrolyzed with comparable rate by PSA and hK2 *in vitro*, although distinct fragmentation patterns were obtained due to cleavage sites differences (Deperthes et al. 1996, Lovgren et al. 1997). Contrary to the degradation of the semenogelins, proteolysis of fibronectin occurs much more efficiently by hK2 (Deperthes et al. 1996). In fact, it has been shown that the hK2 protease activity may be 20.000-fold higher than PSA on the appropriate synthetic substrates (Rittenhouse et al. 1998). Therefore, the *in vivo* consequence of these distinct substrate specificities may be that the semenogelins are largely broken down by the action of PSA, whereas a substantial part of fibronectin might be degraded by hK2, despite the huge differences in seminal plasma concentrations of these kallikreins (Deperthes et al. 1996). However, the relative contribution of PSA and hK2 to gel-lysis *in vivo* still remains to be defined.

PSA and hK2 are both produced in zymogen form or as proenzymes and require proteolytic activation by cleavage of their propeptide sequence. Recently, experimental clues for possible mechanisms underlying their activation have been obtained. It has been demonstrated that hK2, in contrast to PSA, can become catalytically active by autoactivation (Mikolajczyk et al. 1997) and that hK2 additionally can convert pro-PSA into an enzymatically active mature form of PSA (Kumar et al. 1997, Lovgren et al. 1997, Takayama et al. 1997). hK2 thus appears to be the physiological activator of itself as well as of PSA. Recent studies have demonstrated that PSA and hK2 are secreted in their zymogen form into the spent medium of LNCaP or transfected cells, respectively, suggesting that proteolytic activation of the enzymes is an extracellular event (Kumar et al. 1997, Mikolajczyk et al. 1997, Rittenhouse et al. 1998). Since both enzymes are present in their mature active form in seminal plasma directly after ejaculation (Lilja 1985, Deperthes et al. 1996), this may indicate that physiologic activation of both enzymes occurs inside the lumen of the prostate (Lovgren et al. 1997). With the recent identification of prostase, which has structural features of serine proteases, the emerging picture may become even more complex. It has been speculated that this new enzyme is involved in a cascade of reactions together with PSA and hK2, finally culminating in the physiological processes described above (Nelson et al. 1999).

Copulatory plug formation and dissolution in rodents

In rodents, seminal clotting and subsequent dissolving is obviously different from the human gel-forming and liquefaction process (extensively reviewed by Williams-Ashman 1984). In these animals, a copulatory or vaginal plug is swiftly formed after depositon of the ejaculate in the vagina which differs from the soft gel of the human ejaculate in that it is a hard, rubbery lump which can stay for many hours to days and which does not contain spermatozoa (Zaneveld et al. 1974, Williams-Ashman 1984). In contrast to the relative slow release of the entrapped spermatozoa from the coagulum in humans, a substantial portion of

the spermatozoa in rodents is catapulted via the cervix into the uterus within seconds after ejaculation. Dissolution of the plug is thought to result from infiltrating leukocytes and their proteases instead of from lysis by prostatic enzymes (Williams-Ashman 1984). Plug formation and stability is acquired by the strong protein cross-linking activity of dorsal protein 1 (DP1) or prostate-specific TGase [for enzymology of TGases, see below (Williams-Ashman et al. 1972)]. In this regard it is important to note that the morphology of the rat prostate is different from the human prostate. The rat (rodent) prostate is composed of several distinct lobes: the ventral, lateral type 1 and 2, dorsal and anterior lobes (coagulating gland) (Hayashi et al. 1991). DP1 is a major secretory glycoprotein of the rat dorsal prostate, the lateral type 1 prostate and the coagulating gland, and is produced in minor amounts by the lateral type 2 prostate, and not by the ventral prostate (Wilson and French 1980, Romijn 1990, Hayashi et al. 1991, Kinbara and Cunha 1996). The protein is synthesized in large quantities, because approximately 25 % of the total cytosol protein of the dorsal prostate and the coagulating gland consists of DP1, leading to a final contribution to the ejaculated seminal fluid of about 5% of the total protein content (Wilson and French 1980). Substrates of DP1 are synthesized by the seminal vesicles. Rat seminal vesicle secretions contain several proteins, designated seminal-vesicle-secreted protein I-VIII (SVS I-VIII), according to their electrophoretic mobility (Aumuller and Seitz 1990, Seitz and Aumuller 1990). The major plug component and substrate for DP1 appeared to be the 49 kDa basic SVS II, although other SVS proteins may be integrated in the copulatory plug and can be substrates for DP1 as well (Fawell et al. 1986, Fawell and Higgins 1987, Seitz and Aumuller 1990). It has been proposed that plug formation can be modulated by polyamines which occur in high concentrations in seminal fluid (Williams-Ashman 1984) and are established physiological substrates for TGases (Folk et al. 1980).

Concluding remarks

Together, these data show that, although rodents and humans have a completely different coagulation and liquefaction system, in each case the enzymic contribution is derived from the prostate, whereas the corresponding protein substrates are produced by the seminal vesicles. Both in humans and rodents, the prostate contributes high amounts of polyamines to seminal plasma (Mann and Lutwak-Mann 1981, Williams-Ashman 1984, Romijn 1990). It also illustrates the different nature of the seminal coagulum. The human gel is essentially formed by non-covalent interactions in which TGase-specific bonds, if present at all, probably do not play an important role (Chaistitvanich and Boonsaeng 1983, Lilja and Laurell 1985, Lilja et al. 1987), whereas large quantities of isopeptide bonds due to TGase activity are present in the vaginal plug of rodents (Williams-Ashman et al. 1972). Therefore, the existence of a human prostate-specific TGase in semen, as recently has been demonstrated (Dubbink et al. 1999b), might be expected to play a different role in reproductive physiology than rat DP1.

2.5.2 Immunomodulatory mechanisms in semen

Immunological aspects of semen

Only limited knowledge has been obtained so far about the contribution of the prostate to reproductive immunology. For reviews on semen immunology, see Alexander and Anderson 1987, Marshburn and Kutteh 1994, Naz and Menge 1994, Kelly 1995, and Kelly and Critchley 1997. Two important aspects can be considered. First, the male reproductive tract is open to the external environment and needs to be protected against invading organisms. Seminal plasma contains high concentrations of zinc and spermine, up to 140 µg/ml and 3.5 mg/ml, respectively. These compounds appear to originate primarily from secretions of the prostate, which is the richest source in the body of zinc and spermine. Both zinc and spermine (oxidized) are considered to have antibacterial potency and are possibly involved in local defence mechanisms in the lower male reproductive tract, although this remains to be established (Williams-Ashman and Lockwood 1970, Partin and Coffey 1998). Second, spermatozoa contain components that are antigenic and need to be protected both against the male and female immune system. Anti-sperm antibodies can be directed against multiple spermatozoal epitopes and may affect fertility by interference with sperm motility leading to sperm migration inhibition through the cervical canal and the upper reproductive tract. Alternatively, anti-sperm antibodies may interfere with events essential for the fertilization process such as capacitation or sperm-egg interaction due to sterical hindrance or blocking of the receptor involved in sperm-egg attachment (Mahony and Alexander 1991). It has been suggested that even after fertilization, anti-sperm antibodies may affect implantation and may decrease pregnancy rates and the viability of developing embryos (Menge and Naz 1988, Marshburn and Kutteh 1994, Naz and Menge 1994). Furthermore, because the female is repeatedly exposed to spermatozoal antigens, establishment of anaphylaxis (a sensitized immunological response) must be prevented. It is important to note here that a majority of women do not develop anti-sperm antibodies and become not sensitized despite repeated introduction of millions of sperm cells in the vagina after intercourse (Marshburn and Kutteh 1994). These immunological aspects need to stay in balance to achieve a situation of both minimal chances for invading micro-organisms and maximal spermatozoa survival (Kelly 1995, Kelly and Critchley 1997).

Modulation of immunological responses in the female genital tract

Several mechanisms are proposed to prevent or modulate immunological responses in the female reproductive tract against seminal components. It needs to be stressed that information about the immunomodulating effects suggested for seminal plasma components come from *in vitro* experiments and that the *in vivo* significance of these effects remains to be elucidated. Only those mechanisms will be discussed in which characteristic secretory products of the prostate might be involved.

Direct immunosuppressive mechanisms

Seminal plasma is a potent inhibitor of proliferation and activity of cells of the immune system, e.g. lymphocytes, monocytes and NK cells, and thus may affect cell-mediated and

humoral immunity in both the male and the female genital tract (James and Hargreave 1984, Alexander and Anderson 1987). Several studies point towards an inhibitory role for polyamines, i.e., spermine and spermidine, of T-lymphocyte proliferation after antigen challenge. To display this bioactivity, the polyamines need to be converted to an oxidized form by the action of polyamine oxidase (Byrd et al. 1977, Quan et al. 1990, Imade et al. 1997, Kelly and Critchley 1997). Therefore, some caution has to be taken considering this role of polyamines in seminal immunosuppression, because it is not without discussion whether the observed effects are artifactually caused by direct cytotoxicity of oxidized polyamines (see Alexander and Anderson 1987). Immunosuppressive properties, i.e., inhibition of NK cell activity, have been attributed to a seminal plasma protein fraction containing TGase activity, but it is unclear from these experiments how such inhibition is achieved (Ablin et al. 1990).

Modulation of sperm cell immunogenicity

A more indirect though potentially important modulation of female immune responses is rendering spermatozoa less immunogenic when they leave the epididymis by coating them with seminal plasma components during ejaculation. Several reports suggest that the antigenicity of spermatozoa can be modified by the enzymatic action of seminal plasma TGase, which in humans most probably is hTG_P (Dubbink et al. 1999b). Although no covalent TGase cross-links could be detected in human seminal plasma (Lilja and Laurell 1985), the possibility of TGase catalyzed cross-links at the spermatozoal surface has not intensively been investigated. Most evidence in this direction comes from studies using rat and rabbit spermatozoa. Uteroglobin, which is produced and secreted by epithelial cells of numerous organs, is an excellent substrate for different TGase types *in vitro* (Manjunath et al. 1984, Miele et al. 1994). Pretreatment of rabbit epididymal spermatozoa with prostatic fluid or with pure uteroglobin, but not with TGase alone, decreased the immunological response of allogeneic splenocytes to these cells. However, simultaneous treatment with both uteroglobin and TGase completely inhibited sperm antigenicity. On the other hand, prostatic fluid which had first been exposed to antibodies either against uteroglobin or TGase could not decrease the immunological response against spermatozoa. It has been suggested that these observations could be explained by the covalent TGase-mediated attachment of uteroglobin to proteins of the spermatozoal membrane, thereby masking antigenic determinants (Mukherjee et al. 1983, Manjunath et al. 1984).

Comparable results were described for immunomodulation of rat epididymal spermatozoa by SVS-IV and TGase. SVS-IV has intrinsic immunosuppressive properties which could be increased by the TGase-mediated incorporation of spermidine (Porta et al. 1993). Treatment of epididymal spermatozoa with SVS-IV led to inhibition of splenocyte proliferation in response to these cells, and decreased their ability to induce several aspects of peritoneal macrophage activation, showing a reduced sperm immunogenicity. As described above for uteroglobin, this effect was far greater if the spermatozoa were simultaneously pretreated with TGase, but was absent if incubated with TGase alone (Peluso et al. 1994). In contrast to what has been suggested for uteroglobin, this action of TGase may not be ascribed to the direct covalent binding of SVS-IV to sperm surface proteins, but is suggested to result

indirectly from increasing the sperm binding properties of SVS-IV through branched SVS-IV homopolymer formation (Paonessa et al. 1984, Porta et al. 1991, Peluso et al. 1994). Furthermore, TGase was shown to be able to modify the sperm surface by covalent binding of the polyamines spermine and spermidine (Paonessa et al. 1984). It remains to be studied whether the latter cell surface changes also affect the immunological properties of spermatozoa.

These studies, though performed with spermatozoa from rat or rabbit, may be indicative for a potential function of seminal plasma TGase or hTG_P in immunomodulation of sperm cells in humans. Recently, hTG_P has been demonstrated in human prostatic fluid and seminal plasma by Western blotting with a polyclonal antiserum against the hTG_P C-terminal (Dubbink et al. 1999b). Immunoreactivity has also been found on the spermatozoal neck and tail with a polyclonal antiserum against a purified seminal plasma TGase, which may represent hTG_P (Ablin and Whyard 1991). TGase activity has been found both in human seminal plasma and on the spermatozoal surface (Porta et al. 1986). Indirect evidence suggests that this sperm-bound TGase activity derives from seminal plasma and becomes attached to the spermatozoa during ejaculation. TGase-mediated incorporation of spermidine into human sperm membrane and seminal plasma proteins has been demonstrated *in vitro* (Porta et al. 1986). In addition, several candidate protein substrates for TGase, through which hTG_P may modify the sperm membrane, are present in seminal plasma. Clara cell 10kDa (cc10kDa) is proposed as the human counterpart of uteroglobin. The secretory cc10kDa protein has immunomodulatory activities, is present in prostate epithelium and can serve as acyl donor and acceptor for TGase (Mantile et al. 1993, Miele et al. 1994). β_2-microglobulin (β_2M) is recognized as a TGase substrate. It is present in seminal plasma in high concentrations (James and Hargreave 1984) and has been detected on cell surfaces (Fesus et al. 1981). The gel-forming proteins semenogelin I, semenogelin II, and fibronectin, all can act as TGase substrates (Barsigian et al. 1988, Peter et al. 1998). Although the proteins may not become cross-linked in the gel, it is possible that they are covalently attached to the sperm surface. In fact, semenogelin epitopes have been found on the postacrosomal sheath of the sperm head, the mid region and the sperm tail (Lilja et al. 1989), and fibronectin immunoreactivity on the equatorial segment and several other parts of ejaculated spermatozoa (Glander et al. 1987, Aumuller et al. 1997b). Because binding and cross-linking may represent dissociable events, it remains to be established if TGase cross-linking is involved (Barsigian et al. 1988). For all of these TGase substrates present in human seminal plasma, it needs to be confirmed that they indeed can act as physiological substrates for hTG_P. Evidence for this has been obtained only by using purified tissue type TGase (TG_C) or plasma factor XIIIa (FXIIIa), whereas hTG_P might be the only naturally occurring TGase type in seminal plasma (Dubbink et al. 1999b). In addition to TGase substrates deposited by the male, it cannot be excluded that proteins from the female become cross-linked to the sperm surface, e.g. by sperm bound hTG_P during transport through the female genital tract. With regard to an immunomodulating function of hTG_P, it is of interest that protein modifications brought about by TG_C *in vivo* indeed can change the antigenic properties of proteins. Specific deamidations caused by TG_C in the protein gliadin, a component of wheat gluten, alters its antigenicity and makes the protein a better

target for gut T-cells. The resulting change is suggested to play a role in the pathogenesis of celiac disease (Molberg et al. 1998). Although this shows gain of antigenicity, it obviously illustrates the influence of a TGase enzyme on the antigenic structure of a protein substrate.

Reduction of the antigenic load by extensive proteolysis of seminal plasma proteins
The antigenic load offered by seminal plasma proteins may also need to be reduced. It has been suggested that this occurs by extensive proteolytic degradation of residual seminal plasma proteins in the vagina, which still remain present after liquefaction by PSA and hK2 proteolysis. For this to be achieved, pepsinogen II (also named gastricsin) might be responsible. This enzyme is secreted as a zymogen both by the prostate and seminal vesicles. Pepsinogen II becomes active by autocatalytic activation 2-7 h after semen deposition, after the vaginal pH has decreased back to its physiological pH of 3.4-4.5 (Szecsi and Lilja 1993, Wilson 1995). It still needs to be established whether this degradation indeed reduces the females immune response to seminal plasma proteins.

2.5.3 Sperm motility and further sperm surface changes

Spermatozoa need to acquire progressive motility to be able to move through the cervical mucus and the upper reproductive tract and also need to undergo capacitation to become fully fertile.
There is evidence for the presence of a seminal plasma motility inhibitor (SPMI) factor in semen, which is able to completely immobilize sperm cells at a physiological concentration. SPMI is exclusively produced by the seminal vesicles and has recently been identified as one of the semenogelins, probably semenogelin I (Robert and Gagnon 1995, 1996). Liquefaction, which has been discussed in more detail above, is essential for spermatozoa to become motile. However, this may not simply be caused by the release of entrapped and immobilized spermatozoa from the coagulum meshwork, because purified SPMI at soluble concentrations can completely arrest flagellar beating (Robert and Gagnon 1996). Degradation of SPMI by prostatic secretions or PSA considerably decreases the capacity of SPMI to inhibit sperm motility although SPMI fragments still have residual inhibitory capacity (Robert and Gagnon 1995, 1996). Recently, also fibronectin was suggested to play a role in sperm motility inhibition since fibronectin antibodies increase sperm motility and fibronectin protein decreases sperm motility (Aumuller et al. 1997b). It can be hypothesized that fibronectin proteolysis by PSA and hK2 is necessary for reduction of this biological activity *in vivo*. The mechanisms underlying sperm motility inhibition by SPMI and fibronectin are still unclear, but may result from their attachment to specific areas on the sperm surface, like the tail (SPMI) and the midpiece (fibronectin) of the spermatozoa (Lilja et al. 1989, Aumuller et al. 1997b).
Capacitation is the time-dependent acquisition by spermatozoa to become fully prepared for interaction with the oocyte (Fraser 1995). It renders the spermatozoa capable of undergoing the acrosome reaction and, although this may be a separate event, capable of expressing hyperactivated motility (Mortimer et al. 1998). Among other things, capacitation involves the removal or alteration of factors, referred to as decapacitation factors, from the sperm

surface. These decapacitation factors are of epididymal or seminal plasma origin (Fraser 1995). It cannot be excluded that some of the prostatic enzymes mentioned earlier are involved in this process. Furthermore, the involvement of a fertilization promoting factor (FPP; pGlu-Glu-ProNH_2) has been considered (for reviews on FPP, see Fraser et al. 1997, Fraser 1998a, 1998b). FPP is a tripeptide structurally related to thyrotrophin-releasing hormone, and is released by the prostate into seminal plasma. Physiological concentrations of FPP can accelerate capacitation and inhibit spontaneous acrosomal exocytosis of capacitated spermatozoa, together giving spermatozoa an increased chance of fertilization. FPP is thought to act through a spermatozoal receptor named TCP-11, thereby modulating intracellular adenylyl cyclase and hence cAMP production. Elevated intracellular levels of cAMP are known to stimulate capacitation and may therefore be important for the acquisition of fertilizing ability (for review, see Aitken 1997).

3 The transglutaminase family of proteins: tissue distributions, activation mechanisms and physiological functions

3.1 Enzymology of transglutaminases

Transglutaminases (TGases, protein-glutamine: amine γ-glutamyltransferase, EC 2.3.2.13) are Ca^{2+}-dependent enzymes that catalyze an acyl transfer reaction between peptide-bound glutamine residues and either peptide-bound lysine residues or primary amines (for reviews, see Folk 1980, Lorand and Conrad 1984). The γ-carboxamide of the glutamine serves as acyl donor or amine acceptor and binds to the acyl-acceptor or amine-donor ε-amino group of the lysine or the primary amino group of a polyamine (Figure 2). The reaction occurs by the formation of an acyl-enzyme intermediate through the transient binding of the γ-carboxamide with the sulfhydryl group of the active-site cysteine under simultaneous release of ammonia. Subsequently, the amine donor substrate is bound to the enzyme and cross-linking takes place. After cross-linking of a polyamine a free amine group remains that can be cross-linked to another γ-carboxamide group of a peptide-bound glutamine. The post-translational TGase bonds that hence can be formed are ε-(γ-glutamyl)lysine isopeptide bonds between different protein substrates or within the same protein substrate (Figure 2A), (γ-glutamyl)polyamine bonds yielding protein-polyamine conjugates (Figure 2B) and bis(γ-glutamyl)polyamine bonds consisting of polyamine cross-bridges between two proteins or intramolecularly (Figure 2C). In the absence of an amine substrate the acyl-enzyme intermediate may react with water, resulting in deamidation of the γ-carboxamide group of the peptide-bound glutamine, yielding a glutamic acid residue instead (Figure 2D).

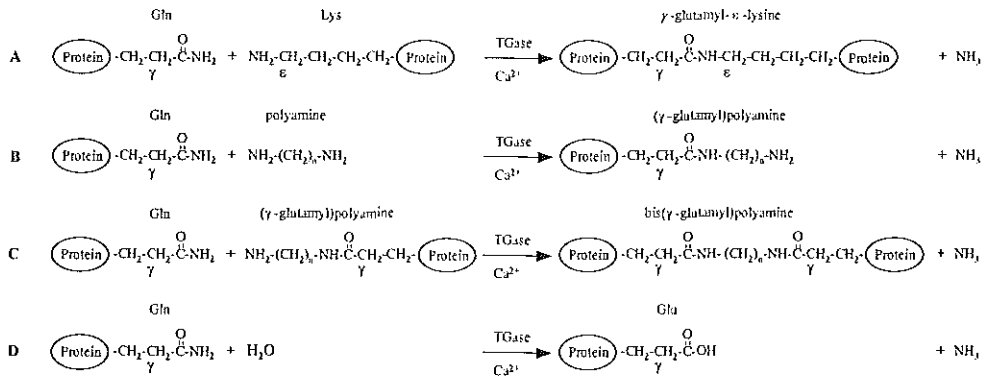

Figure 2. Transglutaminase-catalyzed reactions. Formation of ε-(γ-glutamyl)lysine isopeptide bonds (A), (γ-glutamyl)polyamine bonds (B), and bis(γ-glutamyl)polyamine bonds (C), and deamidation of peptide-bound glutamine.

Many TGase protein substrates have already been identified, that can serve as amine acceptor and/or amine donor, and this list will certainly grow (Steinert and Marekov 1995, McDonagh and Fukue 1996, Zeeuwen et al. 1997). In a particular protein substrate only a limited number of lysine or glutamine residues react with TGases (Murthy et al. 1998). Substrate specificity is dependent on the TGase type involved, and determined by the primary structure flanking both the amine donor and acceptor amino acids, the conformational properties of the substrate protein, and the accessibility of the amino acid residues (Folk 1983, Grootjans et al. 1995, Steinert and Marekov 1995, Tarcsa et al. 1998). TGases possess a differential specificity for peptide-bound lysine- and glutamine residues, being much less selective toward the amine donor lysine residues in proteins than toward peptide-bound glutamine residues (Folk 1980). However, although a broad range of amino acid residues flanking the substrate lysine are tolerated, and thus in theory many lysine residues may participate in the formation of the ε-(γ-glutamyl)lysine isopeptide bonds, amino acid residues directly preceding an accessible lysine can considerably affect their TGase reactivity and therefore may define the final number of amine donor lysines in a given native protein substrate (Grootjans et al. 1995). Polyamines can serve as competitive inhibitors of TGase-catalyzed ε-(γ-glutamyl)lysine cross-link formation by competition with peptide-bound lysines for incorporation into a protein substrate (Folk 1980, Lorand and Conrad 1984, Williams-Ashman 1984).

The action of TGases leads to the formation of high molecular mass homo- or heteropolymeric protein complexes, if protein substrates are involved that can serve both as amine donor and acceptor (Lorand and Conrad 1984). Large macromolecular structures formed by TGase-catalyzed cross-linking obtain an increased rigidity and become less susceptible to mechanical, chemical and enzymatic disruption (Greenberg et al. 1991). It was believed that this was due to the irreversibility of the covalent ε-(γ-glutamyl)lysine

isopeptide bonds, and that hence the complexes could only be dissolved by proteolytic degradation of the protein tails. In agreement with this hypothesis was the failure to isolate an enzyme which could cleave the TGase catalyzed cross-links. Only recently, it has been detected that, utilizing artificial substrates, distinct TGases possess the intrinsic potential also to hydrolyse TGase-generated bonds, suggesting that TGases could play a dynamic biological role in being able to catalyze both the formation and the cleavage of ε-(γ-glutamyl)lysine isopeptide bonds (Parameswaran et al. 1997). Degradation of cross-linked protein complexes may therefore be the consequence of both bond hydrolysis and proteolysis, and stability of the protein complexes may be due to a decreased access of the macromolecular structures resulting in a prolonged proteolysis.
Although the presence of a TGase in a tissue of interest can be determined by immunohistochemistry or by *in vitro* methods, i.e., assaying TGase activity in protein lysates, this does not necessarily implicate that the enzyme is also active *in vivo*. An estimation of *in vivo* functioning of TGases is usually made by measuring the amount of enzyme product in tissue lysates or body fluids. For this purpose, protein isolates are completely degraded by proteolysis, and subsequently the amount of ε-(γ-glutamyl)-lysine isopeptide or (γ-glutamyl)polyamine bonds is evaluated by HPLC (Lorand and Conrad 1984). More recently, it was also found to be possible to detect ε-(γ-glutamyl)-lysine isopeptide bonds by means of immunohistochemistry with a monoclonal antibody or polyclonal antiserum raised against this cross-link, allowing *in situ* localization of the TGase product (el Alaoui et al. 1991, Aeschlimann et al. 1995).

3.2 The TGase protein family

TGases are widely distributed in various organisms, including vertebrates, invertebrates, plants and bacteria. For example, analogues of mammalian TGases have been found in grasshopper embryos (Singer et al. 1992), in horseshoe crab hemolymph (Tokunaga et al. 1993), in uni- and multicellular plants (reviewed by Serafini-Fracassini et al. 1995), and in several bacteria (Kanaji et al. 1993).
In humans, seven distinct TGase types have been described, and their respective cDNAs and deduced primary protein sequences have been resolved. These include keratinocyte TGase [TG_K (Rice et al. 1992)], tissue-type or cellular TGase [TG_C (Gentile et al. 1991)], epidermal TGase [TG_E (Kim et al. 1993)], prostate-specific TGase [hTG_P (Dubbink et al. 1996)], TG_X, identified in keratinocytes (Aeschlimann et al. 1998), plasma factor XIIIa (Ichinose et al. 1990), and the catalytically inactive TGase-like erythrocyte membrane protein band 4.2 (Korsgren et al. 1990). TGase nomenclature is presented in Table 2. The organization of most TGase genes has been resolved. The TGase genes encompass 13 or 15 exons and, with the exception of the *TGM2* and *TGM3* genes, are scattered over different chromosomes. These data and corresponding transcript and protein sizes are summarized in Table 2.

Table 2. The human TGase gene family[a].

Gene name	Chromosomal localization	Gene size (kb)	Exon number	mRNA size (kb)	Protein name	Protein mass (kDa)
TGM1	14q11.2-q13	14.1	15	2.7	TG_K (TGase1)	92/106[b]
TGM2	20q11.2-20q12	32.5	13	4.0	TG_C (TGase2)	85
TGM3	20q11.2-20q12	42.8	13	2.9	TG_E (TGase3)	77
TGM4	3p21.33-p22	35	13	3.5	TG_P (TGase4)	77
TGM5	nd	nd[c]	nd	2.2/2.8	TG_X (TGase5)	81
Factor XIIIa	6p24-p25	>160	15	3.9	FXIIIa	83
Band 4.2	15q15-q21	20	13	2.4	Band 4.2	72

[a] References used: *TGM1*, Kim et al. 1992, 1995b, Phillips et al. 1992, Yamanishi et al. 1992; *TGM2*, Gentile et al. 1991, 1994, Fraij and Gonzales 1997; *TGM3*, Kim et al. 1993, 1994a, Gentile et al. 1994; *TGM4*, Gentile et al. 1995, Dubbink et al. 1996, 1998, 1999b; *TGM5*, Aeschlimann et al. 1998; *FactorXIIIa*, Grundmann et al. 1986, Board et al. 1988, Ichinose and Davie 1988; *Band 4.2*, Sung et al. 1990, 1992, Korsgren and Cohen 1991.

[b] The 106 kDa protein represents the size of the post-translationally modified enzyme

[c] nd, not determined

Human TGases have a molecular mass between 76 and 92 kDa which may be slightly higher in some cases because of post-translational modifications. They share an overall amino acid sequence identity of 25% to 45% (Table 3). Sequence similarities are particularly high in the middle portions or catalytic core domains of the proteins (see below), varying from 33% up to 56% (Table 4). The active-site cysteine of functionally active human TGases is located in the amino acid sequence GQCWVFA (for sequence alignment, see Chapter 2). Site-directed mutagenesis of this cysteine into a serine or alanine completely abolishes TGase activity, as has been shown for TG_C (Lee et al. 1993) and FXIIIa (Hettasch and Greenberg 1994), because of interference with the formation of an acyl-enzyme intermediate. In band 4.2 this cysteine has been replaced by an alanine, explaining why the protein could not be functionally active (Korsgren et al. 1990). Interestingly, however, converting this alanine to cysteine does not lead to an active enzyme (Risinger, M.A. and Cohen, C.M., cited by Hettasch and Greenberg 1994), demonstrating that for catalytic activity also other amino acids are required.

Table 3. Identities of human TGases as calculated from multiple alignment of the corresponding amino acid sequences.

	TG_K	TG_C	TG_E	TG_P	TG_X	FXIIIa	Band 4.2
TG_K	100%						
TG_C	35.8%	100%					
TG_E	36.1%	37.7%	100%				
TG_P	36.4%	32.0%	33.8%	100%			
TG_X	36.8%	42.3%	45.4%	33.1%	100%		
FXIIIa	42.1%	36.6%	34.0%	31.2%	34.1%	100%	
Band 4.2	27.3%	33.6%	31.2%	28.2%	33.0%	25.3%	100%

Table 4. Identities of the core domains of human TGases as calculated from multiple alignment of the corresponding amino acid sequences (see Aeschlimann et al. 1998).

	TG_K	TG_C	TG_E	TG_P	TG_X	FXIIIa	Band 4.2
TG_K	100%						
TG_C	47.2%	100%					
TG_E	48.5%	47.4%	100%				
TG_P	48.6%	46.1%	47.1%	100%			
TG_X	49.8%	56.4%	55.4%	47.3%	100%		
FXIIIa	52.9%	50.9%	46.1%	43.8%	47.4%	100%	
Band 4.2	34.3%	42.1%	34.5%	37.5%	41.4%	33.1%	100%

So far, the three-dimensional structure has been resolved for FXIIIa monomeric and dimeric forms. Because of the high sequence identities among TGases, in particular in the catalytic center region, it seems likely that at least part of the secondary structure is identical for the other family members. FXIIIa is made up of four sequential domains; an N-terminal β-sandwich, a core domain, barrel 1, and barrel 2 (Yee et al. 1994). The central core domain is largest in size and contains the active-site cysteine. The reaction center was found to be made up of a catalytic triad consisting of the active-site Cys, a His and an Asp (corresponding to residue Cys-268, His-327 and Asp-350 in hTG_P; see alignment in Chapter 2) which interact through hydrogen bonds (Cys-His, His-Asp). The catalytic triad residues are located at the base of a cavity bounded by the core and barrel 1 domains. The arrangement of the catalytic triad resembles that of cysteine proteases (Yee et al. 1994). All catalytically active TGases contain these catalytic triad amino acids. Band 4.2 , however, lacks the His residue, which further explains why the Ala to Cys substitution at the active-site position does not result in an active TGase.

3.3 Physiological functions of TGases

TGases are involved in many physiological processes. The next sections are intended to give an impression of the most obvious processes in which distinct TGases are involved, and to describe biochemical mechanisms underlying their activation.

3.3.1 Plasma factor XIIIa (FXIIIa)

Human plasma factor XIII (FXIII) is involved in the final stages of the blood coagulation cascade. It circulates as an inactive heterotetrameric complex consisting of two FXIIIa subunits that are noncovalently associated with two carrier *b* subunits [A_2B_2 (for recent reviews, see Ichinose et al. 1990, Greenberg et al. 1991, Muszbek et al. 1996)]. It has been suggested that the *b* subunits protect FXIIIa against proteolysis and increase its half-life in the circulation. They may also prevent a slow, non-proteolytic activation (Board et al. 1993). In the nonactivated zymogenic form of FXIII, the N-terminal activation peptides of the FXIIIa subunits, i.e., the first 37 amino acids, cross the interface between two *a* subunits and binds to the core domain of the opposite monomer meanwhile precluding substrate binding to the catalytic center (Yee et al. 1994). For activation of FXIII, it is necessary that the peptide bonds between Arg37 and Gly38 of the *a* subunits are cleaved by thrombin. Subsequently, a Ca^{2+}-dependent dissociation of the *b* subunits from the A_2B_2 complex occurs. Crystal structure analysis of thrombin-cleaved FXIIIa suggests that the activation peptide still remains attached to the zymogen while still occluding the active-site cavity. To get an enzymatically active configuration and exposure of the active-site thiol group, it therefore seems necessary that the FXIIIa dimer undergoes large conformational changes which are most likely induced by substrate binding (Yee et al. 1996). Biochemical data indeed indicate that FXIIIa substrates are regulators of the activation process. For example, fibrinogen reduces the Ca^{2+} concentration required for *b* subunit dissociation to plasma levels, enhances the release of the activation peptide and increases the rate of exposure of the active-site thiol (Credo et al. 1978, Janus et al. 1983, Hornyak and Shafer 1991). FXIIIa contains also a second thrombin cleavage site between Lys513 and Ser514 at the junction of the catalytic core domain and barrel 1. Thrombin-cleavage at both sites yields the 4-kDa activation peptide, a 51-kDa FXIIIa core domain polypeptide which has retained its catalytic activity, and a 25-kDa C-terminal peptide (Greenberg et al. 1988, Lai et al. 1994). The major source of FXIIIa appears to be cells of bone marrow origin, being megakaryocytes, monocytes and macrophages. Also blood platelets contain large amounts of FXIIIa, i.e., 100- to 150-fold higher than in the surrounding plasma, which may have been obtained during platelet formation from megakaryocytes. Extrahemopoietic synthesis of FXIIIa has been observed in hepatocytes around the central veins in the liver (Adany 1996, Adany and Antal 1996). Within the cell, FXIIIa is localized in the cytoplasm and present in a homodimeric form of two identical *a* chains (A_2). Activation of intracellular FXIIIa is supposed to occur by a slow non-proteolytic mechanism without the release of the activation peptide (Muszbek et al. 1995).

During blood coagulation, fibrinogen molecules are cleaved by thrombin into fibrin monomers. This is followed by a self-assembly process by which a loose meshwork of hydrogen-bonded fibrin monomers is formed (Mosesson 1992). Activated FXIIIa stabilizes the blood clot by the formation of intermolecular cross-links between adjacent fibrin chains and by cross-linking other plasma proteins to fibrin, such as α_2-antiplasmin and fibronectin. The final result is a clot with greatly enhanced elasticity and mechanical strength and an increased resistance towards degradation by plasmin, the major clot-lysing enzyme (Ichinose et al. 1990, Greenberg et al. 1991, Mosesson 1992). Patients with impaired fibrin cross-linking due to inherited FXIIIa deficiency have lifelong bleeding complications, which can be life-threathening, and severe problems with wound healing. In early life these patients carry a high risk of death from intracanial bleedings. Female patients are unable to carry a pregnancy to term and suffer from recurrent abortions. Part of these problems can be overcome by FXIII replacement therapy (Fisher et al. 1966, Kitchens and Newcomb 1979, Board et al. 1993, Egbring et al. 1996).

3.3.2 Tissue-type or cellular TGase (TG_C)

Enzymatic activities of TG_C

In contrast to most other TGase family members, TG_C is widely expressed throughout the body [another exception might be TG_X (Thomazy and Fesus 1989)]. TG_C is unique in that it can display two enzymatic activities, i.e., cross-linking activity and a Mg^{2+}-dependent GTPase activity (Lee et al. 1989, Lai et al. 1996, 1998). Site-directed mutagenesis has shown that the active-site cysteine, which is indispensable for TGase activity, is not required for GTP hydrolysis activity. TGase and GTPase activity are thus co-ordinated by two separate and independent active sites (Lee et al. 1993). TG_C contains a single GTP-binding site to which the nucleotide can reversibly bind (Achyuthan and Greenberg 1987, Bergamini and Signorini 1993). Both the GTP-binding site and the intrinsic GTPase activity reside within the N-terminal part of the TG_C core domain (Singh et al. 1995, Lai et al. 1996, Iismaa et al. 1997). Binding of GTP (or Mg-GTP complexed forms) induces conformational changes leading to a strong non-competitive inhibition of TGase activity which can be partly reversed by high Ca^{2+} levels (Achyuthan and Greenberg 1987, Lai et al. 1998, Monsonego et al. 1998). Manipulation of the intracellular Ca^{2+} and GTP concentrations in a physiological setting using permeabilized or intact cells has indeed demonstrated that the TGase activity is strictly controlled by these compounds (Smethurst and Griffin 1996, Zhang et al. 1998). In fact, under normal physiological GTP concentrations, the intrinsic TGase activity is strongly repressed and might even be absent. Moreover, induction of TG_C expression is not accompanied with a parallel rise in intracellular TGase activity, unless the intracellular Ca^{2+} concentration is increased and lowering of the intracellular GTP concentration results in increased TGase activity (Smethurst and Griffin 1996, Schittny et al. 1997, Lai et al. 1998, Verderio et al. 1998, Zhang et al. 1998).

TG_C in signal transduction

Intriguingly, TG_C was found to be implicated in signal transduction. The enzyme has been identified as the GTP-binding regulatory protein (G protein), $G\alpha_h$, which is involved in the transmembrane transmission of the ligand-bound α_{1B}-adrenergic receptor signal to its effector enzyme, phospholipase C-δ1 [PLC-δ1 (Nakaoka et al. 1994, Feng et al. 1996)]. Coupling of $TG_C/G\alpha_h$ to PLC activates the hydrolysis of membrane-bound inositol phopholipids leading to the generation of the second messengers 1, 4, 5-triphosphate (IP3) and diacylglycerol and subsequent intracellular Ca^{2+} mobilization and protein kinase C activation (for a review on G proteins and TG_C, see Im et al. 1997). Interaction with and stimulation of PLC occurs via an eight amino acid region near the C-terminus of $TG_C/G\alpha_h$ (Hwang et al. 1995). The C-terminal region also mediates its binding to the α_{1B}-adrenergic receptor (Feng et al. 1999). The function of $TG_C/G\alpha_h$ in signal transduction is independent of its TGase activity (Chen et al. 1996), but its intrinsic GTPase activity is essential. Once the receptor is activated, it stimulates $TG_C/G\alpha_h$ to become GTP-bound and to activate PLC, thus meanwhile negatively regulating its intrinsic TGase activity. The intrinsic GTPase activity is supposed to be involved in the subsequent deactivation of the signalling pathway, i.e., turning the enzyme from a GTP-bound into a GDP-bound state (Nakaoka et al. 1994, Im et al. 1997). Besides mediating signal transduction of the α_{1B}-adrenergic receptor, $TG_C/G\alpha_h$ was also found to act in signal transmission of the oxytocin, the thromboxane and the α_{1D}-adrenergic receptor to PLC (Baek et al. 1996, Chen et al. 1996, Vezza et al. 1999). The oxytocin receptor is also coupled to PLC-δ1 by $TG_C/G\alpha_h$ (Park et al. 1998). $TG_C/G\alpha_h$ might therefore appear to be more generally involved in signal transduction pathways by coupling receptors to PLC-δ1.

TG_C functions in cellular processes

It has been difficult to come to a decisive clue on the function(s) of TG_C. It is conceivable that a function of TG_C is determined by the relative contribution of its cross-linking activity and the receptor mediator function. The enzyme has been implicated in a diverse array of cellular processes including apoptosis or programmed cell death (Fesus et al. 1987), cell growth and adhesion (Gentile et al. 1992), wound healing (Upchurch et al. 1991 , Raghunath et al. 1996), extracellular matrix formation (Aeschlimann et al. 1995), and axonal growth and regeneration (Eitan and Schwartz 1993, Eitan et al. 1994). Some of the functions initially ascribed to its cross-linking activity might need to be reconsidered with respect to its actions in the signal transduction pathway. In agreement with the obvious functional diversity is that intracellularly TG_C can be found in cytosol and plasma membrane fractions (Singh and Cerione 1996) as well as in the nucleus (Singh et al. 1995, Lesort et al. 1998, Piredda et al. 1999). Although the nuclear-bound TG_C may represent only a minor fraction of the total amount, i.e., 7% in the neuroblastoma cell line SH-SY5Y (Lesort et al. 1998), there is evidence that it is functionally active in the nucleus and thus significant. A number of nuclear proteins can be modified by TG_C as determined for the core histones H2A and H2B *in vitro* (Ballestar et al. 1996) and for the retinoblastoma gene product (pRB) *in vivo* (Oliverio et al. 1997). In addition, histone H2B was recently also

identified as one out of a few proteins that specifically interact with TG_C (Piredda et al. 1999) and nuclear TGase activity has been measured *in situ* (Lesort et al. 1998).

TG_C and apoptosis

Although the exact role of TG_C in apoptosis is still not fully understood a relation with this cell death process is suggested by several *in vitro* and *in vivo* studies. Expression of TG_C is frequently induced in cells which are committed to apoptosis and usually occurs early in the apoptotic pathway (for reviews, see Fesus et al. 1991, Piacentini 1995, Melino and Piacentini 1998). Activation of the intrinsic TGase activity is thought to be a late event during the effector phase of the apoptotic pathway, once the intracellular Ca^{2+} level rises. This will lead to the assembly of a protein shell or apoptotic envelope by cross-linking intracellular proteins (Fesus et al. 1989, Knight et al. 1991), of which the major contributor may be cytoplasmic actin (Nemes et al. 1997, Oliverio et al. 1997). Ultimately, the highly cross-linked protein shells may prevent leakage of intracellular compounds before clearance of the dying cells by phagocytosis, resulting in an immunologically silent process without inflammation and thus maintaining the structural integrity of the tissue in which apoptosis occurs (Piredda et al. 1997). In transfection studies, overexpression of TG_C in distinct cell lines was found to increase their susceptibility towards apoptosis, whereas antisense TG_C cDNA was reported to repress the apoptotic process (Gentile et al. 1992, Melino et al. 1994). In this respect it is of particular interest to note the well documented influence of retinoic acids on TG_C expression. Distinct retinoic acids were found to be able to induce both apoptosis and TG_C expression in various cell lines, through retinoic acid receptor and/or retinoic X receptor signaling pathways (Zhang et al. 1995, Melino et al. 1997, Joseph et al. 1998). Retinoids were found to regulate TG_C expression at the transcriptional level probably via a complex retinoid response element in the *TGM2* gene promoter (Chiocca et al. 1988, Nagy et al. 1996, 1997). The effect of retinoic acids may not be confined to enhancement only of transcription of *TGM2*, but may also include activation of pre-existing TGase enzyme (Melino et al. 1994), enhancement of the ability of TG_C to bind GTP, association with the plasma membrane, and stimulation of phospholipase C activity (Singh and Cerione 1996). Experiments with transgenic mice containing 3.8 kb of the *TGM2* promoter linked to a reporter gene in a retinoic acid receptor-deficient genetic background suggest that a direct modulation of TG_C expression by retinoids also occurs *in vivo*. These mice showed a decreased transgene expression in the interdigital web of the embryonic limb, thus coinciding with a region which is destined to undergo apoptosis. Moreover, the lack of retinoic acid receptors in these animals causes a severe and fully penetrant interdigital webbing which, at least in part, seems to be due to a decrease in cell death in the interdigital mesenchym during embryogenesis (Dupe et al. 1999). In line with a role of TG_C in physiological tissue remodeling by apoptosis during development is that genetically normal mice as well as chickens show high expression of the enzyme in the interdigital web of the developing limb, but also in morphogically apoptotic myoblasts (Nagy et al. 1997, Thomazy and Davies 1999). A close association between increased TG_C expression and apoptosis has further been shown in several induced and/or physiological processes *in vivo*, resulting in tissue involution such as lead nitrate-induced hyperplasia of

the liver (Fesus et al. 1987), androgen withdrawal from the prostate (Guenette et al. 1994b, Rittmaster et al. 1995), forced weaning of the mammary gland (Guenette et al. 1994a, Nemes et al. 1996), and post partum involution of the uterus (Piacentini and Autuori 1994). In a number of these studies, TG_C was confirmed to become activated, as shown by the presence of increased levels of ε-(γ-glutamyl)lysine isopeptide bonds.

TG_C in cell adhesion and extracellular matrix formation

Several lines of evidence indicate a role of TG_C in cell adhesion and extracellular cell matrix (ECM) formation. A prerequisite for functioning in ECM formation is that the enzyme can be expressed on the cell surface or secreted into the extracellular space. Although the secretory pathway for TG_C is unclear, the protein has been identified in the ECM of several tissues, e.g. differentiating cartilage (Aeschlimann et al. 1995), lung during postnatal development (Schittny et al. 1997), and breast cancer tissues (Hettasch et al. 1996). Upon deposition of the enzyme in the ECM, TG_C will meet optimal conditions for its activation (Ca^{2+} and GTP concentrations high and low, respectively) and many potential substrates (Aeschlimann and Paulsson 1994). These include fibronectin, fibrinogen (Barsigian et al. 1988), collagen II, osteonectin (Aeschlimann et al. 1993, 1995), collagen V and XI (Kleman et al. 1995), and osteopontin (Kaartinen et al. 1997). Binding of TG_C to fibronectin was found to occur via its most N-terminal 7 amino acids (Jeong et al. 1995). Proof for an extracellular function comes both from *in vitro* and *in vivo* studies. *In vitro* transfected cells overexpressing TG_C show an increased adhesion to their substratum (Gentile et al. 1992, Johnson et al. 1994). In contrast, antisense transfected cells, expressing reduced levels of TG_C, display a decreased adhesion and decreased ability to cross-link the fibronectin on which the cells were layered (Jones et al. 1997). Contradictory results were obtained with stably transfected cells with TG_C cDNA under control of an inducible promoter which showed no increased adhesion of the cells after TG_C induction, although extracellular fibronectin cross-linking was increased. This discrepancy might be due to endogenous TG_C already present in the noninduced cells, or because of clonal selection of adhesive cells in case TG_C expression is controlled by a constitutive promoter (Verderio et al. 1998). Extracellular polymerization of fibronectin by TG_C reinforced the attachment of cultured human umbilical vein endothelial cells to the subendothelial ECM. Whether this was due to direct cross-linking of cell surface proteins to the ECM or a higher affinity of the cells for cross-linked fibronectin was not determined (Martinez et al. 1994). TG_C was found to mediate both binding of fibrinogen and fibronectin to the extracellular surface of hepatocytes and incorporation of these proteins into large cross-linked protein complexes (Barsigian et al. 1988, 1991). Furthermore, A204 rhabdomyosarcoma cells deposite collagen V/XI fibrils, in culture, which appeared to be highly insoluble due to TG_C cross-linking (Kleman et al. 1995). *In vivo*, TG_C could be co-localized by immunohistochemistry with ε-(γ-glutamyl)-lysine isopeptide bonds in the ECM of postnatal maturing lung. TG_C is intracellularly expressed before birth and becomes externalized and activated 2-3 weeks after birth in the period that mature lung parenchyma is formed. A similar observation was made for the formation of lung blood vessels and large airways, which precedes parenchyma maturation. TG_C may thus stabilize remodeling processes and ECMs in the

lung (Schittny et al. 1997). In the same way, sequential expression and activation of TG_C could be demonstrated in cartilage of maturing trachea and of the hypertrophic zone of long bone growth plate (for review, see Aeschlimann et al. 1996). TG_C is up-regulated in hypertrophic cartilages and externalized upon maturation or terminal differentiation of the chondrocytes, respectively. Subsequent cross-linking of matrix proteins may lay the basis for mineralization of both tracheal and long bone cartilage which is in the latter case succeeded by endochondral ossification (Aeschlimann et al. 1993, 1995, Thomazy and Davies 1999). Post-translational modification by TG_C may promote the mineralization process by clustering Ca^{2+}-binding proteins (Aeschlimann et al. 1996), which may occur indirectly by changing properties of cartilage matrix proteins, as has been shown for osteopontin (Kaartinen et al. 1999). Cross-linking of osteopontin *in vitro* leads to increased affinity for several distinct collagens, probably due to conformational changes. Induction of protein aggregation, caused by cross-linking or otherwise, and leading to enhanced collagen binding ability of phosphoproteins such as osteopontin, might be an essential event in matrix maturation and in the mineralization process in cartilage and bone (Kaartinen et al. 1999, and references therein). In addition to elevated TG_C levels, hypertrophic chondrocytes of chicken also express an increased FXIIIa level, and the protein becomes externalized in significant amounts. In fact, FXIIIa was shown to represent the major TGase type in avian growth region cartilage (Nurminskaya et al. 1998, Thomazy and Davies 1999). This may implicate that FXIIIa is involved in mammalian bone and cartilage formation as well, although so far no clearcut evidence has been obtained for such a role (Aeschlimann et al. 1996). The expression of TG_C in hypertrophic chondrocytes as well as in the apoptotic region of the interdigital web can be directed via a 3.8 kb promoter region 5'-flanking the *TGM2* gene (Nagy et al. 1997). TG_C may also play a role in skin regeneration after transplantation of cultured epithelial autografts by cross-linking of the papillary dermis and the dermo-epidermal junction (Raghunath et al. 1996). Together, the studies described above strongly suggest that TG_C can act both in stabilization of the ECM and in anchoring cells to their basement membranes.

Impact of TG_C modifications on the biological function of proteins

The TGase action of TG_C can also modify the biological function of several proteins such as osteopontin (as already described above, and Kaartinen et al. 1999), the latent form of transforming growth factor-β [TGF-β (Kojima et al. 1993, Nunes et al. 1995)], midkine (Mahoney et al. 1996, Kojima et al. 1997), and interleukin-2 [IL-2 (Eitan and Schwartz 1993)]. TGF-β is secreted in a large latent complex consisting of TGF-β, its propeptide, and latent TGF-β-binding proteins (LTBPs). TG_C mediates anchoring of the latent complex in the pericellular matrix by cross-linking LTBP-1 to ECM components (Taipale et al. 1994, Nunes et al. 1997). This may create a pool of large latent TGF-β complexes and/or modulate subsequent plasmin activation (Nunes et al. 1997). In this respect it is interesting that TG_C expression can be transcriptionally either up- or down-regulated via a TGF-β1 response element in the *TGM2* promoter (Ritter and Davies 1998). Furthermore, dimerization/multimerization of midkine by TG_C cross-linking turns the molecule into a better growth-promoting factor for neurons (Mahoney et al. 1996) and may also be of

biological importance in enhancing plasminogen activator activity (Kojima et al. 1997). Post-translational dimerization of interleukin-2 (IL-2) converts the molecule into an apoptosis-inducing compound for oligodendrocytes (Eitan and Schwartz 1993, Eitan et al. 1994, Eizenberg et al. 1995). In the absence of cross-linked IL-2, oligodendrocytes survive and inhibit axonal outgrowth after injury. These data are further demonstrated by the fact that administration of TGase in injured rat optic nerves leads to recovery of the visual response and concurrent appearance of axons. These findings may have clinical significance, in helping to overcome nerve regeneration blocks that normally occur after injury (Eitan et al. 1994).

TG_C involvement in pathological processes

TG_C might play an important role in a variety of disease processes including cataract formation (Lorand and Conrad 1984), pathogenesis of renal (Johnson et al. 1997) and hepatic fibrosis (Mirza et al. 1997), celiac disease (Dieterich et al. 1997, Molberg et al. 1998, van de Wal et al. 1998), and many neurodegenerative diseases (for reviews, see Lorand 1996, Cooper et al. 1999). Celiac disease patients have severe damage of the small intestine which is initiated by the ingestion of gluten. Recently, it has been demonstrated that these patients developed auto-antibodies against TG_C, in addition to antibodies against gliadin, a component of wheat gluten (Dieterich et al. 1997, Marsh 1997). TG_C expression is increased in affected tissues and TG_C could change the antigenic properties of gliadin by deamidation of selective glutamines (Q to E conversion, Figure 2D). Selective deamidation was found to generate an epitope that efficiently binds and stimulates gliadin-specific T cells. Altogether, these data suggest that TG_C plays a central role in the pathogenesis of celiac disease (Molberg et al. 1998, van de Wal et al. 1998). TG_C is suggested to be involved in the pathologic proteinaceous aggregate formation in several diseases of the central nervous system. These include Alzheimer's disease and neurodegenerative diseases caused by proteins containing expanded polyglutamine stretches such as Huntington's disease (for reviews, see Lorand 1996, Cooper et al. 1999). In the latter group of disorders there is a close association between the length of the polyglutamine domain and disease occurrence. *In vitro* studies have shown that an increased polyglutamine sequence improves the TG_C substrate properties of a protein, supporting a role of TG_C in these diseases (Kahlem et al. 1996, 1998). However, for an ultimate proof of TGase involvement in these diseases, the *in vivo* existence of the ε-(γ-glutamyl)lysine isopeptide bound has to be demonstrated in the affected brain regions or deposits (Lorand 1996).

No disorders have been described for which inherited or acquired TG_C mutations are causative to give additional information on the function of TG_C. Important clues on the physiological role of TG_C in development and adult life may ultimately come from studies of *TGM2* knockout mice which are now in progress (Melino and Piacentini 1998).

3.3.3 Keratinocyte TGase (TG_K)

TG_K is primarily expressed in stratified squamous epithelia. In skin, TG_K is present throughout the entire epidermis, including the basal layer, but predominantly in the granular

layer beneath the stratum corneum. TG_K has been found to be upregulated both at the mRNA and protein level at the time keratinocytes differentiate (Thacher and Rice 1985, Kim et al. 1995b, Steinert et al. 1996a). It is the predominant and most extensively studied TGase in keratinocytes, but these cells also express TG_C, TG_E and TG_X (Thacher and Rice 1985, Kim et al. 1995a, Aeschlimann et al. 1998). TG_C seems not essential for epidermal differentiation since it is mainly expressed in the basal cells and is down regulated during differentiation (Floyd and Jetten 1989, Kim et al. 1995a), Therefore, its putative functions in the skin will not be discussed. Some data on TG_E and TG_X in keratinocyte differentiation are presented in the next sections.

During skin formation, epidermal keratinocytes differentiate into a barrier of cornified cells which protects the organism against pathogens, chemical and physical insults. The skin is also essential for an optimal fluid balance of the organism (for reviews, see Roop 1995, Eckert et al. 1997, Melino et al. 1998). TGases are indispensable for the final stages of skin differentiation, particularly in the formation and assembly of the cornified cell envelope (CE), a 15-nm thick layer of insoluble protein deposited just inside the cell periphery (Thacher and Rice 1985, Rice et al. 1992, Melino et al. 1998). Terminal differentiation of the keratinocytes and CE formation can be considered as a specialized form of apoptosis (Melino et al. 1998). The CE consists of an array of proteins which are covalently connected through TGase cross-links. These include the major CE protein loricrin as well as elafin, filaggrin, involucrin, keratin intermediate filaments and small proline-rich proteins 1 and 2 (SPR1 and 2). Both intrachain cross-links of loricrin and interchain cross-links of loricrins between themselves or with the other proteins have been found. SPRs are suggested to be primarily cross-bridging proteins by mediating links between epidermal proteins resulting in e.g. loricrin-SPR1-loricrin or loricrin-SPR1-keratin 1 complexes. Formation of cross-links in the CE are supposed to occur in a sequential order and only a limited number of glutamine and lysine residues within a given substrate might be of particular importance (Steinert and Marekov 1995, Candi et al. 1998b, Steinert et al. 1998, Tarcsa et al. 1998).

The importance of TG_K in skin differentiation has become clear in patients with autosomal recessive lamellar ichtyosis (LI). LI is a severe congenital disease representing a phenotypically and genetically heterogeneous group of keratinization disorders that are characterized by an abnormally increased thickness of the stratum corneum or hyperkeratosis, generalized large scales, and reddened skin. Newborns with this disease are often encased in a tough and inelastic membrane that breaks easily, resulting in a high risk of sepsis and dehydration. Linkage studies and *TGM1* analysis have shown that a subgroup of LI patients have inherited mutations in this gene causing decrease or absence of TG_K activity (Huber et al. 1995, Parmentier et al. 1995, Russell et al. 1995) and unability to form cross-linked envelopes (Jeon et al. 1998). Transgenic *TGM1*-null mice have identical symptoms which substantiate the findings in LI patients. At birth, these mice have an erythematous and shiny skin with abnormal keratinization and defective cell envelope assembly. Although the mice survive pregnancy, they die within several hours after birth due to dehydration (Matsuki et al. 1998).

TG_K is acylated at its amino-terminus by myristate and palmitate, through which most of the enzyme is attached to the inner surface of the keratinocyte plasma membrane (Chakravarty and Rice 1989, Steinert et al. 1996b). Only a small proportion of the enzyme is present in the cytosol fraction. The intact 106-kDa (zymogenic) form of TG_K displays a low specific activity. After proteolytical processing during terminal keratinocyte differentiation the specific activity of TG_K increases up to 200-fold. Although cleaved, the enzyme remains mainly membrane-bound as a 67/33/10-kDa complex by means of secondary interactions via the acylated amino-terminal 10-kDa membrane anchorage fragment (Kim et al. 1995a, Steinert et al. 1996a). The cleavage positions were found to coincide with the thrombin cleavage sites of FXIIIa, i.e, at the junction of the membrane anchorage domain and the β-sandwich and at the junction of the catalytic core domain and barrel 1. The 67- kDa fragment encompasses the core domain. Although the major TG_K activity resides in the processed 67/33/10-kDa complex, the core domain alone displays TGase activity (like FXIIIa) even at a higher level than the 106-kDa zymogen (Kim et al. 1994b, 1995a, Candi et al. 1998a).

3.3.4 Epidermal TGase (TG_E)

Apart from hair follicle cells, the terminal differentiating epidermis is the principal tissue in which TG_E is expressed. Like TG_K, TG_E expression is induced during keratinocyte differentiation, although mRNA and protein levels are much lower (Kim et al. 1995a). It is a pro-enzyme that requires proteolytic activation by cleavage in a flexible hinge region located between the predicted core domain and barrel 1 which is in the same region as described for the second cleavage site for FXIIIa and TG_K. This results in a 50-kDa, still catalytically active, fragment containing the active center and a C-terminal 27-kDa fragment (Kim et al. 1990, 1993). Contrary to TG_K, TG_E is a cytosolic enzyme and appears to occur only in the granular epidermal layer (Kim et al. 1990, Polakowska et al. 1994).
TG_E is believed to be essential for CE assembly. No diseases have been associated with a TG_E deficiency or defect which makes it more difficult to address its specific function in skin formation. It is obvious from the phenotype of LI and the *TGM1*-null mice that TG_E cannot replace or compensate for the lack of cross-links usually performed by TG_K. TG_E and TG_K may therefore cross-link in a complementary manner in CE formation. This is supported by the fact that both enzymes possess distinct preferences for glutamine and lysine residues within the same substrates as has been observed for loricrin, SPR1 and SPR2. From *in vitro* cross-linking assays performed with these substrates, it has been proposed that TG_E is primarily responsible for intrachain cross-linking of loricrin and for initial cross-linking of loricrin and SPRs together or of loricrin alone to form small interchain oligomers. Large polymeric structures only occur after further cross-linking by TG_K. Interchain polymerization may direct the oligomers from the cytoplasm to the cell periphery and anchor them into the developing CE. Such a model is consistent with the phenotypes seen in cases of TG_K deficiency (Candi et al. 1995, 1999, Matsuki et al. 1998, Tarcsa et al. 1998). Because there are many substrates known to be cross-linked *in vivo* in

the CE, the final picture is expected to be very complex. For a recent review on protein cross-links in the CE and the involvement of TG_E and TG_K therein, see Melino et al. 1998. Ca^{2+} is required for full catalytic activity of TG_E and TG_K. Interestingly, the Ca^{2+} concentration in the epidermis is higher in the suprabasal layers than in the basal layer. During the final stages of skin differentiation, the keratinocyte plasma membrane becomes permeabilized, resulting in increased cytosolic Ca^{2+} concentrations. This change, together with increased TG_E and TG_K expression levels and Ca^{2+} dependent activation of proteases, may lead to a substantial increase of TGase activity and cross-linking reactions at final stages of keratinocyte differentiation (Thacher and Rice 1985, Eckert et al. 1997).

3.3.5 TG_X

TG_X is only recently discovered and therefore little data are available at present (Aeschlimann et al. 1998). It has been identified by means of RT-PCR on human keratinocyte mRNA with degenerated primers deduced from the active-site region. The enzyme is expressed in the keratinocytes at a slightly higher level than TG_C and is, like TG_E and TG_K, induced during keratinocyte differentiation. In addition to TG_E and TG_K, TG_X might therefore participate in the formation of the cornified cell envelope. TG_X expression is, however, not restricted to keratinocytes and hence may function in other tissues as well.

3.3.6 Band 4.2

Band 4.2 is one of the major constituents of erythrocyte membranes, where it comprises about 5% of the total protein mass (for reviews, see Cohen et al. 1993, Yawata 1994b). There are contradictory data concerning its expression in nonerythroid tissues. By immunohistochemistry as well as Northern blotting, expression was found in kidney, heart, brain and liver (White et al. 1992, Cohen et al. 1993). However, in a recent study in mice, using Northern blot analysis and *in situ* hybridization, band 4.2 was found to be restricted to cells of the erythroid lineages in adult spleen and bone marrow. The embryonal liver was found to express band 4.2 during the period that erythropoiesis occurs in this organ. In fact, these authors found a complete temporal coincidence of band 4.2 expression and erythropoiesis during embryogenesis and after birth, i.e., embryonal expression in the yolk sac (E7.5-E11.5 days) is followed by synthesis in the liver (E12.5-16.5), and around and after birth, expression is fully taken over by spleen and bone marrow (Zhu et al. 1998).
Although the exact function of band 4.2 remains to be established, several data point towards a role in maintaining the shape and stability of erythrocytes (Cohen et al. 1993, Yawata 1994b). Band 4.2 is myristylated at its N-terminal glycine by which it might become associated to other proteins or the erythrocyte membrane (Risinger et al. 1992). The protein is firmly associated with the cytoplasmic domain of the major integral erythrocyte membrane protein and anion exchanger band 3. In addition, binding to the erythrocyte membrane skeletal proteins ankyrin, band 4.1 and spectrin was described (Cohen et al. 1993, Rybicki et al. 1995, Golan et al. 1996). Biophysical studies suggest that band 4.2 serves as an accessory linking protein to strengthen the interaction between band 3

and the cytoskeletal network (Golan et al. 1996, Rybicki et al. 1996). In accordance with a function in stabilization of the membrane skeleton of erythrocytes, band 4.2 deficiencies are associated with mild to intermittently severe hemolytic anemia. In these cases, erythrocytes have abnormal shapes and may have an increased osmotic fragility, possibly due to membrane skeletal instability or to weakening of cytoskeleton-membrane associations. Band 4.2 deficiencies can be inherited via mutations in the corresponding gene, but may also occur as a secondary effect due to defects in other erythrocyte proteins, e.g band 3 (Cohen et al. 1993, Yawata 1994a, 1994b).

3.3.7 Prostate-specific TGase (hTG_P)

Rodent TG_P has been described as early as 1896 by Gamus and Gley (see Aumuller and Seitz 1990), and thus is the longest known TGase. Most research has been done so far on rat TG_P or dorsal protein 1 (DP1). Much less is known on its human analogue hTG_P since in fact evidence for its presence has only been obtained a few years ago by the demonstrated cross-reactivity of an anti-DP1 antiserum with human seminal plasma (Enderle-Schmitt et al. 1989, Aumuller and Seitz 1990) and the isolation of its corresponding cDNA (Grant et al. 1994, Dubbink et al. 1996). In an earlier study, TGase activity had already been detected in seminal plasma (Porta et al. 1986) which was most probably due to hTG_P (Dubbink et al. 1999b). Because DP1 and hTG_P have been discussed above in relation to their possible functions in reproductive biology, only some additional information on DP1 will be presented here.

rTG_P/DP1 is highly glycosylated and possesses a complex lipid anchor that is retained during secretion. Close examination of the lipid and sugar components of the lipid anchor suggest that it might be a glycosylphosphatidylinositol-like structure [GPI-anchor (Seitz et al. 1991a, Esposito et al. 1996)]. The function of this GPI-anchor is unclear so far, but there is some evidence that it protects the enzyme from autocatalytic self-aggregation (Seitz et al. 1991a, Steinhoff et al. 1994). It has also been hypothesized that it might anchor the enzyme to the sperm surface (Esposito et al. 1996). Like other TGases, DP1 is Ca^{2+}-dependent even though it requires significantly less Ca^{2+} for optimal activation than other TGases (Seitz et al. 1991a). Furthermore, in *in vitro* experiments the enzyme is strongly activated by sodium dodecyl sulfate (SDS) and a variety of phosphatidic acids (Esposito et al. 1996). It is therefore tempting to speculate that DP1 activity becomes enhanced after limited proteolysis, like is described for TG_K (see above). Immunohistochemical studies have demonstrated DP1 in apical blebs of the dorsal prostate and coagulating gland epithelium, suggesting that it is released via the apocrine pathway (Seitz et al. 1990, Steinhoff et al. 1994, and see above). Secretion of DP1 is a rapid process that occurs within 40 min after its synthesis and may not involve the Golgi apparatus, whereas another abundant prostate secretory protein (DP2) requires more than 8 h while following a traject through the Golgi apparatus (Bartlett et al. 1984, Groos et al. 1999). Several *in vivo* studies with rats have shown that DP1 synthesis is androgen-dependent. DP1 expression is down-regulated, in both dorsal prostate and coagulating gland, by castration, and subsequent re-addition of androgen restores the expression level back to normal (Wilson and French 1980, Bartlett et

al. 1984, Ho et al. 1992). In explant cultures of rat prostatic tissues, DP1 expression can be induced by a broad panel of natural and synthetic androgens, which is blocked by simultaneous incubation with anti-androgen, whereas in the absence of hormone no expression could be observed (Lopes et al. 1996). Androgen regulation is thought to occur via stabilization of the DP1 mRNA transcript through decreasing its degradation rate and prolonging its half-life (Ho et al. 1992). The expression of DP1 in the dorsal prostate is age-dependent since only few cells express the enzyme 12 days after birth while the number of DP1-positive cells substantially increase during day 13 and 14. The first appearance of DP1 in the coagulating gland is one day later but further follows the same induction pattern (Lopes et al. 1996).

4 Scope of this thesis

At the start of the study presented in this thesis, it was hypothesized that the human prostate could express at least two TGases, TG_C and the human analogue of rTG_P/DP1. To obtain evidence for the presence of human TG_P and to be able to discriminate between these two TGases in future experiments, we set out to identify the corresponding hTG_P cDNA (Chapter 2). During the course of the project, the research was focussed on increasing our knowledge of the expression and function of hTG_P.

Cloning of hTG_P cDNA was achieved based upon the known high identities in the catalytic core domains of human TGases. The isolation of hTG_P cDNA formed the basis for all the other experiments described in this thesis: characterization of the *TGM4* gene and promoter structure (Chapter 3), functional analysis of the promoter (Chapters 3 and 4), development of antisera against the hTG_P C-terminus, which were used to evaluate the expression of the hTG_P protein in human tissues and body fluids (Chapter 5), and demonstration of the prostate-specific expression of the enzyme by both Northern blotting and immunohistochemical staining (Chapters 2, 3, and 5). Research has been done to study if hTG_P could serve as a prostate tumor marker (Chapter 5) and if sequence determinants could be identified that are responsible for the regulation of its (prostate-specific) expression (Chapter 4).

Insight into the expression and regulation of hTG_P may add in understanding prostate function and dysfunction.

Chapter 2

Tissue-Specific and Androgen-Regulated Expression of Human Prostate-Specific Transglutaminase

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Abstract

Transglutaminases (TGases) are calcium-dependent enzymes catalysing the post-translational cross-linking of proteins. In the prostate at least two TGases are present, the ubiquitously expressed tissue-type TGase (TG_C), and a prostate-restricted TGase, (TG_P). This paper deals with the molecular cloning and characterization of the cDNA encoding the human prostate TGase (hTG_P). For this purpose we have screened a human prostate cDNA library with a probe from the active-site region of TG_C. The largest isolated cDNA contained an open reading frame encoding a protein of 684 amino acids with a predicted molecular mass of 77 kDa as confirmed by *in vitro* transcription-translation and subsequent SDS/PAGE. The hTG_P gene was tissue-specifically expressed in the prostate, yielding an mRNA of approx. 3.5 kb. Furthermore, a 3-fold androgen-induced up-regulation of hTG_P mRNA expression has been demonstrated in the recently developed human prostate cancer cell line, PC346C. Other well established human prostate cancer cell lines, LNCaP and PC-3, showed no detectable hTG_P mRNA expression on a Northern blot. The gene coding for prostate TGase was assigned to chromosome 3.

Introduction

Transglutaminases (TGases, EC 2.3.2.13) are calcium-dependent enzymes that catalyse the acyl transfer reaction between peptide-bound glutamine residues and primary amine groups. This reaction results in the post-translational modification of proteins either by the incorporation of amines (such as polyamines) into proteins or by the cross-linking of proteins if the amine is a peptide-bound lysine. The cross-links are resistant to proteolytic enzyme degradation and thus contribute to the formation of highly insoluble macromolecules, which can be catabolized only by proteolysis of the protein chains (Greenberg et al. 1991, Aeschlimann and Paulsson 1994).

Products of the enzymatic actions of TGases can be found in most tissues (Thomazy and Fesus 1989) and body fluids (Williams-Ashman 1984, Romijn 1990, Aeschlimann and Paulsson 1994). A number of different TGases, with different structural properties and cellular origin, account for the formation of such reaction products. Well documented examples of TGases are plasma factor XIIIa (Ichinose et al. 1990), keratinocyte TGase [TG_K (Kim et al. 1991, Rice et al. 1992)], epidermal TGase [TG_E (Kim et al. 1993)], tissue-type or cellular TGase [TG_C (Gentile et al. 1991, 1992)] and prostatic TGase [TG_P (Williams-Ashman 1984, Seitz and Aumuller 1990, Seitz et al. 1991a, Ho et al. 1992)]. While the physiological functions of factor XIIIa (blood clotting), TG_E and TG_K (squamous differentiation of the epidermis by formation of cornified cell envelopes) are evident, the roles of TG_C and TG_P are less well established. The ubiquitously expressed TG_C was suggested to be involved in processes associated with cell growth regulation, differentiation and programmed cell death (el Alaoui et al. 1992, Fesus 1993, Johnson et al. 1994). This enzyme may take part in such diverse events as apoptotic body formation (Thomazy and Fesus 1989, Knight et al. 1990, Fesus et al. 1991, Piacentini et al. 1992), extracellular

matrix stabilization (Thomazy and Fesus 1989, Knight et al. 1990, Fesus et al. 1991, Piacentini et al. 1992, Aeschlimann et al. 1993, Aeschlimann and Paulsson 1994) and signal transduction (Nakaoka et al. 1994). TG_P was, until very recently, demonstrated only in rodents. In these animals, the enzyme is involved in the formation of copulatory plugs in the female genital tract after coitus (Williams-Ashman 1984), and may play a role in masking the antigenicity of the male gamete, thereby suppressing an immune response in the female genital tract against the sperm cells (Mukherjee et al. 1983, Paonessa et al. 1984). Isolation of the cDNA encoding rat prostate TGase (rTG_P) happened to be achieved when Ho et al. (1992) attempted to clone the gene for DP1, an abundantly expressed rat dorsal prostate protein. DP1 occurred only in the dorsal rat prostate and the coagulating gland, where it comprised up to 25% of the total cellular protein, and its expression was found to be higher in intact than in castrated animals (Ho et al. 1992).
Also in the human prostate two types of TGases (TG_C and TG_P) might potentially be expressed. To isolate the human TGase similar to the rat DP1 enzyme, we have searched for human TG_P (hTG_P) cDNA clones in a human prostate cDNA library. Here we present the molecular cloning and characterization of hTG_P cDNA. In addition, this paper deals with chromosome assignment, organ specificity of hTG_P expression and androgenic regulation of hTG_P in human prostate cancer cells.

Materials and Methods

Cell culture
LNCaP (Horoszewicz et al. 1983) and PC-3 (Kaighn et al. 1979) cells were cultured in RPMI medium supplemented with 7.5% (v/v) fetal-calf serum (FCS) and antibiotics. The PC346C cell line was recently established in our laboratory (Romijn et al. 1995). Cells were cultured in a Dulbecco's modified Eagle's medium (DMEM)/F12-based growth medium (GIBCO, Grand Island, NY, U.S.A.), essentially as described by Limon et al. (1990), but dihydrotestosterone was replaced by the synthetic androgen R1881 (NEN, Boston, MA, U.S.A.) and with further supplementation of 2% (v/v) FCS. The growth of PC346C cells was stimulated by androgens (Romijn et al. 1995). To study androgen regulation of gene expression, the cells were cultured in the absence and presence of 0.1 nM R1881 in growth medium containing dextran-charcoal treated (DCC) serum. The cells were harvested from near confluent cultures (after 7 days for LNCaP and 10 days for PC346C), and subjected to RNA isolation as described below.

Screening of a human prostate cDNA library
For the isolation of hTG_P cDNA, an oligo(dT) and random-primed λgt11 human prostate cDNA library (Clontech, Palo Alto, CA, U.S.A.) was screened with a random ^{32}P-labelled 380 bp probe from the active-site region of TG_C. This probe was obtained by PCR amplification with primer TRAGLU 1 and TRAGLU 2 (for list of primers, see Table 1) of this area from pSG5.hTG, a plasmid containing the entire cDNA sequence of human TG_C, which was kindly provided by Dr. P.J.A. Davies (Gentile et al. 1992).

Table 1. Oligonucleotides used for isolation of cDNA clones and chromosomal localization of the hTG_P gene.

Primer Name	Sequence [a,b]	Length	Orientation	Source	Position
λGT11.for [c]	5'-GGTGGCGACGACTCCTGGAGCC-3'	22-mer	sense	λGT11	-
λGT11.rev [c]	5'-GACACCAGACCAACTGGTAATG-3'	22-mer	anti-sense	λGT11	-
TRAGLU 1	5'-TA/TTGGCCAGTGCTGGGTTT-3'	19-mer	sense	TG_C	955 – 973
TRAGLU 2	5'-TTC/GACCTCC/GGTGAAG/CACGA-3'	19-mer	anti-sense	TG_C	1328 - 1310
TRAGLU R1	5'-GCTTTGGCCAGTGCTGGGTT-3'	20-mer	sense	Rat DP1	820 - 839
TRAGLU R2	5'-CACCTCCGTGAACACAAATGT-3'	21-mer	anti-sense	Rat DP1	1196 - 1176
HPSTG 1	5'-CTCATTCACATAGGTGTCCAC-3'	21-mer	anti-sense	hTG_P	974 - 954
HPSTG 2	5'-TCATAGCACACATGGCCCT-3'	19-mer	anti-sense	hTG_P	702 - 684

[a] The primers were designed from their corresponding mRNA sequences as reported by Gentile et al. 1991 (TRAGLU 1 and TRAGLU 2), by Ho et al. 1992 (TRAGLU R1 and TRAGLU R2), and in this paper (HPSTG 1 and HPSTG 2)

[b] TRAGLU 1 and TRAGLU 2 are degenerated primers as is indicated by a slash.

[c] λGT11.for and λGT11.rev represent the flanking λGT11 primers used for PCR.

Hybond-N+ filters (Amersham, Aylesbury, Bucks, U.K.) were prehybridized with 100 µg/ml denatured salmon sperm DNA in poly(ethylene glycol) (PEG)-hybridization mix, composed of 0.25 M $Na_2HPO_4.2H_2O$ (pH7.2), 0.25 M NaCl, 7% (w/v) SDS, 10% PEG 6000 and 1 mM EDTA, for 2 h. Hybridizations were carried out overnight at 50°C, followed by two washes with 2 x SSC/0.1% SDS and two washes with 1 x SSC/0.1% SDS, each for 15 min at 50°C. Washed blots were covered with plastic film and exposed to RX medical X-ray film (Fuji) with intensifying screen for 4 days at -80°C.

Positive plaques were picked up and rescreened at least twice until the selected plaques were completely purified. The inserts of these clones were amplified by PCR (see below), and directly sequenced with primer TRAGLU R2 (Table 1). The selected phage clone was purified and treated as described below under 'DNA sequencing'. Since this procedure resulted in the isolation of a clone containing only part of the hTG_P cDNA sequence (see the Results section), a 2.2 kb *Sac* I-*Hpa* I fragment of this clone was used as a probe to rescreen the same cDNA library under more stringent conditions with the purpose of isolating the entire hTG_P cDNA. Hybridizations and washes were performed at 65°C in stead of 50°C. Selected recombinant phage clones were purified and treated as described below.

PCR amplification and primer removal

PCR amplifications of phage suspension aliquots, or of isolated DNA, were performed with superTaq polymerase (Sphaero Q, HT Biotechnology, U.K.) in the presence of 100 ng of each primer. All samples were first denatured at 94°C for 5 min. Amplification followed in 35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 2 min; and a final extension at 72°C for 10

min. PCR products were analysed by agarose gel electrophoresis. If necessary, primers were removed with the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

DNA sequencing

DNA was isolated from the selected and purified recombinant phage clones following standard procedures (Sambrook et al. 1989) and the cDNA fragments were subcloned into the *Eco*RI site of pGEM-7Zf(+) (Promega, Madison, WI, U.S.A.) for sequencing. Plasmid isolations were performed with a Qiagen plasmid kit. Double-stranded DNA sequence analysis was performed by dideoxy nucleotide chain termination using a T7 sequencing kit (Pharmacia, LKB Biotech, Uppsala, Sweden). Oligonucleotide primers used for walking primer sequencing were designed and synthesized on the basis of the obtained sequences (Pharmacia).

In vitro transcription-translation of hTG_P and TG_C cDNA

For *in vitro* transcription-translation studies the entire open reading frame of hTG_P cDNA was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen Corporation, San Diego, CA, U.S.A.). For comparison the entire 3.3 kb cDNA for TG_C (Gentile et al. 1992) was subcloned into the *Eco*RI site of pcDNA3 (pcDNA3-TG_C). An empty pcDNA3 vector was used as a negative control. Subsequently, a one tube, coupled transcription-translation was performed with the TNT® coupled reticulocyte lysate system from Promega using the T7 RNA promoter present in pcDNA3 for *in vitro* transcription of sense RNA. The reactions were performed on 1 µg of circular template DNA in a final volume of 25 µl according to the manufacturer's protocol. The transcription-translation was carried out at 30°C for 90 min in the presence of ^{35}S-labelled methionine (Amersham). Labelled products were separated by SDS/10%-PAGE. The gel was dried and the synthesized proteins were visualized by exposure to RX medical X-ray film (Fuji) for 4 h. Prestained markers (Novex, San Diego, CA, U.S.A.) were used as size standards.

Northern Hybridization

Isolation of total RNA was carried out by the guanidine isothiocyanate/cesium chloride centrifugation method (Sambrook et al. 1989). Total RNA (20 µg) was electrophoresed in a 1 % agarose formaldehyde gel and transferred to Hybond-N+. RNA was fixed to the membrane by UV cross-linking. The blot was hybridized overnight at 60°C with the 2.2 kb probe mentioned above in hybridization mix composed of 0,5 M $Na_2HPO_4.2H_2O$ (pH7.2), 7% SDS, 1% BSA, and 1 mM EDTA. The final wash step was in 0.3 x SSC/0.1% SDS at 60°C for 30 min. The blot was exposed to RX medical X-ray film (Fuji) with intensifying screen for 4 days at -80°C. The signals were quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

A human multiple-tissue Northern blot (MTN II, Clontech) was used to determine the tissue specificity of hTG_P expression. Hybridization was performed with the same cDNA fragment as above using conditions recommended by the manufacturer. Washes were performed under high stringency conditions (up to 0.1 x SSC/0.1% SDS at 63°C for 30

min). The integrity of the blotted RNA was determined by hybridization with human β-actin cDNA.

Chromosomal localization of the gene encoding hTG_P

A human/hamster-human/mouse somatic cell hybrid panel (BIOS Research Laboratories, New Haven, CT, U.S.A.) was screened by PCR analysis with the HPSTG1-TRAGLU R1 primer set (Table 1). PCR reactions were performed on 50 ng of somatic cell hybrid DNAs as described above. An annealing temperature of 60°C was used. The resulting products were analysed by agarose gel electrophoresis. The specificity of the reaction products was confirmed by blotting and subsequent hybridization with a ^{32}P random-labelled 2.2 kb *Sac* I-*Hpa* I hTG_P fragment. Hybridization and washes were as described above for Northern hybridization.

Results

Isolation of cDNA clones

Screening under low-stringency conditions of approx. 1.2 x 10^6 p.f.u. of a λgt11 human prostate cDNA library with a 380 bp fragment from the active-site region of TG_C, yielded nine positive clones. Partial sequence analysis with primer TRAGLU R2 (Table 1) in the vicinity of the active site revealed that one clone (4.2) had high sequence similarity to rat DP1 (Ho et al. 1992). The insert size was approx. 2.8 kb. The other clones appeared to be parts of TG_C cDNA. Sequence analysis of the entire insert of clone 4.2 showed an open reading frame encoding a protein of 574 amino acids. Comparison with the amino acid sequences of other TGases, suggested that part of the 5' end of hTG_P cDNA was missing, probably due to a cloning artefact. To obtain further cDNA information, the same prostate cDNA library was rescreened under high stringency conditions with the 2.2 kb *Sac* I-*Hpa* I fragment from clone 4.2 (see Figure 1 for restriction map). This resulted in a large number of positive plaques (about 500) of which 24 were isolated. As our main interest concerned the 5' end of the hTG_P cDNA, a further selection was made by PCR with combinations of primer HPSTG 1 or HPSTG 2 and one of the flanking λGT11 primers. In this way, three overlapping clones, clone 1.8, 1.12 and 1.17, were selected with inserts varying from 2.4 kb to 3.3 kb. The relative positions of the cDNAs of these clones are shown in Figure 1. *Eco*RI fragments of the three clones were sequenced. Whereas clone 4.2 appeared to lack a coding region for 130 amino acids at its N-terminus, both clone 1.8 and clone 1.17 contained the entire open reading frame of hTG_P cDNA. Further analysis showed a number of cloning artefacts (see Figure 1). Clone 1.17 contained amplifications of central parts of the hTG_P cDNA sequence at its 5' and 3' terminal ends, and 1.12 appeared to have a 5' nucleotide stretch similar to that of clone 4.2 but situated more to the 5'-end.

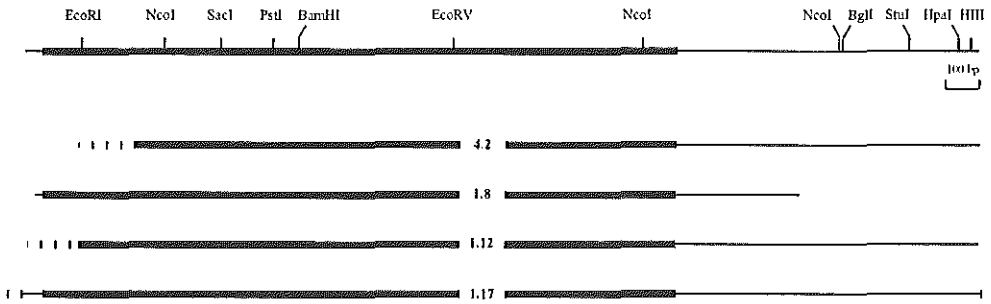

Figure 1. Restriction map and relative positions of cDNA clones for hTG$_P$. Black bars represent coding regions. Cloning artefacts are indicated by broken black bars. Clone 4.2 and 1.12 contained at their 5' ends the same artificial nucleotide sequence but at different positions. Clone 1.17 contained amplifications of central parts of the hTG$_P$ cDNA at its terminal ends. Clone 1.8 had only three nucleotides in front of its start codons.

cDNA sequence of hTG$_P$

Figure 2 shows the composite nucleotide sequence of the hTG$_P$ cDNA as well as its deduced amino acid sequence. Its total length is 2983 bp with an open reading frame that corresponds to a protein of 684 amino acids with a predicted molecular mass of 77 kDa and a pI of 6.94.

Two start codons are present at the beginning of the open reading frame, preceded by an in-frame stop codon. Which one of the start codons is used for translational start cannot be deduced from the sequence. However, the DNA sequence surrounding the second methionine agrees well with the Kozak consensus sequence (Kozak 1994) with an A at position -3 and a G in position +4 (as the A of the ATG codon is designated +1). The 3' non-coding region of hTG$_P$ cDNA contains the variant polyadenylation signal, ATTAAA [Figure 2 (Wickens 1990).

The active-site amino acid sequence GQCWVF (residues 266-271) is present, suggesting that the clone encodes for an active TGase. Also other amino acids recently shown to be crucial for catalytic activity of TGases, but not part of the active centre, are present. These amino acids, Arg-37 (R), Arg-38 (R) [Huber et al. 1995, Russell et al. 1995], His-296 (H), His-327 (H) and Asp-350 (D) [Hettasch and Greenberg 1994], occur in all human TGases. The amino acid sequence shows no typical Ca^{2+}-binding site-motif, like an EF-hand structure, but two regions enriched with acidic residues (residues 144-154 and 433-453) may reflect Ca^{2+}-binding sites. In addition, the putative hTG$_P$ protein has seven potential N-glycosylation sites (sites are indicated in Figure 2).

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                  AGAGATAGAGTCTTCCCTGGCATTGCAGGAGAGAATCTGAAGGG ATG ATG GAT GCA     56
                           •                                    M   M   D   A      4

TCA AAA GAG CTG CAA GTT CTC CAC ATT GAC TTC TTG AAT CAG GAC AAC GCC GTT    110
 S   K   E   L   Q   V   L   H   I   D   F   L   N   Q   D   N   A   V     22

TCT CAC CAC ACA TGG GAG TTC CAA ACG AGC AGT CCT GTG TTC CGG CGA GGA CAG    164
 S   H   H   T   W   E   F   Q   T   S   S   P   V   F   R   R   G   Q     40

GTG TTT CAC CTG CGG CTG GTG CTG AAC CAG CCC CTA CAA TCC TAC CAC CAA CTG    218
 V   F   H   L   R   L   V   L   N   Q   P   L   Q   S   Y   H   Q   L     58

AAA CTG GAA TTC AGC ACA GGG CCG AAT CCT AGC ATC GCC AAA CAC ACC CTG GTG    272
 K   L   E   F   S   T   G   P   N   P   S   I   A   K   H   T   L   V     76

GTG CTC GAC CCG AGG ACG CCC TCA GAC CAC TAC AAC TGG CAG GCA ACC CTT CAA    326
 V   L   D   P   R   T   P   S   D   H   Y   N   W   Q   A   T   L   Q     84

AAT GAG TCT GGC AAA GAG GTC ACA GTG GCT GTC ACC AGT TCC CCC AAT GCC ATC    380
 N   E   S   G   K   E   V   T   V   A   V   T   S   S   P   N   A   I    112
 ▲
CTG GGC AAG TAC CAA CTA AAC GTG AAA ACT GGA AAC CAC ATC CTT AAG TCT GAA    434
 L   G   K   Y   Q   L   N   V   K   T   G   N   H   I   L   K   S   E    130

GAA AAC ATC CTA TAC CTT CTC TTC AAC CCA TGG TGT AAA GAG GAC ATG GTT TTC    488
 E   N   I   L   Y   L   L   F   N   P   W   C   K   E   D   M   V   F    148

ATG CCT GAT GAG GAC GAG CGC AAA GAG TAC ATC CTC AAT GAC ACG GGC TGC CAT    542
 M   P   D   E   D   E   R   K   E   Y   I   L   N   D   T   G   C   H    166
                                                 ▲
TAC GTG GGG GCT GCC AGA AGT ATC AAA TGC AAA CCC TGG AAC TTT GGT CAG TTT    596
 Y   V   G   A   A   R   S   I   K   C   K   P   W   N   F   G   Q   F    184

GAG AAA AAT GTC CTG GAC TGC TGC ATT TCC CTG CTG ACT GAG AGC TCC CTC AAG    650
 E   K   N   V   L   D   C   C   I   S   L   L   T   E   S   S   L   K    202

CCC ACA GAT AGG AGG GAC CCC GTG CTG GTG TGC AGG GCC ATG TGT GCT ATG ATG    704
 P   T   D   R   R   D   P   V   L   V   C   R   A   M   C   A   M   M    220

AGC TTT GAG AAA GGC CAG GGC GTG CTC ATT GGG AAT TGG ACT GGG GAC TAT GAA    758
 S   F   E   K   G   Q   G   V   L   I   G   N   W   T   G   D   Y   E    238
                                           ▲
GGT GGC ACA GCC CCA TAC AAG TGG ACA GGC AGT GCC CCG ATC CTG CAG CAG TAC    812
 G   G   T   A   P   Y   K   W   T   G   S   A   P   I   L   Q   Q   Y    256

TAC AAC ACG AAG CAG GCT GTG TGC TTT GGC CAG TGC TGG GTG TTT GCT GGG ATC    866
 Y   N   T   K   Q   A   V   C   F   G   Q   C   W   V   F   A   G   I    274
                                       *
CTG ACT ACA GTG CTG AGA GCG TTG GGC ATC CCA GCA CGC AGT GTG ACA GGC TTC    920
 L   T   T   V   L   R   A   L   G   I   P   A   R   S   V   T   G   F    292

GAT TCA GCT CAC GAC ACA GAA AGG AAC CTC ACG GTG GAC ACC TAT GTG AAT GAG    974
 D   S   A   H   D   T   E   R   N   L   T   V   D   T   Y   V   N   E    310
                                 ▲
AAT GGC AAG AAA ATC ACC AGT ATG ACC CAC GAC TCT GTC TGG AAT TTC CAT GTG   1028
 N   G   K   K   I   T   S   M   T   H   D   S   V   W   N   F   H   V    328

TGG ACG GAT GCC TGG ATG AAG CGA CCG GAT CTG CCC AAG GGC TAC GAC GGC TGG   1082
 W   T   D   A   W   M   K   R   P   D   L   P   K   G   Y   D   G   W    346

CAG GCT GTG GAC GCA ACG CCG CAG GAG CGA AGC CAG GGT GTC TTC TGC TGT GGG   1136
 Q   A   V   D   A   T   P   Q   E   R   S   Q   G   V   F   C   C   G    364

CCA TCA CCA CTG ACC GCC ATC CGC AAA GGT GAC ATC TTT ATT GTC TAT GAC ACC   1190
 P   S   P   L   T   A   I   R   K   G   D   I   F   I   V   Y   D   T    382

AGA TTC GTC TTC TCA GAA GTG AAT GGT GAC AGG CTC ATC TGG TTG GTG AAG ATG   1244
 R   F   V   F   S   E   V   N   G   D   R   L   I   W   L   V   K   M    400

GTG AAT GGG CAG GAG GAG TTA CAC GTA ATT TCA ATG GAG ACC ACA AGC ATC GGG   1298
 V   N   G   Q   E   E   L   H   V   I   S   M   E   T   T   S   I   G    418

AAA AAC ATC AGC ACC AAG GCA GTG GGC CAA GAC AGG CGG AGA GAT ATC ACC TAT   1352
 K   N   I   S   T   K   A   V   G   Q   D   R   R   R   D   I   T   Y    436
     ▲
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GAG TAC AAG TAT CCA GAA GGC TCC TCT GAG GAG AGG CAG GTC ATG GAT CAT GCC  1406
 E   Y   K   Y   P   E   G   S   S   E   E   R   Q   V   M   D   H   A   454

TTC CTC CTT CTC AGT TCT GAG AGG GAG CAC AGA CGA CCT GTA AAA GAG AAC TTT  1460
 F   L   L   L   S   S   E   R   E   H   R   R   P   V   K   E   N   F   472


CTT CAC ATG TCG GTA CAA TCA GAT GAT GTG CTG CTG GGA AAC TCT GTT AAT TTC  1554
 L   H   M   S   V   Q   S   D   D   V   L   L   G   N   S   V   N   F   490
                                                           ▲
ACC GTG ATT CTT AAA AGG AAG ACC GCT GCC CTA CAG AAT GTC AAC ATC TTG GGC  1568
 T   V   I   L   K   R   K   T   A   A   L   Q   N   V   N   I   L   G   508

TCC TTT GAA CTA CAG TTG TAC ACT GGC AAG AAG ATG GCA AAA CTG TGT GAC CTC  1622
 S   F   E   L   Q   L   Y   T   G   K   K   M   A   K   L   C   D   L   526

AAT AAG ACC TCG CAG ATC CAA GGT CAA GTA TCA GAA GTG ACT CTG ACC TTG GAC  1676
 N   K   T   S   Q   I   Q   G   Q   V   S   E   V   T   L   T   L   D   544
 ▲
TCC AAG ACC TAC ATC AAC AGC CTG GCT ATA TTA GAT GAT GAG CCA GTT ATC AGA  1730
 S   K   T   Y   I   N   S   L   A   I   L   D   D   E   P   V   I   R   562

GGT TTC ATC ATT GCG GAA ATT GTG GAG TCT AAG GAA ATC ATG GCC TCT GAA GTA  1784
 G   F   I   I   A   E   I   V   E   S   K   E   I   M   A   S   E   V   580

TTC ACG TCT TTC CAG TAC CCT GAG TTC TCT ATA GAG TTG CCT AAC ACA GGC AGA  1838
 F   T   S   F   Q   Y   P   E   F   S   I   E   L   P   N   T   G   R   598

ATT GGC CAG CTA CTT GTC TGC AAT TGT ATC TTC AAG AAT ACC CTG GCC ATC CCT  1892
 I   G   Q   L   L   V   C   N   C   I   F   K   N   T   L   A   I   P   616

TTG ACT GAC GTC AAG TTC TCT TTG GAA AGC CTG GGC ATC TCC TCA CTA CAG ACC  1946
 L   T   D   V   K   F   S   L   E   S   L   G   I   S   S   L   Q   T   634

TCT GAC CAT GGG ACG GTG CAG CCT GGT GAG ACC ATC CAA TCC CAA ATA AAA TGC  2000
 S   D   H   G   T   V   Q   P   G   E   T   I   Q   S   Q   I   K   C   652

ACC CCA ATA AAA ACT GGA CCC AAG AAA TTT ATC GTC AAG TTA AGT TCC AAA CAA  2054
 T   P   I   K   T   G   P   K   K   F   I   V   K   L   S   S   K   Q   670

GTG AAA GAG ATT AAT GCT CAG AAG ATT GTT CTC ATC ACC AAG TAG CCTTGTCTGAT  2110
 V   K   E   I   N   A   Q   K   I   V   L   I   T   K   -              684

GCTGTGGAGCCTTAGTTGAGATTTCAGCATTTCCTACCTTGTGCTTAGCTTTCAGATTATGGATGATTAAA  2181
TTTGATGACTTATATGAGGGCAGATTCAAGAGCCAGCAGGTCAAAAAGGCCAACACAACCATAAGCAGCCA  2252
GACCCACAAGGCCAGGTCCTGTGCTATCACAGGGTCACCTCTTTTACAGTTAGAAACACCAGCCGAGGCCA  2323
CAGAATCCCATCCCTTTCCTGAGTCATGGCCTCAAAAATCAGGGCCACCATTGTCTCAATTCAAATCCATA  2394
GATTTCGAAGCCACAGAGTCTCTCCCTGGAGCAGCAGACTATGGGCAGCCCAGTGCTGCCACCTGCTGACG  2465
ACCCTTGAGAAGCTGCCATATCTTCAGGCCATGGGTTCACCAGCCCTGAAGGCACCTGTCAACTGGAGTGC  2536
TCTCTCAGCACTGGGATGGGCCTGATAGAAGTGCATTCTCCTCCTATTGCCTCCATTCTCCTCTCTCTATC  2607
CCTGAAATCCAGGAAGTCCCTCTCCTGGTGCTCCAAGCAGTTTGAAGCCCAATCTGCAAGGACATTTCTCA  2678
AGGGCCATGTGGTTTTGCAGACAACCCTGTCCTCAGGCCTGAACTCACCATAGAGACCCATGTCAGCAAAC  2749
GGTGACCAGCAAATCCTCTTCCCTTATTCTAAAGCTGCCCCTTGGGAGACTCCAGGGAGAAGGCATTGCTT  2820
CCTCCCTGGTGTGAACTCTTTCTTTGGTATTCCATCCACTATCCTGGCAACTCAAGGCTGCTTCTGTTAAC  2891
TGAAGCCTGCTCCTTCTTGTTCTGCCCTCCAGAGATTTGCTCAAATGATCAATAAGCTTTAAATTAAACTC  2962
TACTTCAAGAAAAAAAAACCG                                                     2983
```

Figure 2. Nucleotide and deduced amino acid sequence of hTG$_P$ cDNA. The deduced amino acid sequence is numbered from the first methionine. The active-site sequence GQCWVF is bold typed and the active-center cysteine is marked with an asterisk. A putative polyadenylation signal is double underlined. An in-frame stop codon preceding the start codons is indicated by a closed circle. Two putative Ca^{2+}-binding regions are underlined. Potential N-glycosylation signals (N-X-S/T) are indicated by closed triangles. The sequence deviates at several positions from the sequence recently published by Grant et al. (1994). A remarkable difference was found at position 338-342 of the deduced amino acid sequence. In all four isolated clones we here found an insertion of the five amino acids DLPKG, which is also present at this position in all other TGases. Other amino acid substitutions were found at position K313E, R466Q and F584N. In addition, the 23 nucleotides at the 3'-terminal end were found to be different (similar in clone 4.2, 1.12 and 1.17).

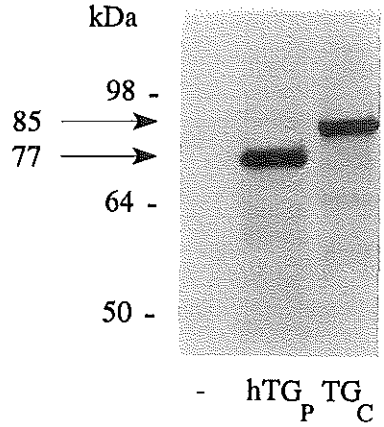


Figure 3. *In vitro* transcription-translation of hTG_P and TG_C cDNA. The entire open reading frames of the cDNAs encoding hTG_P (pcDNA3-hTG_P) an TG_C (pcDNA3-TG_C) were subcloned into th eukaryotic expression vector pcDNA3. One µg of each vector was used for a one tube *in vitro* transcription-translation reaction. As a control pcDNA3 without insert (-) was used. Protein products were separated by SDS/10%-PAGE. O the left-hand side the molecular mass of the translated proteins and of the molecular mas standards are indicated.

In vitro transcription-translation of hTG_P and TG_C cDNA

To verify whether the isolated cDNA was able to produce a protein product and whether the size of the encoded protein corresponds to its predicted molecular mass, the entire coding region of hTG_P was cloned into pcDNA3 for *in vitro* transcription-translation. For this purpose, we first subcloned the 3' *Eco*RI-*Eco*RI fragment of the largest clone, clone 1.17 (Figure 1), into the *Eco*RI site of pcDNA3. Subsequently, removal of the 3' *Eco*RI site (including the 3' artefact of clone 1.17) was achieved by cleavage with *Hpa* I (insert) and *Xba* I (vector). Because of the presence of a 5' artefact in clone 1.17 (see above), we used the 5' 184 bp *Eco*RI-*Eco*RI fragment from clone 1.8 for ligation to the *Eco*RI-*Hpa* I fragment of clone 1.17. In this way the entire coding region of hTG_P was cloned into pcDNA3 (pcDNA3-hTG_P) with only three nucleotides left in front of its start codon.

Figure 3 shows that both pcDNA3-hTG_P and pcDNA3-TG_C produced a single protein. The molecular mass of the hTG_P protein was established to be approx. 77 kDa which is in agreement with its predicted molecular mass. The TG_C construct yielded a protein of approx. 85 kDa which size is somewhat larger than its predicted molecular mass of 77 kDa. This observation is in agreement with the *in vitro* translation results of Gentile et al. (1991). The control plasmid produced no protein bands.

Expression of hTG_P in the prostate and in prostate derived cell lines

To determine the expression pattern of hTG_P a human multiple-tissue Northern blot with poly $(A)^+$ RNA from eigth different tissues including prostate was hybridized under high stringency conditions with a 2.2 kb *Sac* I-*Hpa* I fragment from clone 4.2. As shown in Figure 4 hTG_P mRNA was exclusively expressed in the prostate. A single mRNA was detected with a length of approx. 3.5 kb. Prolonged exposure of the Northern blot did not result in a positive signal in either of the other tissues tested. The hTG_P mRNA could be clearly distinguished from the approx. 4 kb TG_C mRNA as was determined by Northern-blot analysis of total RNA from benign prostate hyperplasia tissue (results not shown).

Next it was investigated whether hTG$_P$ was also expressed in prostate cancer cell lines. For this purpose we performed Northern-blot analysis of total RNA from the well-known human prostate carcinoma cell line LNCaP as well as from a prostate cancer cell line, PC346C, recently developed in our laboratory [Romijn et al. 1995 (see the Materials and methods section)]. While no hTG$_P$ mRNA in LNCaP could be detected, PC346C expressed one mRNA of the same length (3.5 kb) as has been found in prostatic tissue. The Northern blot results for LNCaP and PC346C are shown in Figure 5. We could not detect hTG$_P$ mRNA in the androgen-independent cell line PC-3 [results not shown (Kaighn et al. 1979)].

Androgen regulated expression of hTG$_P$

To investigate androgen regulation of hTG$_P$ expression, we used the androgen-responsive prostate cancer cell lines LNCaP and PC346C. Both cell lines were grown in the presence or absence of 0.1 nM R1881. Northern-blot analysis of total RNA from treated and untreated cells, using the 2.2 kb *Sac* I-*Hpa* I fragment from clone 4.2 as probe, showed that in PC346C cells the amount of hTG$_P$ mRNA was 3-fold increased in the presence of R1881, demonstrating that the hTG$_P$ expression can be upregulated by androgen. However, even after induction with R1881, no hTG$_P$ mRNA could be visualized in the LNCaP cells (Figure 5). The same blot was also hybridized with a probe for TG$_C$, but neither in LNCaP nor in PC346C cells was TG$_C$ expression detected.

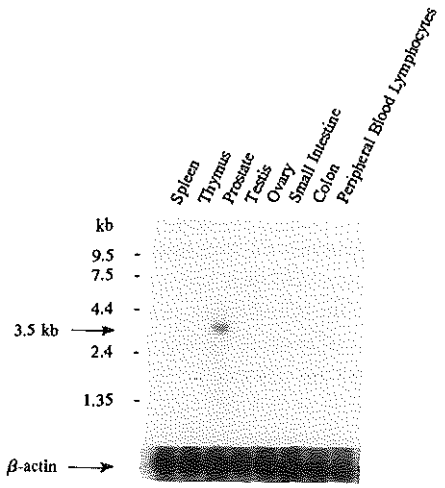


Figure 4. Northern-blot analysis of TG$_P$ expression in human tissues. A human multiple-tissue Northern blot was hybridized under high stringency conditions (see the Materials and methods section) with a random ^{32}P-labelled 2.2 kb *Sac* I-*Hpa* I fragment from clone 4.2. Each lane contained 2 μg poly (A)$^+$ RNA. The integrity of the blot was determined by hybridization with human β-actin cDNA.

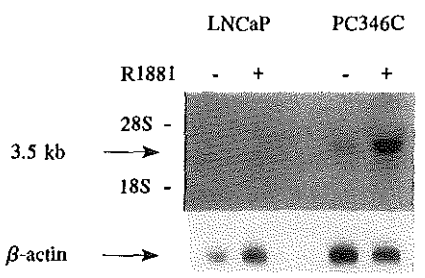


Figure 5. Androgen-regulated expression of hTG$_P$ mRNA. Northern-blot analysis of PC346C and LNCaP grown in the presence (+) or absence (-) of 0.1 nM of the synthetic androgen R1881 and further treated as described in the Materials and methods section. Each lane contains 20 μg of total RNA. The blot was hybridized under high stringency conditions (see the Materials and methods section) with a random ^{32}P-labelled 2.2 kb *Sac* I-*Hpa* I fragment from clone 4.2. Hybridization with a β-actin probe was performed as a control.

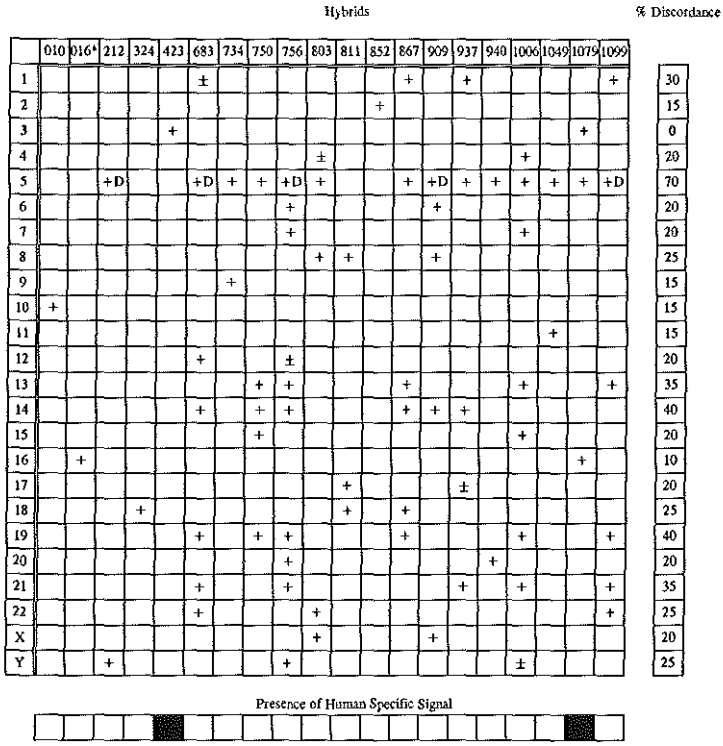


Figure 6. Assignment of the hTG$_P$ gene to human chromosome 3. A panel of human/hamster and human/mouse somatic cell hybrid DNAs was screened by PCR with the HPSTG1-TRAGLU R1 primer set (see Table 1). PCR products generated by this primer pair were separated on a 1% agarose gel (not shown). DNA was amplified from somatic hybrid cell lines (cell line designations are indicated above figure), as well as human, hamster and mouse genomic DNA. The figure shows correlation between the presence of human chromosomes in the somatic cell line panel (as described by the manufacturer), and the presence or absence of a specific PCR product. On the left side the human chromosome number is indicated. The somatic cell lines in which a specific PCR product could be detected are indicated in the lower bar by the shaded areas. Percentage discordance is shown at the right. Assignment of the hTG$_P$ gene to chromosome 3 had a discordance of 0%, while all other human chromosomes were excluded by ≥ 10% discordance. Symbols: An asterisk indicates human/mouse somatic cell hybrid, all others are human/hamster hybrids; D, multiple deletions; +, >30% of the cells contain the given chromosome; ±, 5-30% of the cells contain the given chromosome.

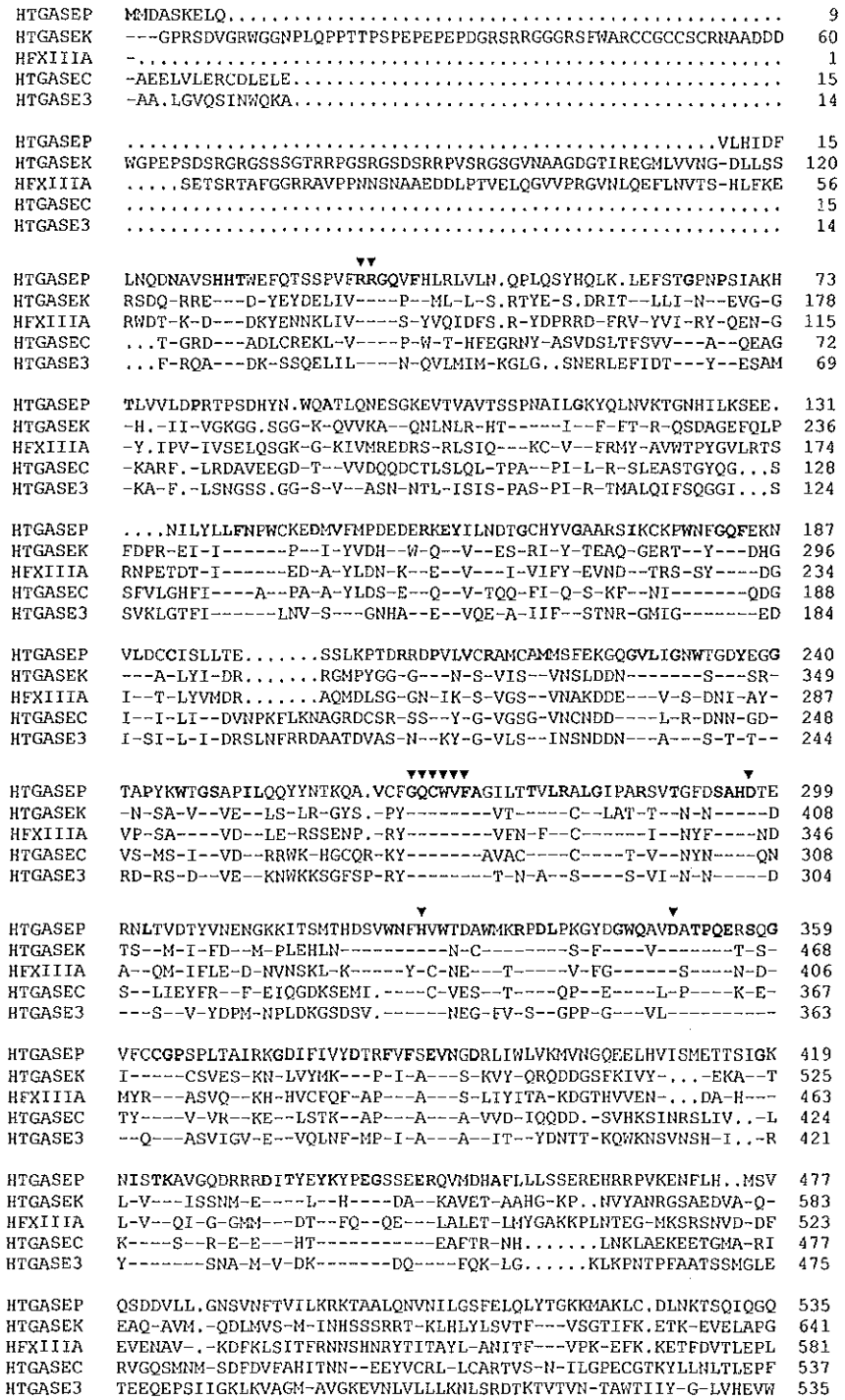
Chromosomal localization of the hTG$_P$ gene

Chromosomal localization of the gene encoding hTG$_P$ was performed by screening by PCR a panel of 20 human/hamster and human/mouse somatic-cell-hybrid cell lines that contain each of the human chromosomes in one or more of the hybrid DNAs (Figure 6). For this approach the primer pair HPSTG1-TRAGLU R1 was used (Table 1).

PCR analysis on chromosomal DNA isolated from PC346C cells (results not shown) resulted in a product of 250 bp, while PCR on hTG$_P$ cDNA would have yielded a product of 150 bp (see Table 1). This difference in size is most probably due to the presence of an intron of approx. 100 bp in this part of the gene. Comparison with the organization of other TGase genes in that region is in favour of this assumption (see Kim et al. 1994a).
As presented in Figure 6 only the presence or absence of human chromosome 3 is concordant with the presence of a PCR product, whereas at least two discordancies were observed for all other chromosomes. The authenticity of the obtained 250 bp products was proven by hybridization with the 2.2 kb *Sac* I-*Hpa* I fragment from clone 4.2. No cross-reactivity was found with hamster or mouse chromosomal DNA present in the DNA hybrids.

Discussion

In this paper, we describe the molecular characterization of the cDNA encoding a prostate TGase, hTG$_P$. The composite cDNA sequence consists of 2983 nucleotides, containing an open reading frame encoding a protein of 684 amino acids. The deduced amino acid sequence contains significant similarity with other members of the human TGase family (Figure 7). Especially in the region of the active site these sequences display a high degree of similarity not only concerning the deduced primary amino acid sequences, but also the hydrophobicity directly C-terminal to the active site (Ichinose et al. 1990, Kim et al. 1991). The amino acid sequences of hTG$_P$ and rTG$_P$ share an identity of 53%. The overall identity of hTG$_P$ with the other TGases is approx. 30%. The degree of similarity is very low C-terminal to the putative Ca^{2+}-binding site at position 433-453. Essential amino acids are present in the deduced amino acid sequence, such as the active-site cysteine which is an absolute prerequisite for TGase activity as well as the amino acids directly surrounding this cysteine (Hettasch and Greenberg 1994). Also other amino acids which are away from the active centre in the primary sequence but are required for catalytic function of TGases, because of their putative influence on the protein conformation, are present (Hettasch and Greenberg 1994, Huber et al. 1995, Russell et al. 1995). Together, these results suggest that we have isolated a cDNA encoding an active prostatic TGase.
We assigned the gene encoding hTG$_P$ unambiguously to chromosome 3. No other human genes encoding TGases known so far are localized on this chromosome. The other TGase genes are assigned to chromosome bands 6p24-p25, FXIIIa (Board et al. 1988), 14q11.2-q13, TG$_K$ (Kim et al. 1992) and TG$_C$ as well as TG$_E$ to 20q11.2-20q12 (Gentile et al. 1994, Wang et al. 1994). While this paper was in preparation, a report by Gentile et al. confirmed our results by sublocalizing the hTG$_P$ gene to chromosome band 3p21.33-p22 (Gentile et al. 1995).



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HTGASEP   VSEVTLTLDSKTYINSLAILDDEPVIRGFIIAEIVESKEIMASEVFTSFQYPEFSIELPN  595
HTGASEK   A-DRVTMPVA..-KEYRPH-V-QGAMLLNVSGHVK--GQVL-KQHTFRLRT-DL-LT-LG  699
HFXIIIA   SFKKEAV-IQ..AGEYMGQ-LEQASLHF-VT-R-N-TRDVL-KQKS-VLTI--II-KVRG  639
HTGASEC   SEKSVPLCILYEK..YRDC-TESNL-KVRALLVEPVINSYLLA-RDLYLEN--IK-RILG  595
HTGASE3   KDSA-MS--PEEE..AEHPIKISYAQYERYLKSDNMIRITAVCK-PDESEVVVERDIILD  593

HTGASEP   TGRIGQLLVCNCIFKNTLAIPLTDVKFSLESLGISSLQTSDHGTVQPGET..IQSQIKCT  653
HTGASEK   AAVV--ECEVQIV---P-PVT--N-V-R--GS-LQRPKILNV-DIGGN--..VTLRQSFV  757
HFXIIIA   -QVV-SDMTVTIQ-T-P-KET-RN-WVH-DGP-VTRPMKKMFREIR-NS-..V-WEEV-R  697
HTGASEC   EPKQKRK--AEVSLQ-P-PVA-EGCT-TV-GA-LTEE-KTVEIPDPVEAGEEVKVRMDLV  655
HTGASE3   NPTLTLEVLNEARVRKPVNVQMLFSNPLD-PVRDCV-MVEGS-LLLGNLKIDVPTLGPKE  653

HTGASEP   PIKTGPKKFIVKLSSKQVKEINAQKIVLITK                               684
HTGASEK   -VRP--RQL-AS-D-P-LSQVHGVIQ-DVAPAPGDGGFFSDAGGDSHLGETIPMASRGGA  817
HFXIIIA   -WVS-HR-L-ASM--DSLRHVYGELD-Q-QRRPSM                           732
HTGASEC   -LHM-LH-LV-NFE-DKL-AVKGFRN-I-GPA                              687
HTGASE3   RSRVRFDILPSRSGT--LLADFSCNKFPAI-AMLSIDVAE                      693
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Figure 7. Comparison of the deduced amino acid sequence of hTG$_P$ with other human TGases. HTGASEP, K, C, 3 and HFXIIIA represent hTG$_P$, TG$_K$ (Phillips et al. 1990), TG$_C$ (Gentile et al. 1991), TG$_E$ (Kim et al. 1993), and plasma Factor XIIIa (Ichinose et al. 1986), respectively. Bold type residues represent amino acids present in all sequences. Hyphens indicate identities with the amino acids of hTG$_P$. Dots represent gaps introduced for optimal alignment. Closed triangles indicate amino acids, mentioned in the text, important for the catalytic function of TGases.

In vitro transcription-translation of hTG$_P$ cDNA demonstrated that the core protein without any post-translational modifications behaves as a protein of approx. 77 kDa on SDS/PAGE, which is slightly larger than its 65 kDa rat DP1 (Seitz et al. 1991b, Ho et al. 1992). Results of Seitz and co-workers (Enderle-Schmitt et al. 1989, Seitz et al. 1990) suggested that a protein of about the same molecular mass, with cross-reactivity to an antibody against rTG$_P$, is present in human semen.

The putative hTG$_P$ protein contains seven N-glycosylation consensus sequences distributed over the entire protein. Four of these are at identical sites in hTG$_P$ and rTG$_P$. However, this does not necessarily result in glycosylation at these sites. Both TG$_C$ and FXIIIa contain several potential N-glycosylation sites, but no glycosylation has been observed (Ichinose et al. 1990, Greenberg et al. 1991). In the rat, TG$_P$ was reported to be glycosylated (Seitz et al. 1991a, Ho et al. 1992) and to contain a glycosylphosphatidylinositol (GPI) anchor which may protect the protein from autoaggregation (Seitz et al. 1991a). Until now, the identified GPI-anchored proteins were demonstrated to contain a hydrophobic sequence at their C-terminal sequence (Englund 1993). Based upon these results one would not expect a GPI-anchor for either rTG$_P$ or hTG$_P$, because of the lack of a hydrophobic stretch of amino acids at their C-terminus. It cannot be excluded, however, that for the attachment of the GPI-anchor to rTG$_P$ other sequence determinants might be used. It would be of interest to know whether hTG$_P$ is also glycosylated and has a protective GPI-anchor.

Androgens are essential for the growth and maintenance of prostate tissue and control the formation of prostatic secretions. Immunohistochemical and mRNA studies have shown that RatDP1 or rTG$_P$ is androgen-regulated (Ho et al. 1992, Steinhoff et al. 1994). The expression of rTG$_P$ was demonstrated to be restricted to the dorsal lobe of the prostate and the coagulation gland. Expression of the hTG$_P$ gene leads to an mRNA of approx. 3.5 kb

both in prostatic tissue and in the androgen-responsive prostate cancer cell line PC346C. The androgen-independent prostate cancer cell line PC-3 (results not shown) as well as the androgen-responsive prostate carcinoma cell line LNCaP showed no expression of hTG_P mRNA as determined by Northern-blot hybridization. As far as the tissues tested are concerned, hTG_P is exclusively expressed in the prostate. Presently we are examining a more extended number of tissues to confirm prostate-specificity of hTG_P expression. Androgen-dependent expression of hTG_P was observed in PC346C cells. The level increased 3-fold in the presence of 0.1 nM of the synthetic androgen R1881. The question remains open as to whether the androgen-up-regulated expression of hTG_P is due to a direct regulation at the transcriptional level or is a consequence of stabilization of the mRNA. Indirect evidence obtained by Ho et al. (1992) suggests that the latter is the case for rTG_P and that androgen acts through prolongation of the rTG_P mRNA half-live. At present, only a few human genes have been described, for example the prostate-specific genes hGK-1 and PSA, as being under androgenic control at the transcriptional level. Both hGK-1 and PSA contain androgen response elements in their promoters (Riegman et al. 1991a, 1991b). Kinetic studies of the androgen-regulated expression of hTG_P mRNA and isolation and characterization of the promoter region of hTG_P may elucidate at which level androgens regulate its expression. Additionally, comparison of the promoter regions of genes specifically expressed in the prostate may give some clues to tissue-specific regulatory elements.

Unlike hTG_P, TG_C is up-regulated by androgen withdrawal in the rat ventral prostate (Guenette et al. 1994b). However, this may be an indirect consequence of the induction of the apoptotic pathway by removal of androgens from the circulation.

Contrary to rTG_P, for which there is a very obvious function in the formation of the copulatory plug (Williams-Ashman 1984), little is known about the physiological role of hTG_P. Most likely the function of hTG_P is in semen. However, in contrast to PSA, for example, which is also excreted by the prostate, the deduced protein sequence does not contain an obvious signal sequence for excretion (Riegman et al. 1991a). The same is true for rTG_P (Ho et al. 1992). For this protein evidence has been obtained that secretion might occur via an alternative pathway (Steinhoff et al. 1994), which is also suggested for two other TGases, FXIIIa and TG_C (Aeschlimann and Paulsson 1994). Some effort has been made to obtain evidence for the presence and function of hTG_P in human semen. A report of Lilja and Laurell (1985) suggests that, in contrast to rat seminal clotting, TGase activity may not be essential for human semen coagulation. They hypothesized that, besides disulphide bridges in particular, non-covalent protein interactions are important for the clot structure. In addition, they did not find TGase activity in human semen, while Porta et al. (1986), demonstrated TGase activity in human seminal plasma of normal individuals. Others found only TGase activity in infertile patients with semen having prolonged liquefaction time (Enderle-Schmitt et al. 1989, Seitz et al. 1990). Besides a role of hTG_P in coagulation, it may function in suppressing the sperm surface antigenicity, thereby preventing an immunological response in the female genital tract. Suppression of antigenicity through the involvement of the cross-linking activity of TGase has been

reported for rat and rabbit, as well as for human sperm (Mukherjee et al. 1983, Manjunath et al. 1984, Paonessa et al. 1984). At last, an intracellular role of hTG_P cannot be excluded.
Presently, prostate cancer is the most common cancer in the male population of the U.S.A. and Europe. A main problem in the treatment of clinically localized prostate cancer is the presence of micrometastasis, at time of radical prostatectomy (Zincke et al. 1994). Monitoring the presence of prostate cancer cells in tissues and blood before and after treatment may offer the possibility to optimize treatment methods (Katz et al. 1995). Given its prostate-restricted expression, as suggested by our results, hTG_P may represent a valuable alternative to PSA as a marker for detection of prostate cancer cells, for instance by reverse-transcription PCR analysis.
We are interested in prostatic TGases and their possible role in the regulation of human prostate tumor growth and metastasis (TG_C) and their usefulness as differentiation or tumor markers (hTG_P) in prostate cancer. The isolation of the cDNA for hTG_P and some first clues to the specificity and regulation of its expression may help us to discriminate between both prostatic TGases, and to get more insight into the putative function(s) of the hTG_P protein. Currently, antisera against the C-terminal region of either hTG_P or TG_C are being generated to enable us to discriminate at the protein level as well.

Acknowledgements

We are indebted to Mrs. Sigrun Erkens-Schulze and Mrs. Wilma Teubel for cell culture work and RNA isolations, respectively, and Henri van Rooij and Jos Veldscholte for using their cDNA library filters and assistance during library screening. We would like to thank Dr. P. J. A. Davies for supplying us with TG_C cDNA.

Chapter 3

The Human Prostate-Specific Transglutaminase Gene (*TGM4*): Genomic Organization, Tissue-Specific Expression, and Promoter Characterization

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Genomics (1998): **51**, 434-444

Abstract

Human prostate-specific transglutaminase (hTG_P) is a cross-linking enzyme secreted by the prostate. In this study, we performed dot blot analysis of 50 normal human tissues to demonstrate unambiguously the prostate-specific expression of hTG_P. Furthermore, we elucidated the genomic organization of the *TGM4* gene, the gene encoding hTG_P. The structure of this gene displays striking similarity to that of other transglutaminase (TGase) genes. The *TGM4* gene spans approximately 35 kb of genomic DNA and consists of 13 exons and 12 introns. The main transcription initiation site is located 52 bp upstream of the translational start codon. A hTG_P splice variant of intron 1 was detected. This splice variant contains an in-frame antisense *Alu* element insertion. The *TGM4* promoter was analyzed by sequencing and transfection experiments. At positions -1276 to -563, the promoter harbors a cyclophilin pseudogene with 94% similarity to the cyclophilin A cDNA. Deletion mapping of the *TGM4* promoter in the transiently transfected human prostate cancer cell line PC346C showed comparable activity of 2.1-, 1.5-, and 0.5-kb promoter fragments.

Introduction

Human prostate-specific transglutaminase (hTG_P) belongs to a family of enzymes that all have the ability to catalyze the irreversible cross-linking of peptide-bound glutamine residues either with peptide-bound lysines or with primary amines [such as polyamines (Greenberg et al. 1991, Aeschlimann and Paulsson 1994, Dubbink et al. 1996)]. Transglutaminases (TGase, EC 2.3.2.13) can be found throughout the body, but each TGase type is characterized by its own typical tissue distribution, which can be highly tissue-specific or without any tissue restriction at all. At present six distinct human TGases have been described. These include plasma factor XIIIa (Ichinose et al. 1990), keratinocyte TGase [TG_K (Rice et al. 1992)], epidermal TGase [TG_E (Kim et al. 1993)], tissue-type or cellular TGase [TG_C (Gentile et al. 1991)], the catalytically inactive TGase-like erythrocyte membrane protein band 4.2 (Korsgren et al. 1990), and hTG_P (Dubbink et al. 1996).
In contrast to the other types of TGases, little is known about the physiological function of prostatic TGase. Most information on this TGase type has been obtained from studies on rat prostatic TGase (rTG_P or dorsal protein 1, DP1). DP1 is an abundantly expressed secretory protein of the dorsal prostate, the coagulating gland, and the lateral type 1 prostate of the rat (Wilson and French 1980, Romijn 1990, Hayashi et al. 1991). DP1 expression is androgen-regulated as has been demonstrated *in vivo* by castration and androgen replacement experiments (Ho et al. 1992). Also in explant culture studies, the expression of DP1 can be induced by a broad range of natural and synthetic androgens (Lopes et al. 1996). The enzyme is believed to be responsible for the cross-linking process during the formation of the copulatory plug after mating (Williams-Ashman 1984).
The function of hTG_P, which is expressed at a considerably lower level than DP1, has not been unambiguously established. Whereas a role in semen coagulation cannot be excluded, other functions of hTG_P, related to the appropriate sperm transfer to the female, have been

proposed. These include the suppression of sperm cell immunogenicity and maturation, which could be achieved by modifying the surfaces of the male gametes with seminal plasma components, e.g., polyamines and the human uteroglobin analogue, the Clara cell 10-kDa protein (Manjunath et al. 1984, Porta et al. 1986, Mantile et al. 1993). With the cloning of the hTG_P cDNA, several characteristics of the enzyme could be resolved; additionally hTG_P mRNA expression could be monitored (Grant et al. 1994, Dubbink et al. 1996). The cDNA encodes a protein of 77 kDa, and its deduced amino acid sequence shares a 53% similarity with DP1. The overall identity with the other human TGases is approximately 30%, with the highest similarity in the active site region.

hTG_P mRNA with a length of 3.5 kb could be demonstrated in prostatic tissues and in the human prostate cancer cell line PC346C, but was undetectable in other human prostate cancer cell lines (Dubbink et al. 1996). Evidence that hTG_P expression is limited to the prostate and can be stimulated by androgens has been obtained (Dubbink et al. 1996). With the purpose of studying the regulatory mechanisms underlying hTG_P expression, we isolated the *TGM4* gene, the gene encoding hTG_P, and its promoter.

The complete genomic structures of most other human TGases have been described (Ichinose and Davie 1988, Korsgren and Cohen 1991, Phillips et al. 1992, Kim et al. 1994a, Fraij and Gonzales 1997). Although the sizes of the various TGase genes range from approximately 14 kb [*TGM1*, the gene encoding TG_K (Kim et al. 1992)] to over 160 kb [*FXIIIa* (Ichinose and Davie 1988)], their basic organization and localization of introns are largely the same. In this study, we further confirmed the prostate specificity of hTG_P and present the *TGM4* gene structure and its 5'-flanking sequences. Functional activity of the promoter region was demonstrated after its transfection to PC346C cells.

Materials and Methods

Screening of a human genomic cosmid library

A cosmid library was prepared by subcloning a partial *Mbo*I human genomic DNA digest from peripheral blood lymphocytes into the *Bam*HI site of cosmid vector pTCF (Pan et al. 1994). The genomic library was not amplified before use. Clones ($1.2x10^6$) were screened with a random ^{32}P-labeled 2.2-kb *Sac*I-*Hpa*I hTG_P cDNA fragment (Dubbink et al. 1996). For selection of cosmid clones containing the *TGM4* promoter region, a ^{32}P-labeled 184-bp hTG_P cDNA fragment corresponding to nucleotides 42-225 was used (Dubbink et al. 1996). Rescreening of the same library with a 500-bp *Nsi*I-*Nsi*I fragment, isolated from one of the selected cosmid clones, containing the 5'-untranslated region (UTR) of the hTG_P cDNA and the proximal promoter of the *TGM4* gene, was performed to obtain a larger segment of the *TGM4* promoter. Filters were prehybridized for 2 h in a mix containing 5 µg/ml denatured pTCF. Hybridizations and wash steps were performed at 65°C, as previously described (Dubbink et al. 1996). Cosmid DNA from purified clones was isolated following standard methods (Sambrook et al. 1989) or with a Qiagen plasmid isolation kit (Qiagen, Hilden, Germany). Clone inserts were characterized by standard restriction digestion followed by Southern blotting and hybridization with ^{32}P-labeled hTG_P cDNA probes spanning the

entire coding region. Finally, the *TGM4* exon contents of the selected clones were determined by intronic PCR amplification as described below.

Exon-intron structure of the TGM4 gene

Oligonucleotides were chosen based upon the structural similarities between the other known TGase genes. PCR amplifications of cosmid DNA were performed with 1-5 U of Goldstar DNA polymerase (Invitrogen Corp., San Diego, CA), depending on the size of the fragment to be amplified, in the presence of 100 ng of each primer in a final volume of 50 µl. All samples were first denaturated at 94°C for 5 min. Amplification followed in 35 cycles of 94°C for 1 min; 60°C for 1 min; 72°C for 2 min; and with a final extension at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis and hybridization using the entire hTG_P cDNA as probe (Dubbink et al. 1996). The intron sizes were determined by subtraction of exon sequences from the corresponding fragments amplified on genome DNA. The size of intron 1 was assessed by a combination of restriction enzyme analysis and hybridization experiments.

Cloning of PCR fragments was performed with a TA Cloning kit (Invitrogen) into the pCR2.1 vector by a shotgun procedure or by using PCR products isolated from gel according to the manufacturer's protocol. Appropriate clones were selected based upon insert sizes. Sequence analysis was performed by dideoxy nucleotide chain termination using a T7 sequencing kit (Pharmacia, Uppsala, Sweden).

5'-Rapid amplification of cDNA ends

The transcription initiation site was determined by 5'-rapid amplification of cDNA ends (5'-RACE) of human prostate Marathon-Ready cDNA (Clontech, Palo Alto, CA) with three gene-specific primers, HPSTG 2 [702-684, numbering according to the published hTG_P cDNA sequence (Dubbink et al. 1996)], 5'-TCATAGCACACATGGCCCT-3'; HPSTG 16 (322-303), 5'-AGGGTTGCCTGCCAGTTGTA-3'; and HPSTG 17 (548-529), 5'-CACGTAATGGCAGCCCGTGT-3' in combination with adaptor primer 1 (AP1, Clontech). PCR was performed as described above. Products obtained were analyzed by agarose gel electrophoresis and subsequent Southern blotting and hybridization with the exon I-specific oligonucleotide HPSTG 13, (2-22) 5'-GAGATAGAGTCTTCCCTGGCA-3', and reprobed with primer HPSTG 34 (see Results), 5'-GTTACTCGGGAGACTGAGGT-3'. Oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase (Sambrook et al. 1989). Hybridizations were performed in 5x SSC/1% SDS (1x SSC:0.15 M NaCl/0.015 M sodium citrate). The final wash step was performed in 0.1x SSC/0.1% SDS. Both hybridizations and washes were performed at 37°C. The amplified fragments obtained with primer HPSTG 16 were cloned in pCR2.1. Twelve clones were partly or entirely sequenced as described above.

Isolation of the TGM4 gene promoter and promoter constructs

A 3.0-kb *Pst*I-*Pst*I fragment of one of the selected cosmid clones, which hybridized with exon I primer HPSTG 13 and contained the 5'-flanking region of the gene, exon I, and part of the first intron of the *TGM4* gene, was subcloned into the *Pst*I site of pBluescript SK

(Stratagene, La Jolla, CA). The promoter region present in this fragment was entirely sequenced using a double-stranded nested deletion kit and the T7 sequencing kit (Pharmacia). For the identification and classification of repetitive sequences, the CENSOR program was used (http://charon.lpi.org/~server/).

TGM4 gene promoter constructs were prepared as follows. A *Bam*HI site was introduced at the translational start site by PCR amplification using primers 5'-CATGCCTAGCTGGATTGCAGAG-3' and 5'-CGTAGGATCCCTTCAGATTCTCTCCTG-3' (46-26). The PCR product was digested with *Bam*HI and *Bgl*II, yielding a product of 560 bp, which was then subcloned into the *Bgl*II site of pGL3-basic (Promega, Madison, WI) in front of the luciferase reporter gene. This construct, pTGM4-0.5Luc (-510 to +54), was checked by double-stranded sequence analysis. Two longer constructs were prepared by subcloning a *Pvu*II-*Bgl*II fragment and a *Sma*I-*Bgl*II fragment, derived from the above-described construct containing the 3.0-kb *Pst*I-*Pst*I fragment, into pTGM4-0.5Luc digested with *Sma*I and *Bgl*II. This resulted in the constructs pTGM4-1.5Luc (-1508 to +54) and pTGM4-2.1Luc (-2077 to +54), respectively. Plasmid isolations were performed with a Qiafilter plasmid isolation kit (Qiagen).

Cell culture, transient transfections and luciferase assay

PC346C cells were cultured in a Dulbecco's modified Eagle's medium (DMEM)/F12-based growth medium (Gibco, Grand Island, NY), as used previously (Dubbink et al. 1996). The cells were transfected according to the calcium phosphate method essentially as described by (Chen and Okayama 1987), using $1x10^6$ cells per 25-cm^2 flask and 5 µg of the appropriate construct. After a 4-h incubation with the precipitate, the culture medium was replaced by 15% glycerol in PBS for 90 s at room temperature. Subsequently, transfected cells were incubated in culture medium for 24 h. Transfections were performed at least three times with two independent plasmid isolates.

Cells were harvested in 400 µl lysis buffer, composed of 25 mM Tris-phosphate, pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 15% glycerol. Subsequently, 100 µl luciferin [0.25 µM (Sigma, St. Louis, MO)]/ 0.25 µM ATP was added to 150 µl cell lysate, and luciferase activity was measured in a LUMAC 2500 M Biocounter (LUMAC, Landgraaf, The Netherlands).

Northern hybridization

To analyze the expression of hTG_P mRNA in normal human tissues, a human RNA master blot (Clontech) containing poly(A)$^+$ mRNA of 50 different tissues was used. Under conditions recommended by the manufacturer, hybridization was performed with a ^{32}P-labeled 184-bp fragment of hTG_P cDNA corresponding to nucleotides 42-225 (Dubbink et al. 1996). Final washes were performed under high-stringency conditions (up to 0.1x SSC/0.1% SDS at 65°C).

RNA isolation and RT-PCR

Total RNA from prostatic tissue specimens was isolated by the LiCl/urea method as described by Auffray and Rougeon (1980). Subsequent first-strand cDNA synthesis was

performed at 42°C on 0.5 μg RNA using 25 U M-MLV reverse transcriptase (Perkin-Elmer Cetus, Norwalk, CT) and random hexamer primers in the presence of 5 mM $MgCl_2$. PCR was carried out essentially as described above. Primers used were HPSTG 13 and HPSTG 14 (145-125), 5'-GGACTGCTCGTTTGGAACTCC-3'. PCR products were separated on a 1% agarose gel and cloned in pCR2.1. Selected clones were entirely sequenced on both strands as described above. For determining the exon-intron boundaries of the described splice variant, a 4.5-kb *Eco*RI-*Eco*RI fragment of one of the selected cosmid clones, containing part of exon II and intron 1, was subcloned into the *Eco*RI site of pGEM-7Zf(+) (Promega). Subsequent sequence analysis of the splice boundaries was performed with primers designed from the spliced sequence. The position of the spliced sequence in intron 1 was determined by PCR analysis of selected cosmid clones as well as by analysis of the construct containing the 4.5-kb *Eco*RI-*Eco*RI fragment described above with primers designed from exons I and II in combination with the primers designed from the spliced sequence.

Results

Exon-Intron junctions and intron sizes of the TGM4 gene

Screening of a human genomic cosmid library with two hTG_P cDNA fragments (see the Materials and Methods) yielded clones 1 and 24.1.3 (Figure 1). Restriction mapping and subsequent Southern hybridization with the exon I-specific oligonucleotide HPSTG 13 showed that clone 24.1.3 contained approximately 650-bp *TGM4* promoter sequences. To obtain a larger part of the promoter, a 500-bp *Nsi*I-*Nsi*I fragment from cosmid clone 24.1.3, comprising the proximal *TGM4* promoter and most of exon I, was used for rescreening of the genomic library. This led to the isolation of cosmid clone 3.1.1 (Figure 1). Combined, the three selected clones cover the whole *TGM4* gene and a substantial part of the 5'-flanking region.

These clones were used to determine the intron sizes and localizations. Based upon the assumption that the *TGM4* gene had a genomic organization similar to that of other identified TGase genes, specific primer pairs were designed in the predicted neighboring exons. All introns (Table 1), except intron 1, could be PCR amplified. Intron-containing amplified fragments were subcloned, and each exon-intron boundary was sequenced. The length of intron 1 was determined by restriction mapping, and the sequences of its exon-intron junctions were resolved by direct sequence analysis of cosmid clone 24.1.3. All exon-intron junctions conformed to the gt/ag rule of splice donor/acceptor sites [Table 1 (Shapiro and Senapathy 1987)]. Figure 1 and Table 1 show that the *TGM4* gene spans approximately 35 kb of genomic DNA and basically consists of 13 exons and 12 introns. The exons range in size from 63 to 279 bp; the large exon XIII (1026 bp) includes the 3'-UTR. Exon I encodes 5'-UTR information and the first six amino acids of the hTG_P protein. The intron sizes vary from 94 bp (intron 6) to approximately 10.5 kb (intron 1).

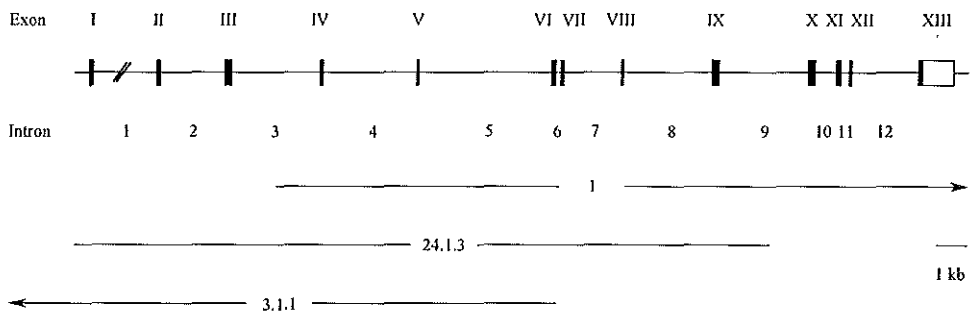

Figure 1. Structural organization of the *TGM4* gene. The 5'- and 3'-untranslated regions are indicated as open boxes, and the coding exons are indicated as black boxes. The introns are represented by the lines between the exons. The positions of the cosmid clones are shown at the bottom. With the exception of intron 1, the drawing is to scale.

Table 1. Exon-Intron Structure and Splice Junction Sequences of the *TGM4* gene.

Intron	cDNA position	Splice donor	Intron length (kb)	Splice acceptor
1	63	GCATCAAAAGgtgagtgggt	10.5	ttgctgacagAGCTGCAAGT
2	237	TTCAGCACAGgtgaagcctc	2.0	ggtgtctcagGGCCGAATCC
3	474	TGGTGTAAAGgtcatgtgaa	2.6	cttgcttcagAGGACATGGT
4	593	CTTTGGTCAGgtaacgattt	2.8	cccaaaatagTTTGAGAAAA
5	701	GTGTGCTATGgtaggtatgg	4.6	tgtgtggcagATGAGCTTTG
6	876	CTGACTACAGgtaagtggca	0.1	atcatttcagTGCTGAGAGC
7	1015	ACTCTGTCTGgtagggttcc	1.9	tttgcggcagGAATTTCCAT
8	1119	CGAAGCCAGGgtgagtgggt	2.7	ttcccctcagGTGTCTTCTG
9	1371	TATCCAGAAGgtgctagcat	2.7	tctctttcagGCTCCTCTGA
10	1650	CAAGGTCAAGgtaccagaac	0.6	tctttggcagTATCAGAAGT
11	1820	CTCTATAGAGgtgagcttcc	0.2	cctactacagTTGCCTAACA
12	1957	CTGACCATGGgtgagtctgc	2.1	aattttccagGACGGTGCAG

Note The capital letters represent coding sequences and lower case letters represent intron sequences. cDNA positions are according to Dubbink et al. (1996).

Identification of a major transcription start site

The transcription start site of the *TGM4* gene was identified by 5'-RACE using Marathon-Ready human prostate cDNA. For this purpose three antisense gene-specific primers, HPSTG 2, HPSTG 16, and HPSTG 17, were chosen in combination with anchored primer AP1, as schematically drawn in Figure 2A. The most abundant bands obtained by PCR amplification and subsequent Southern blot analysis with exon I-specific oligonucleotide

HPSTG 13 are approximately 760, 380, and 600 bp in size, respectively, suggesting that the transcription start site is located close to the 5'-end of the published hTG_P cDNA sequence [(Grant et al. 1994, Dubbink et al. 1996) see also Figs. 2A and 2B].
Twelve independent clones obtained from the primer HPSTG 16 PCR products were sequenced. Figure 2D shows the 5' sequences of these clones and the frequency at which they occurred. We determined the major transcription start site, found in five of the clones, at the C residue eight nucleotides upstream of the AG 5' end of the published hTG_P cDNA sequence (Dubbink et al. 1996, and Figure 2D), i.e., 52 bp upstream of the translational start codon.

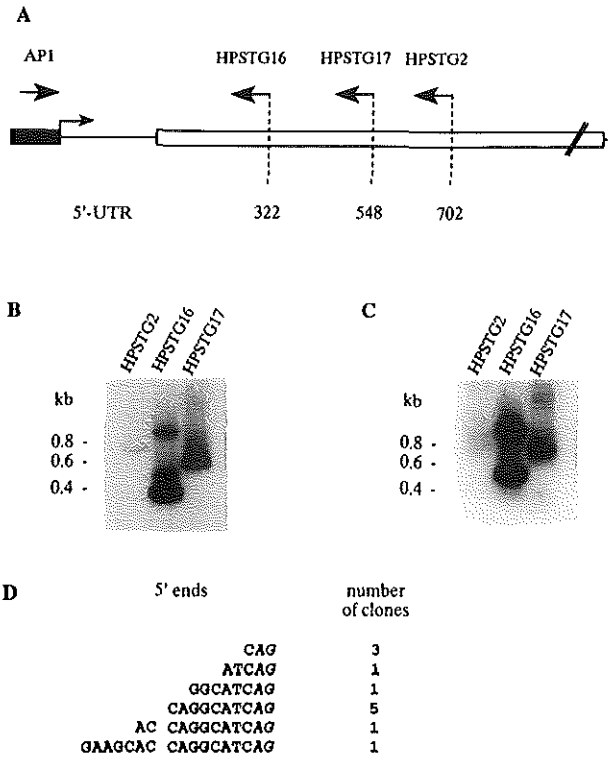

Figure 2. Determination of the transcription initiation site. (A) The transcription initiation site was determined by performing 5'-RACE on Marathon-Ready human prostate cDNA. The positions of the gene-specific primers HPSTG 2, HPSTG 16, and HPSTG 17 are indicated and numbered according to the published cDNA sequence (Dubbink et al. 1996). The adaptor, which is ligated to the cDNA ends by the manufacturer, is indicated as a black bar. AP1 is adaptor primer 1. The open bar represents the hTG_P cDNA open reading frame. (B) Southern blot analysis of the 5'-RACE products with exon I-specific oligonucleotide HPSTG 13. (C) Reprobing the same blot with *Alu*-specific primer HPSTG 34. (D) The 5' sequences of 12 5'-RACE products and the number of clones containing this sequence. The two nucleotides in italic letters indicate the 5' nucleotides of the previously published hTG_P cDNA sequence (Dubbink et al. 1996).

A

```
     yyyyyyncag G   splice acceptor consensus

1    tttttttag AGTTGAGGTC TCACTTTGTT GCCCAGGCTG GCGTGTTGTG GCTATTCACA
61   GCTGCAATCA TAGCTCACTG CAGCCTCAAA CTCCTGGGCT CAAGTGATTC TCCCACCTCA
121  GTCTCCCGAG TAACCGGGAT TACAGgtatg tgact

     splice donor consensus   AGgtrag t
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B

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1    AGAGATAGAGTCTTCCCTGGCATTGCAGGAGAGAATCTGAAGGGATGATGGATGCATCAAAAGAGT
1                                                  M  M  D  A  S  K  E  L

67   TGAGGTCTCACTTTGTTGCCCAGGCTGGCGTGTTGTGGCTATTCACAGCTGCAATCATAGCTCACT
9      R  S  H  F  V  A  Q  A  G  V  L  W  L  F  T  A  A  I  I  A  H  C

133  GCAGCCTCAAACTCCTGGGCTCAAGTGATTCTCCCACCTCAGTCTCCCGAGTAACCGGGATTACAG
31     S  L  K  L  L  G  S  S  D  S  P  T  S  V  S  R  V  T  G  I  T  E

199  AGCTGCAAGTTCTCCACATTGACTTCTTGAATCAGGACAACGCCGTTTCTCACCACACATGGGAGT
53     L  Q  V  L  H  I  D  F  L  N  Q  D  N  A  V  S  H  H  T  W  E  F
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Figure 3. Alternative splicing of intron 1 of the *TGM4* gene. (A) Nucleotide sequence of the intron 1 *Alu* sequence and the exon-intron boundaries that are involved in alternative splicing of intron 1. Consensus splice donor and acceptor sequences are indicated. N, any nucleotide; Y, pyrimidine; R; purine (Shapiro and Senapathy 1987). The alternative exon sequence is shown in capital letters, and the intron sequences are shown in lowercase letters. The splice consensus ag and gt residues are shown in boldface type. (B) Nucleotide and deduced amino acid sequence of the alternative spliced hTG$_P$ cDNA. The *Alu* insertion is underlined.

A hTG$_P$ splice variant

During the experiments for the identification of the transcription start site, several extra bands became visible after hybridization with exon I primer HPSTG 13 (Figure 2B). Using primer HPSTG 16 for 5'-RACE, which should lead to the smallest PCR products, in addition to the 380-bp fragment, bands of 550 bp and 1 kb were present. Sequence analysis of the entire 380- and 550-bp fragments showed that the largest band contained an in-frame insertion of 135 bp at cDNA position 63, the site where we determined the position of intron 1 (Table 1, Figs. 3A en B). The inserted sequence was part of an *Alu*-J repeat (Jurka and Smith 1988). We did not further study the 1-kb fragment. However, reprobing the blot with primer HPSTG 34, a primer designed from the *Alu* sequence, demonstrated that the 1-kb band also contained this sequence. The *Alu* repeat was also found to be present in larger fragments produced with primers HPSTG 2 and HPSTG 17 (compare Figs. 2B and 2C). The *Alu* repeat was also present in hTG$_P$ cDNA from prostatic tissues as deduced from RT-PCR with primers designed from exon I and II (data not shown). The 135-bp spliced *Alu* sequence was localized within a 4.5-kb *Eco*RI-*Eco*RI fragment of cosmid clone 3.1.1, the

A

	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cerebellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	sub-thalamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	
H	yeast total RNA 100 ng	yeast tRNA 100 ng	*E. coli* rRNA 100 ng	*E. coli* DNA 100 ng	Poly r(A) 100 ng	human C_0t1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

B

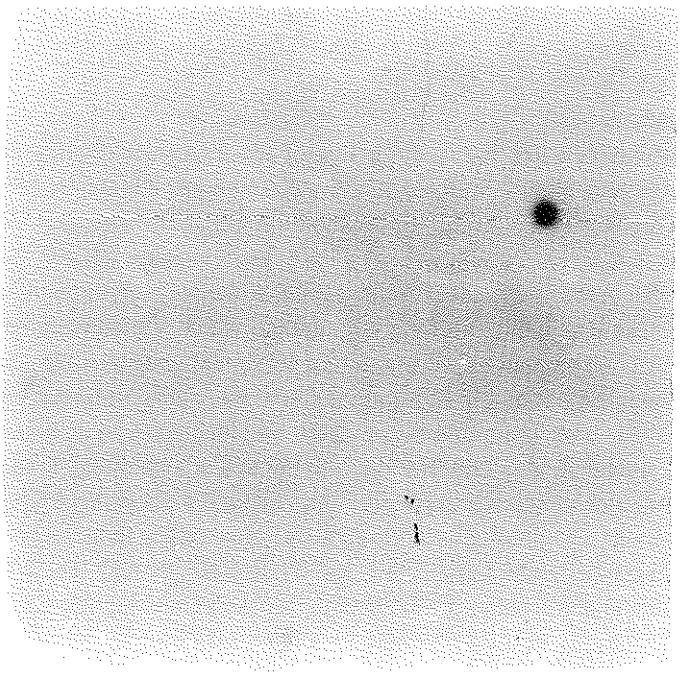

Figure 4. Dot blot analysis of hTG_P expression in human tissues. (A) Loading pattern of the human RNA master blot used. (B) Result of the dot blot analysis. The hybridization was performed under high-stringency conditions (see Materials and Methods) with a 184-bp hTG_P cDNA fragment corresponding to nucleotides 42-225 of its previously published sequence (Dubbink et al. 1996). Each spot contained 85-542 ng of poly(A)$^+$ mRNA.

only band hybridizing with primer HPSTG 34. Sequence analysis of this genomic fragment with primers designed from the *Alu* sequence showed that the 135-bp *Alu* sequence is located within a 163-bp *Alu* repeat. The splice junctions conform to the gt/ag rule and nearly perfectly match the splice donor and acceptor consensus sequences (Figure 3A). PCR analysis of cosmid clones 24.1.3 and 3.1.1 as well as the 4.5-kb *Eco*RI-*Eco*RI fragment with *Alu* primers in combination with an exon II primer demonstrated that the *Alu* sequence is localized approximately 3.1 kb upstream of exon II in the orientation opposite to that of the *TGM4* gene (Figure 5B).

Prostate-specific expression of hTG_P

The results from a previous study on a limited number of tissues suggested that hTG_P is prostate-specifically expressed (Dubbink et al. 1996). To analyze the hTG_P expression in a larger series of normal human tissues, a human RNA master blot, containing poly(A)$^+$ mRNA from 50 different tissues (Figure 4A), was hybridized with a hTG_P cDNA probe that was chosen from a region with very low similarity to other TGases. Figure 4B shows that hTG_P expression is restricted entirely to the prostate. The faint signals present in the kidney mRNA and the *Escherichia coli* DNA spots seem to represent nonspecific reactivity. RT-PCR analysis of kidney mRNA could not confirm expression in the kidney (data not shown).

Structural and functional analysis of the TGM4 gene promoter

To analyze the promoter region of the *TGM4* gene, a 3.0-kb *Pst*I-*Pst*I fragment of cosmid clone 3.1.1, which hybridized with exon I primer HPSTG 13, was isolated. It contained approximately 2.1 kb of the 5'-flanking region of the gene, exon I, and the beginning of intron I. The sequence of the promoter region is shown in Figure 5A. The 5'-UTR cDNA sequence is completely present in this genomic fragment, indicating the absence of more upstream exons. Computer analysis revealed that the *TGM4* proximal promoter harbors a CATAA motif, 49 bp upstream from the major transcription start site, but no typical CCAAT box. Just upstream of the CATAA motif, at positions -96 to -87, the promoter contains an Sp1 binding site. Comparison of the sequences obtained from the proximal promoter regions present in cosmids 24.1.3 and 3.1.1 showed a T/G polymorphism at position -127.

A processed cyclophilin A pseudogene with 94% similarity to cyclophilin A cDNA (Haendler et al. 1987) was present at position -1276 to -563. Because of the absence of a correct start codon (position -1263, Figure 5A), and the interruption of the open reading frame by several deletions and insertions, this gene cannot lead to a functional protein product. Further analysis of the *TGM4* promoter showed 132 bp of an *Alu* repeat sequence (-1452 to -1320) as well as part of a *LINE-1* repetitive element (-1720 to -1492, Figs. 5A

A

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-2077     CTGCAGA ACAGCAAAAA CCTCTGACTT CATTATTGTT ATTCAGAGTA ACCAGGGTCA TTGCTCTCTG GCTTGCTTAA
-2000  AGCCAGCAAA GGCAATTGCC CATCCCTGGG CTTTTGGTCT GGAAGAGACC GTTGGCATCA TTATTCAGCC CCCTCACTTT
-1920  TCAGGTAAGG AGCTAATGAA GACAACATTC CCAGTGACAC ACGGCAAGTT ACTGTGGTGT TGGGGTTTCA GTGTCAGGAG
-1840  TCTCCTCTCT TCCTCTGGCA TTTCTGCTAC TATGCCAGGC TGGCTCCTTT CAG1AGACAT AACATCATTG AAAAATACCA
-1760  CAAGTGCCCC TGTGGAGAGC AGAGCAGATA GCCATTAAAA TTACAAAAGC TTATGCTTTT TGGCCCAACA TTTCACTTCA
-1680  AGGCATTCAA TTCTTCCTAT AGATACACTC ATGTGAGGGT AAAATGATCT CTGTATAACC TTATTCTTTG CAACACTGAT
-1600  TGTAATAAGG AAATATTGGA AGCAACCCAG ATGCCTATGG GTGGGAGGTG AGTTACATAA ATTAGGGACC ATCCACACAA
-1520  ACAGAATATG TGCAGCTGTG AAAAGACTGG GCCACCTCTC CATGTGCAAT AGGAACAGCT CCATCTTGCC AGGCGTGGTG
-1440  GCTCACACCT GTAATCCCAG TACTTTGGGA GGTGAAGGTG GGGCGGATCC CTTGAGCCTA GGCATTCCAG ACCAGCCTGG
-1360  GCAATGCAGT GATATGCTCT CATTGTGTAA ATAAAACAAA ATAATACATT ATTTGCTTTG CAGATGCCAC TGCCGCCAGA
-1280  GCCCTGTAAC ATCAGCCATC GTCAACCCCA CCGTGTTCTT CAACATCACC GTTGACGGCA AGCCTTTAGA CCTGCGTCTC
-1200  CTTCAAGCTG TTTGCAGACA AGGTTCCAAA GCCAGCAGAA AACTTTTGTG CTCTGAGCAC TGGAGAGAAA GGATTTGGTT
-1120  ATAAGAGTTC CTGCTTTCAC AGAATTATTC CAGGGTTTAT GTGTCAGGGT GGTGACTTCA TACGCCATAA TGGCACTGGT
-1040  GGCAAGTCCA TCTATGGGGA GAAATTTGAT GATGAGAACT TCATCCTAAA GCATACAGGT CCTGGCATGT TGTCCATGGC
-960   AAATGCTGGA CCCAATACAA ATGGTTCCCA GTTTTTAATC TGCACTGCCA AGACTGAGTG GTTGGATGGC AAGCTTGTGG
-880   TCTTTGGCAA GGTGAAGGCA TGAATATTGT GGAGGCCATG GAGTGCTTTG TGTCCAGGAA TGGCAAGACC GGCCAGAAGA
-800   TCACCATTGC TGACTGTGGA CAGCTCTTAT AAGTTTGACT TGTGTTTTAT CTTAACCACC AGACCATTCC TTCTGTAGCT
-720   CAGGGGAGCA CCCTCCACCC CATTTGCTCC CAGTATCCTA GAATCTTTGT GCTCTCGCTG CGGTTCCCTT TGGGTTCCAT
-640   GTTTTCCTTG TTCCCTTCCA TGCCTAGCTG GATTGCAGAG TTAAGTTTAT GATTATGAAA TAAAAACTAA ATAACAAAAT
-560   AATAATAATA GTACATTATT TGCTTCTCAG TGCATACAAC ATCTCTGGAA AGATCTAAAA GAAACTGGTA GTAGGATTCA
-480   ACAAGGTGGA CAGGGGGCAT TTTTTTCCAT TTTGAACCAT GTGGATGCAT TATATATTCA AAATGTAAAA CAAAAAACTC
-400   ACAAGCATCT CTTACTTTTT CAACTTGAAG AAAAGAGAAG AAGAAGAAAA CCCTAAAGTC AGTAAAGGTT AGCCTGGGTT
-320   CCTAGACTTA AGCTTGATAG TAACCAGAAT GTCAGGCCAC ATGTGGTGTA GGTCATAGGG TCCTGGCTTT TGGTCACCCA
-240   CTGTTAGGCT CATATAACAG ACAAGGAGGT GCCATTGTCC GTTGTCTGTC CCTTTTACAT CAGCTCATCT TGGCTCCTGA
-160   TTTCCTGGGT CTGATATGGA AGATTCCGGG ATTTTATTAT TTCTAATCAA CATCGCCTTC CCAAACCCCG CCCCTTGGCA
                                    G
-80    GCCATAGCAA GGCCACATCT AGCCCAGGAT CACATAAAAA GGGCTGTTTC CTCCCCTGAG GACCGACTGT GTGGAAGCAC
+1     CAGGCATCAG AGATAGAGTC TTCCCTGGCA TTGCAGGAGA GAATCTGAAG GGATGATGGA TGCATCAAAA Ggtgagtggg
                                  Exon I                                          Intron 1
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B

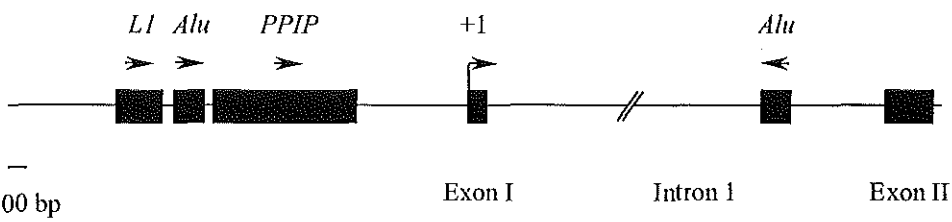

Figure 5. The 5'-region of the *TGM4* gene. (A) Nucleotide sequence of the *TGM4* promoter with adjacent exon I and part of intron 1. The experimentally determined major transcription start site (+1) is indicated by a double-underlined boldface type letter. The CATAA box (-48) is shown in boldface type and is underlined. The translational start sites are shown in boldface, italics. The first nucleotides of intron 1 are represented by lowercase letters and the splice consensus gt residues in boldface type. The *PPIP* pseudogene is underlined and its mutated start site is double-underlined. The *Alu* and *LINE-1* repeats are shown in italics. At position -127, a T/G polymorphism is present. (B) Schematic presentation of the localization and orientation of the *PPIP* pseudogene, the *Alu* repeats, and the *LINE-1* repeat (L1) in the *TGM4* promoter and intron 1. With the exception of intron 1, the drawing is to scale.

and 5B). The *Alu* repeat corresponds to the *Alu*-J subtype and the *LINE-1* repeat to the L1ME3A subtype (Jurka and Smith 1988, Smit et al. 1995).
For functional analysis of the *TGM4* gene promoter, three promoter constructs, pTGM4-0.5Luc, pTGM4-1.5Luc, and pTGM4-2.1Luc, were generated, containing 0.5, 1.5, and 2.1 kb of the promoter, respectively. pTGM4-0.5Luc contains the proximal promoter, pTGM4-1.5Luc additionally contains the entire *PPIP* gene and the *Alu* repeat, and pTGM4-2.1 includes these sequences as well as the *L1* repeat and more distal promoter information (Figure 6). Transient transfections were performed to the human prostate cancer cell line PC346C, the only *in vitro* growing prostatic cell line in which endogenous hTG_P expression has been demonstrated so far (Dubbink et al. 1996). The results presented in Figure 6 show that pTGM4-0.5Luc harbors a functional promoter. The two other constructs containing a larger part of the *TGM4* promoter show the same level of activity.

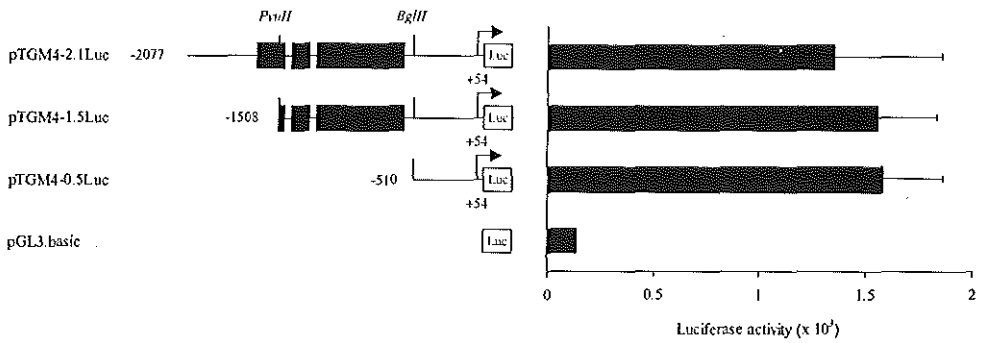

Figure 6. Functional analysis of the *TGM4* promoter. PC346C cells were transiently transfected with the *TGM4* promoter constructs pTGM4-0.5Luc, pTGM4-1.5Luc, and pTGM4-2.1Luc, as described under Materials and Methods. (Left) Schematic representations of the constructs. The promoter fragments were subcloned in front of a luciferase reporter gene using the indicated restriction enzymes as described under Materials and Methods. The *PPIP* pseudogene and the repetitive elements are indicated as black bars (see also Figure 5B). Standard errors of three experiments are indicated.

Discussion

In this report we describe the organization of the *TGM4* gene and identified 2.1 kb of its promoter. This completes the isolation of all currently known human TGase-encoding genes.
The *TGM4* gene is about 35 kb in length and is contained in 13 exons and 12 introns. There is a striking structural similarity between the *TGM4* gene and the other TGase genes including the gene for band 4.2 (Table 2). Only small variations occur in the size of corresponding exons, with the exception of the exons encoding the 5'- and 3'-UTRs. Whereas there is a huge variation in intron sizes and thus in gene sizes (varying from 14.1 to over 160 kb), the number of introns and their localization are largely the same for the

Table 2. Intron and exon sizes of the TGase gene family

	Intron size[a]					Intron size	
Intron	TGM4 (35)[a]	TGM2[b] (32.5)	TGM3[b] (42.8)	Band 4.2[b] (20)	Intron	TGM1[b] (14.1)	FXIIIa[b] (>160)
					1	0.7	nd[c]
1	10.5	2.6	13.4	6.5	2	0.2	nd
2	2.0	5.1	0.3	0.9	3	1.0	nd
3	2.6	1.5	0.6	0.6	4	0.4	nd
4	2.8	2.9	1.8	0.3	5	0.1	nd
5	4.6	1.1	1.3	0.9	6	0.5	nd
6	0.1	4.6	0.1	0.3	7	0.4	nd
7	1.9	0.7	8.6	0.7	8	0.1	nd
8	2.7	1.6	2.2	0.5	9	2.2	nd
					10	0.5	nd
9	2.7	1.0	4.0	0.4	11	0.1	nd
10	0.6	5.4	3.3	2.6	12	0.1	nd
11	0.2	1.1	4.0	2.2	13	0.4	nd
12	2.1	0.9	0.5	3.1	14	4.6	nd

	Exon size[a]					Exon size	
Exon	TGM4 (35)[a]	TGM2[b] (32.5)	TGM3[b] (42.8)	Band 4.2[b] (20)	Exon	TGM1[b] (14.1)	FXIIIa[b] (>160)
					I	89	63
I	63	144	48	?[d]	II	321	148
II	174	180	174	186	III	189	189
III	237	243	240	234	IV	249	252
IV	119	119	119	119	V	119	119
V	108	129	129	105	VI	108	108
VI	175	178	178	178	VII	175	175
VII	139	136	136	139	VIII	139	139
VIII	104	104	104	104	IX	104	104
					X	89	89
IX	252	243	246	243	XI	154	154
X	279	273	309	300	XII	282	288
XI	170	161	158	161	XIII	161	161
XII	137	137	134	134	XIV	137	137
XIII[e]	1026	1209	645	?	XV	403	1688

[a] Gene and intron size is given in kb, the exon size in bp.
[b] Data obtained from Ichinose and Davie 1988, Korsgren and Cohen 1991, Kim et al. 1992, 1994a, Phillips et al. 1992, Yamanishi et al. 1992, and Fraij and Gonzales 1997.
[c] Intron sizes of the *FXIIIa* gene are not determined (nd) yet.
[d] Exon I of the *band 4.2* gene is subjected to alternative splicing.
[e] Includes 3'-UTR.

different TGase genes. The intron phases (Sharp 1981) of corresponding introns are identical in all characterized TGase genes (Ichinose and Davie 1988, Korsgren and Cohen 1991, Phillips et al. 1992, Kim et al. 1994a, Fraij and Gonzales 1997). Comparison of their organization shows that the TGase genes can be divided into two subclasses. The *TGM2*, *TGM3*, *TGM4*, and *band 4.2* genes all encompass 13 exons and 12 introns. The *FXIIIa* and *TGM1* genes contain two extra exons. Exon IX of the former group is separated into two exons, X and XI, and their larger 5' untranslated region is encoded by two exons, I and II.

The *TGM4* promoter contains a cyclophilin A processed pseudogene (*PPIP*). Cyclophilin A is a peptidyl-prolyl *cis-trans* isomerase (PPIase) and as such is involved in protein folding (Fischer et al. 1989, Takahashi et al. 1989). It is encoded by a gene of approximately 5.5 kb with an exon-intron structure (Haendler et al. 1987, Haendler and Hofer 1990). The human genome further contains at least 15 *PPIP* retropseudogenes, which share an identity of 75-95% with the cyclophilin A cDNA sequence (Haendler and Hofer 1990, Willenbrink et al. 1995). The *PPIP* pseudogene in the *TGM4* promoter shares 94% identity with the cyclophilin A cDNA sequence. As in most of the *PPIP* pseudogenes, the *PPIP* pseudogene upstream of the *TGM4* gene is disrupted by deletions and insertions leading to premature stop codons. The *PPIP* pseudogenes are dispersed over at least seven chromosomes. One of the *PPIP* pseudogenes, *PPIP6*, is localized at chromosome 3 (Willenbrink et al. 1995). Recently, the *TGM4* gene has also been mapped to chromosome 3 (Gentile et al. 1995, Dubbink et al. 1996). Comparison of the *PPIP6* sequence with the *PPIP* pseudogene in the *TGM4* promoter showed significant differences (89% identity), which indicates that at least two *PPIP* pseudogenes are located at chromosome 3.

There are at least two other genes with a *PPIP* pseudogene in their promoter regions: the genes encoding cholesterol 7α hydroxylase (Thompson et al. 1993) and angiotensine I converting enzyme (GenBank Accession No. X94359). Both pseudogenes are located further upstream in the 5'-flanking sequence. The pseudogene upstream of the cholesterol 7α hydroxylase gene is in the opposite orientation and at -3.4 kb (Thompson et al. 1993); the pseudogene in the angiotensine I converting enzyme is at 5.4 kb upstream and is positioned in the same orientation as the gene (Villard et al. 1996). At present, there is no regulatory function ascribed to these pseudogenes. Functional analysis of the *TGM4* gene promoter, presented in this study, in the human prostate cancer cell line PC346C showed that basal activity is localized within the proximal 500 bp of the *TGM4* promoter and that no additional activity is transferred by the larger constructs containing the *PPIP* pseudogene, the repetitive elements, and more distal promoter information. The presence of the *PPIP* pseudogene and the repetitive elements in the *TGM4* promoter do not necessarily indicate the 5' border of the regulatory region. As an example, the prostate-specific antigen (PSA) promoter contains an upstream enhancer responsible for androgen-regulated and tissue-specific expression (Schuur et al. 1996, Cleutjens et al. 1997b, Pang et al. 1997) that is separated from the proximal promoter by several *Alu*-type repeats encompassing 1.1 kb of DNA.

Although only one transcript of 3.5 kb can be detected by Northern blot analysis of hTG_P mRNA in the prostate cancer cell line PC346C (Dubbink et al. 1996) and in prostatic tissues (unpublished data), we found a complex splicing pattern of intron 1. The main

transcript is spliced following the exon structure as depicted in Figure 1. One of the less abundant transcripts contains an in-frame insertion of a 135-bp antisense *Alu* repeat encoding 45 extra amino acids. The same *Alu* element also seems to be involved in other splice variants of intron 1, since the *Alu*-specific primer hybridized with several RT-PCR products obtained from prostatic tissues, but reacted with only one band of an *Eco*RI digest of cosmid clone 3.1.1. The boundaries of the 135-bp *Alu* sequence perfectly reflect the splice consensus sequences and are therefore suitable for splice-mediated insertion of this fragment into the hTG_P mRNA.

Splice-mediated insertion of intronic *Alu* elements into mature transcripts has been described for several RNAs. Most often this concerns the introduction of *Alu* sequences into the 5'- or 3'-UTR of mRNAs (Yulug et al. 1995), but insertions of *Alu* cassettes into protein-coding regions have also been reported (Makalowski et al. 1994). Cases have been described in which an inherited mutation in a cryptic splice site of an *Alu* element resulted in the acquisition of an additional exon in all mature transcripts, thereby causing a pathological situation (Mitchell et al. 1991, Knebelmann et al. 1995). The splice variant described here seems to be a normal event in the prostate, since it was detected in all prostatic tissues examined. At present, little is known about the influence of *Alu* cassettes, which are not deleterious, on the function of the host protein. One possibility is that it influences the localization of the protein within the cell (Makalowski et al. 1994). It is therefore tempting to speculate that the *Alu* insertion may interfere with mechanisms underlying the excretion of the hTG_P protein.

Prostate cancer is the most commonly diagnosed cancer in males in Western countries. Metastatic prostate cancer is a fatal disease for which there is no curative treatment at present (Isaacs 1997). Therefore, new treatment strategies based upon newly available methods must be developed. One such alternative approach is to target cytotoxic genes to cells derived from the prostate by means of prostate-specific gene promoters. To date the only human gene promoter that has been extensively characterized for its ability to direct tissue-specific expression is the promoter of the *PSA* gene (Schuur et al. 1996, Cleutjens et al. 1997a, 1997b, Pang et al. 1997). Previously, we obtained preliminary evidence for the prostate specificity of hTG_P (Dubbink et al. 1996). In this report, we clearly demonstrate that the expression of hTG_P is strictly confined to the prostate. Now that we have identified the *TGM4* gene structure and isolated a large part of the *TGM4* promoter, it is possible to investigate the mechanisms underlying this prostate-specific expression. Elucidation of the different mechanisms playing a role in prostate-specific gene expression may finally lead to the development of a well-defined and highly specific anti-cancer treatment based on a molecular approach.

Acknowledgements

We thank Kitty Cleutjens for initial support with the transfection experiments and Frank van der Panne for photography.

Chapter 4

An Sp1 binding site is essential for basal activity of the human prostate-specific transglutaminase gene (*TGM4*) promoter

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Gene, **in press**

Abstract

Human prostate-specific transglutaminase (hTG_P) is a cross-linking enzyme encoded by the *TGM4* gene. The *TGM4* gene promoter was characterized by deletion mapping and mutational analysis. Promoter constructs, containing the minimal promoter requirements, could efficiently drive transcription in the prostate cancer cell lines PC346C and LNCaP and the hepatic cancer cell line Hep3B. The region between positions -113 and -61 was demonstrated to be essential for core promoter activity. Further analysis revealed the functional importance of an Sp1 binding motif, 5'-ACCCCGCCCC-3', at positions -96 to -87. This sequence is a binding site of the ubiquitous transcription factors Sp1 and Sp3.

Introduction

Human prostate-specific transglutaminase (hTG_P) belongs to the transglutaminase (TGase, EC 2.3.2.13) family that comprises at least seven members (Aeschlimann and Paulsson 1994, Aeschlimann et al. 1998). TGases are involved in the formation of stable protein-protein or protein-polyamine bonds through the catalysis of the acyl transfer reaction between a peptide bound glutamine and primary amine groups. hTG_P is supposed to cross-link seminal proteins and polyamines, for instance to the surface of sperm cells, which could result in a decreased immunogenic response in the female urogenital tract (Porta et al. 1986). The expression of hTG_P is entirely restricted to the prostate and regulated by androgens (Dubbink et al. 1996, 1998). In contrast to other prostatic markers, e.g. prostate-specific antigen (PSA) and prostatic acid phosphatase (Lilja and Abrahamsson 1988), hTG_P is only produced by a subset of the luminal prostatic epithelial cells (Dubbink et al. 1999b). At present, the molecular mechanism underlying hTG_P expression is unknown.

hTG_P is encoded by the *TGM4* gene. Recently, we resolved the genomic structure of the *TGM4* gene and determined the sequence of 2.1 kb of its promoter (Dubbink et al. 1998). We demonstrated the functional activity of the *TGM4* promoter by transient transfection experiments using the human prostate cancer cell line PC346C, the only cell line in which hTG_P expression was found (Dubbink et al. 1996). Basal activity was shown to reside within the proximal 0.5 kb of the *TGM4* promoter.

To further characterize the molecular mechanisms underlying the expression of hTG_P, we performed transfections to prostatic and non-prostatic human cell lines with several promoter deletion constructs. The region between positions -113 and -61 was shown to be essential. We demonstrated the importance of an Sp1 binding motif within this region. Transcription factors Sp1 and Sp3 were shown to bind to this motif.

Materials and Methods

Cell culture

PC346C cells were cultured in a Dulbecco's modified Eagle's medium (DMEM)/F12-based growth medium (Gibco, Grand Island, NY, U.S.A.), as described previously (Dubbink et al. 1996). LNCaP and Hep3B cells were cultured in RPMI 1640 and DMEM (Gibco), respectively, both supplemented with 5% (v/v) fetal calf serum and antibiotics. Prior to transfections, RPMI medium was substituted by DMEM with 5% (v/v) FCS.

Generation of TGM4 promoter constructs

For transfections, *TGM4* promoter fragments were cloned in pGL3.basic (Promega, Madison, WI, U.S.A.). pTGM4-0.5Luc (-510 to +54), pTGM4-1.5Luc (-1508 to +54) and pTGM4-2.1Luc (-2077 to +54) have been described previously (Dubbink et al. 1998). Deletion constructs of the proximal promoter were prepared using a nested deletion kit (Pharmacia, Uppsala, Sweden). For this purpose, the pTGM4-0.5Luc construct was digested with *Kpn*I and *Bgl*II and processed according to the manufacturer's protocol. Clones were selected by *Hin*dIII digestions followed by sequencing analysis with a T7 sequence kit (Pharmacia).

A CC to AA mutation at positions -93 and -92 of the *TGM4* promoter, within the Sp1 binding motif, was introduced by PCR (pTGM4-113Luc.mut). PCR amplification was performed with primer 5'-ACGGGGTACCAACATCGCCTTCCCAAACC*AA*GCCCCTTGGCAG-3' (position -113 to -80, mutation indicated in boldface, italics) and a primer designed from pGL3.basic as described previously (Dubbink et al. 1996). For cloning purposes, the promoter primer was provided with a *Kpn*I site (underlined). pTGM4-113Luc (see the Results section) was used as a template for PCR amplification. The PCR product was purified from gel, digested with *Kpn*I and *Hin*dIII and ligated into pGL3.basic. The introduction of the mutation was verified by sequencing. Plasmid isolations for transfections were performed with a Qiafilter plasmid isolation kit (Qiagen, Hilden, Germany).

Transient transfections and luciferase assay

The cells were transfected according to the calcium phosphate method essentially as described (Chen and Okayama 1987), using $1x10^6$ cells per 25-cm^2 flask and 5 μg of the appropriate construct. After a 4-h incubation with the precipitate, the culture medium was replaced by 15% glycerol in PBS and incubated for 90 sec at room temperature. Subsequently, transfected cells were cultured in medium for 24 h. Transfections were performed at least four times in duplicate with two independent plasmid isolates.

Cells were harvested in 400 μl lysis buffer (25 mM Tris-phosphate pH7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100 and 15% glycerol). Subsequently, 100 μl luciferin (0.25 μM) (Sigma, St. Louis, MO, U.S.A.)/ 0.25 μM ATP was added to 10 μl (LNCaP and Hep3B) or 150 μl (PC346C) cell lysate. Luciferase activity was measured in a LUMAC 2500 M Biocounter (LUMAC, Landgraaf, The Netherlands).

Electrophoretic mobility shift assay (EMSA)
Nuclear extracts were prepared as described (Dignam et al. 1983). Double stranded oligonucleotides used for gel retardation were as follows:

-113/-61.wt:
```
5' tcgaCAACATCGCCTTCCCAAACCCCGCCCCTTGGCAGCCATAGCAAGGCCACATCg      3'
3'      GTTGTAGCGGAAGGGTTTGGGGCGGGGAACCGTCGGTATCGTTCCGGTGTAGcagct 5'
```

-113/-61.mut:
```
5' tcgaCAACATCGCCTTCCCAAACCAAGCCCCTTGGCAGCCATAGCAAGGCCACATCg      3'
3'      GTTGTAGCGGAAGGGTTTGGTTCGGGGAACCGTCGGTATCGTTCCGGTGTAGcagct 5'
```

-101/-80.wt:
```
5' tcgaCCCAAACCCCGCCCCTTGGCAG      3'
3'      GGGTTTGGGGCGGGGAACCGTCagct 5'
```

The sequences of these oligonucleotides correspond to human *TGM4* promoter sequences -113 to -61 (wild-type and mutant, mutation in boldface, italics) and -101 to -80 (wild-type), respectively. 5'-overhanging sequences, introduced for labelling purposes, are indicated in lower case letters. The oligonucleotides were labelled by the incorporation of α-^{32}P-dATP with MMLV-reverse transcriptase. For EMSA's, 20-50x10^3 cpm of each probe was added to 20 µl reaction mixture, containing 2 µg poly (dI-dC), 2 µg BSA, 10 µM $ZnCl_2$, 1 mM DTT and 2 µl 10x binding buffer (100 mM Hepes pH 7.6, 300 mM KCl, 62.5 mM $MgCl_2$ and 40% ficoll), and 10 µg LNCaP nuclear protein. Competition experiments were performed in the presence of a 200-fold excess of the indicated non-labelled oligonucleotides. The double stranded Sp1 and NF1 consensus oligonucleotides (5'-ATTCGATCGGGGCGGGGCGAGC-3' and 5'-CCTTTGGCATGCTGCCAATATG-3') were purchased from Promega. In supershift experiments, the reaction mixture was incubated for 30 min at 4 °C with 2 µl mouse monoclonal antibody against Sp1 and/or Sp3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Oligonucleotide-protein complexes were separated from free probes by 4% non-denaturing polyacrylamide gel electrophoresis.

Results

Characterization of the TGM4 promoter in different human cell lines
Previously we localized the main transcription start site of the *TGM4* gene at 52 bp upstream of the ATG translational start codon and demonstrated that the 5'-flanking -510/+54 sequence harbors a functional promoter in transiently transfected PC346C cells (Dubbink et al. 1998). The proximal promoter contains a CATAA motif at positions -48 to -44.
To study the activity of the *TGM4* promoter in different cell lines and the *cis*-acting elements involved, eight luciferase reporter gene constructs were prepared with progressive 5' to 3' deletions. All constructs contained the entire 5'-untranslated region of the *TGM4* gene.

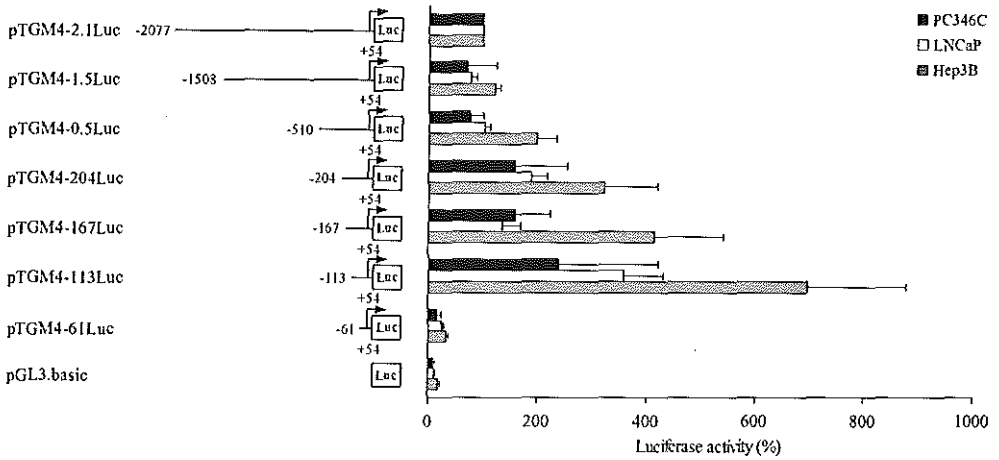


Figure 1. Functional analysis of the *TGM4* promoter in several human cell lines. Transfections of PC346C (solid bars), LNCaP (open bars) and Hep3B (shaded bars) cells with several deletion constructs were performed as described in Materials and Methods. A schematic representation of the deletion constructs is shown at the left-hand side. Relative luciferase activities are presented as percentages of the activities of pTGM4-2.1Luc, which was set to 100%. Standard errors of the mean of four independent, duplicate experiments are shown. Mean luciferase activities, which are not corrected for transfection efficiency, of PC346C, LNCaP and Hep3B obtained with reporter construct pTGM4-2.1Luc were 6 x 10^3, 5 x 10^4 and 2 x10^3 light units, respectively. These values were obtained from 150 µl, 10 µl and 10 µl cell lysate, respectively.

The longest construct, pTGM4-2.1Luc, contained 2.1 kb of the *TGM4* promoter (-2077 to +54), the shortest contained 61 bp upstream of the transcription start site (pTGM4-61Luc, -61 to +54) (Figure 1). Two human prostate cancer cell lines, PC346C and LNCaP, and the human liver cancer cell line Hep3B were transiently transfected with the different constructs. Because the luciferase activities showed large variations among the cell lines due to differences in transfection efficiencies (for pTGM4-2.1Luc activities in each cell line, see legend to Figure 1), the activities of pTGM4-2.1Luc in the different cell lines were set to 100% for better comparison.

Deletion mapping showed that constructs containing between 2.1 kb and 0.5 kb of the *TGM4* promoter had similar relative activities (Figure 1). In all cell lines, the luciferase activities gradually increased by further promoter deletion to -113. Promoter truncation to -61 led in all cases to a dramatic drop in activity, almost to background level, demonstrating that essential regulatory elements are located between -113 and -61.

EMSA of the minimal TGM4 promoter

Between -113 and -61 the *TGM4* promoter sequence contains a candidate Sp1 binding motif, 5'-ACCCCGCCCC-3', at positions -96 to -87 (Figure 2A). To investigate the proteins that can bind between -113 and -61, EMSAs were performed with LNCaP nuclear protein extracts (Figure 2B). EMSAs with oligonucleotide -113/-61.wt demonstrated several retarded protein-oligonucleotide complexes, C1, C2, C3 and C4 (Figure 2B, lane 2, and below), which could be

A

```
                                    AA
-113          CAA CATCGCCTTC CCAAACCCCG CCCCTTGGCA GCCATAGCAA GGCCACATCT
-60     AGCCCAGGAT CACATAAAAA GGGCTGTTTC CTCCCCTGAG GACCGACTGT GTGGAAGCAC
+1      CAGGCATCAG AGATAGAGTC TTCCCTGGCA TTGCAGGAGA GAATCTGAAG GGATG
```

B

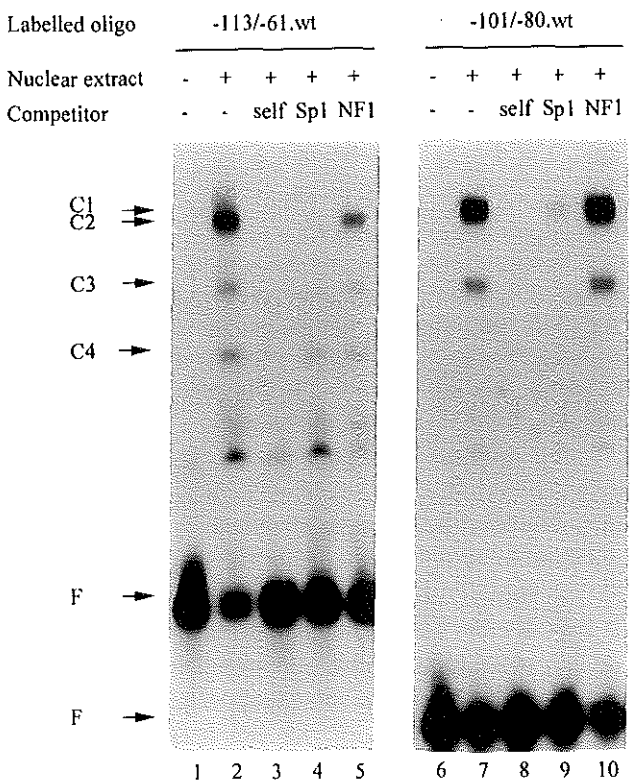
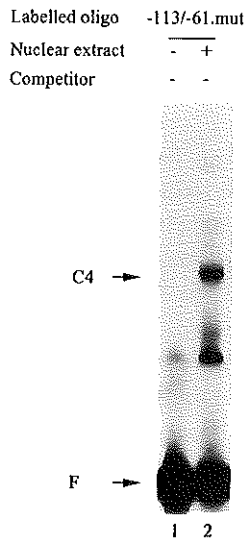


Figure 2. (A) Sequence of the *TGM4* promoter. The major transcription start site (+1) is double underlined and in boldface. The CATAA box (-48) is in boldface. The translational start site is shown in boldface, italics. The putative Sp1 binding site is underlined and in boldface. At position -93 and -92 the functional CC to AA mutation is indicated. (B) EMSAs of the *TGM4* promoter with LNCaP nuclear extracts. LNCaP nuclear extracts were incubated with labelled oligonucleotide -113/-61.wt spanning the *TGM4* promoter between -113 and -61 or with oligonucleotide -101/-80.wt just overlapping the Sp1 binding motif, and further analyzed as described in Materials and Methods. Oligonucleotides are indicated above the panels: lanes 1-5, oligonucleotide -113/-61.wt; lanes 6-10, oligonucleotide -101/-80.wt. Lanes 1 and 6, controls without LNCaP nuclear extracts. Lanes 2 and 7, no competitor added; lanes 3 and 8, competition with the same oligonucleotide (unlabelled); lanes 4 and 9, competition with consensus Sp1 oligonucleotide and lanes 5 and 10, competition with consensus NF1 oligonucleotide. C1, C2, C3 and C4, specific protein-oligonucleotide complexes; F, free oligonucleotides.

A



B

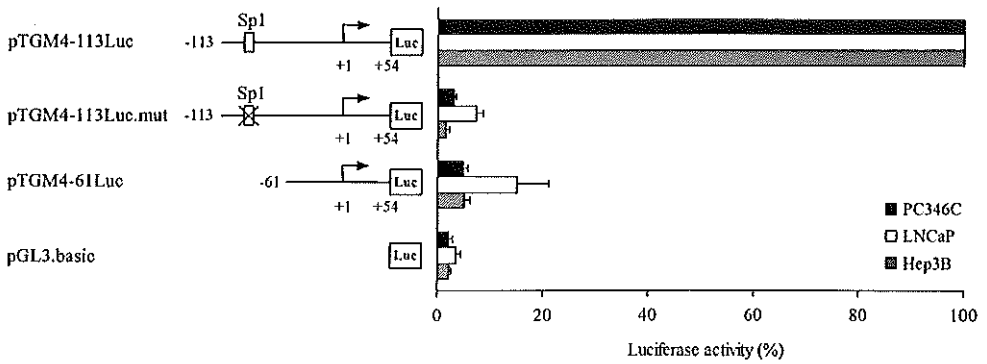


Figure 3. (A) EMSA of an oligonucleotide mutated in the putative Sp1 binding motif present in the *TGM4* promoter with LNCaP nuclear extracts. LNCaP nuclear extracts were incubated with labelled oligonucleotide -113/-61.mut, which contains a CC to AA mutation at positions -93 and -92 (see Figure 2A), and further analyzed as described in Materials and Methods. Lane 1, control without nuclear extract; lane 2, incubation in the presence of nuclear extract. C4, retarded complex which does not contain proteins binding to the Sp1 binding motif; F, free oligonucleotides. (B) Functional analysis of the Sp1 binding site in the *TGM4* core promoter. Transfections of PC346C (solid bars), LNCaP (open bars) and Hep3B (shaded bars) cells with the indicated constructs were performed as described in Materials and Methods. Both pTGM4-113Luc and pTGM4-61Luc represent wild-type constructs; pTGM4-113Luc.mut represents the -93, -92 mutant construct. Luciferase activities are presented as percentages of the activities of the pTGM4-113Luc construct which is set to 100%. Standard errors of the mean of four independent, duplicate experiments are shown.

competed by an excess of unlabelled oligonucleotide -113/-61.wt (lane 3). Complexes C1, C2 and C3 could also be competed by an excess Sp1 consensus oligonucleotide (lane 4), but not by an excess of an NF1 consensus oligonucleotide (lane 5). EMSAs with oligonucleotide -101/-80.wt confirmed the involvement of the Sp1 binding site in complexes C1, C2 and C3 (lanes 6-10).

EMSA and functional analysis of the putative Sp1 binding site within the TGM4 core promoter
To confirm whether the complexes, which could be specifically competed by the Sp1 consensus oligonucleotide, indeed represented protein interactions with the candidate Sp1 binding site, EMSAs with mutant oligonucleotide -113/-61.mut were performed (Figure 3A). This oligonucleotide contained a CC to AA mutation at positions -93 and -92 (mutation indicated in Figure 2A). Contrary to the wild-type oligonucleotides, retarded complexes C1, C2 and C3 were absent when oligonucleotide -113/-61.mut was used (Figure 3A).
To prove the functional significance of the Sp1 binding motif, a pTGM4-113Luc.mut construct was generated which contained the same mutation as described for oligonucleotide -113/-61.mut. The mutant *TGM4* promoter was tested in parallel with the wild type constructs pTGM4-113Luc and pTGM4-61Luc by transient transfections in PC346C, LNCaP and Hep3B cells (Figure 3B). The results clearly demonstrated that mutation of the Sp1 binding site completely abolished promoter activity in all three cell lines, showing the importance of the Sp1 binding motif in transcriptional regulation of the *TGM4* gene.

Transcription factors Sp1 and Sp3 bind to the Sp1 binding motif in the TGM4 core promoter
Several proteins which can direct their regulatory actions through the Sp1 binding motif are known (Hagen et al. 1992, Kingsley and Winoto 1992, Sogawa et al. 1993). To identify which of these proteins bind to the Sp1 sequence in the *TGM4* promoter, supershift experiments with specific antibodies against the Sp1 and Sp3 transcription factors were performed. In the EMSA with oligonucleotide -101/-80.wt (Figure 4, lane 2) the upper band (C1) was supershifted by the Sp1 antibody (lane 3), whereas the two lower bands (C2 and C3) were supershifted by the Sp3 antibody (lane 4). Addition of both Sp1 and Sp3 antibodies to the reaction mixtures resulted in supershifts of all three specific bands (lane 5). Experiments with oligonucleotide -113/-61.wt gave identical results (data not shown). These findings confirmed that the upper, apparently single, retarded band in the EMSA shown in Figure 2B (lane 2) consisted of two protein-oligonucleotide complexes with comparable mobility.

Discussion

We studied the *TGM4* promoter in the prostate cancer cell lines PC346C and LNCaP and in the liver cancer cell line Hep3B. Of these cell lines, only the PC346C cell line is known to express hTG_P (Dubbink et al. 1996). In all cell lines, the *TGM4* promoter showed a high basal transcriptional activity, which gradually increased if shorter promoter fragments were analyzed. This demonstrates that the upstream region negatively influenced basal transcription

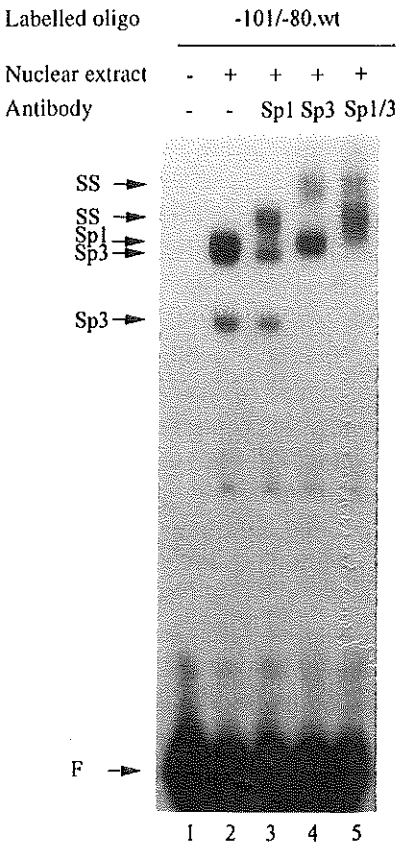


Figure 4. Sp1 and Sp3 bind to the Sp1 binding motif i the *TGM4* core promoter. LNCaP nuclear extracts wer incubated with labelled oligonucleotide -101/-80.wt i the absence (lanes 1 and 2) or presence (lanes 3-5) of the specified antibodies and further analyzed as described in Materials and Methods. Lane 1, prob control; lane 2, probe and extract; lane 3, probe, extract and Sp1 antibody; lane 4, probe, extract and Sp3 antibody; lane 5, probe, extract and both Sp1 and Sp antibodies. F, free oligonucleotide; SS, supershifted protein-oligonucleotide complexes with the specifie antibodies indicated above the lanes.

levels in a non cell-type specific fashion. Our findings suggest that the prostate-specific activity of the TGM4 promoter is located outside the 2.1 kb proximal *TGM4* promoter.

So far, PSA is the only human prostate marker of which regulation has been extensively studied at the molecular level. The *PSA* promoter fragment which appears to be most important for prostate-specific expression of PSA, both *in vitro* and in transgenic mice, is located within a complex 400 bp enhancer region at -4.3 to -3.9 kb, showing that prostate restriction can be coordinated by upstream elements far from the proximal promoter (Schuur et al. 1996, Cleutjens et al. 1997a, 1997b, Wei et al. 1997). Essential determinants for the tissue-specific expression of hTG_P might also be located further upstream or downstream of the *TGM4* transcription start site.

A difference between the *TGM4* and the *PSA* proximal promoter is the much lower basal activity of the latter in different cell lines, including LNCaP. Androgens increased the activity of the *PSA* promoter (Riegman et al. 1991b, Cleutjens et al. 1996, 1997b, Schuur et al. 1996), but not of the proximal *TGM4* promoter (data not shown). This suggests that the previously found androgen regulation of hTG_P (Dubbink et al. 1996) is not directed via the proximal *TGM4* promoter. In this respect it is important to note that the upstream element of the *PSA* promoter conferring prostate specificity is also the most essential region for androgen-regulated

expression of PSA (Schuur et al. 1996, Cleutjens et al. 1997b), which might implicate that prostate specificity and androgen regulation interact with each other. Further studies are necessary to determine the mechanisms of tissue specificity and androgen regulation of the *TGM4* gene expression.

The results presented in this report demonstrated the importance of the Sp1 binding motif 5'-ACCCCGCCCC-3' at position -96 to -87 for the high basal activity of the *TGM4* promoter. This Sp1 sequence has previously been described as a medium affinity binding site of the Sp1 multigene family (Kadonaga et al. 1986). Several proteins have been identified which can bind to Sp1 binding motifs (Hagen et al. 1992, Kingsley and Winoto 1992, Sogawa et al. 1993). The nuclear Sp1 and Sp3 proteins which bind to the Sp1 binding motif in the *TGM4* core promoter are both widely distributed transcription factors with similar binding specificities and affinities which is reflected in the structural identity of their DNA-binding domains (Hagen et al. 1992, Kingsley and Winoto 1992). The presence of several Sp3-oligonucleotide complexes in the EMSA may be due to different translation products of Sp3 mRNA and was also described for other promoters (Kingsley and Winoto 1992, Hagen et al. 1994).

The precise role of Sp1 and Sp3 in *TGM4* gene regulation cannot be deduced from the experiments described here. Sp1 is a transcriptional activator, whereas Sp3 can either repress or activate transcription through interaction with the Sp1 binding motif. Whether Sp3 acts as stimulator or repressor depends on the promoter, the number of DNA binding sites involved and the cellular context (Hagen et al. 1994, Majello et al. 1997). Functional studies in cells lacking endogenous proteins of the Sp1 family may elucidate the relative contribution of both factors to *TGM4* promoter activity. Although Sp1 and Sp3 are ubiquitous transcription factors, they are involved in expressional regulation of many genes with a tissue-specific distribution (Sartorelli et al. 1990, Noti et al. 1996, Ohtaka-Maruyama et al. 1998). One possible mechanism, which has been proposed for the myeloid-specific transcription factor PU.1, might be that a tissue-specific factor changes the chromatin structure rendering the Sp1 binding site accessible to the corresponding proteins (Chen et al. 1993). The expression of hTG_P is not only tissue-specific, but is also extremely heterogeneous in the prostate (Dubbink et al. 1999b). One might speculate that the relative cellular amounts of Sp1 and Sp3 contribute to the final *TGM4* transcription level.

Acknowledgements

We thank Frank van der Panne for photography.

Chapter 5

Human Prostate-Specific Transglutaminase: A New Prostatic Marker with a Unique Distribution Pattern

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Lab Invest (1999): 79, 141-150

Abstract

Human prostate-specific transglutaminase (hTG_P) is a cross-linking enzyme, the physiologic function of which has not been established unequivocally yet. To gain insight into its distribution, we raised antisera against hTG_P. By using Western blotting analysis, we found that these antisera specifically recognize a 77-kDa protein in prostatic fluids, seminal plasmas, and prostatic tissues. The concentrations of hTG_P in these fluids and tissues were found to be highly variable among individuals. Immunohistochemical examination of several formalin-fixed paraffin-embedded human tissues revealed an exclusive expression in the prostate. The histologic localization and distribution of hTG_P within the prostate was assessed by studying multiple sections from tumor-containing prostatectomy specimens and needle biopsies. hTG_P expression was entirely restricted to luminal epithelial cells. No basal epithelial cells or stromal cells were stained. Within the prostate, large areas without any hTG_P-positive cells were seen. Immunopositive cells were present either in a scattered pattern or concentrated in single or multiple glands in which all luminal epithelial cells expressed hTG_P. The latter staining pattern occurred frequently, but not exclusively, in the peripheral zone, whereas scattered expression was most often observed in the transition zone. Expression of the hTG_P protein could occasionally be observed in high grade prostatic intraepithelial neoplasia, but was not detected in prostate carcinoma cells. The expression pattern as observed for hTG_P has not been found thus far for any other prostate-specific marker.

Introduction

Prostate cancer is the most frequently diagnosed cancer in men in Western countries, and once metastasized it is a fatal disease (Isaacs 1997). Recent research on the prostate, therefore, has been focussed mainly on its deregulated growth. As a consequence, a relatively small contribution was made to the further elucidation of the basic physiologic functions of the prostate. For that purpose, the identification and characterization of prostate-specific proteins could be of crucial importance. Human prostate-specific transglutaminase (hTG_P) might represent such a new prostatic marker (Dubbink et al. 1996, 1998).

On the basis of its cDNA sequence, hTG_P belongs to the transglutaminase family [TGase, EC 2.3.2.13 (Greenberg et al. 1991, Aeschlimann and Paulsson 1994, Dubbink et al. 1996)]. These calcium-dependent enzymes catalyze the formation of irreversible ε-(γ-glutamyl)lysyl protein-protein or protein-polyamine bonds throughout the body. TGase-mediated cross-links can stabilize large protein complexes and for example account for the mechanical strength of the skin (Eckert et al. 1997) and for optimal wound healing, eg, blood coagulation (Muszbek et al. 1996). More subtle protein modifications brought about by TGases seem to be important for extracellular matrix formation and may influence cell adhesion properties (Gentile et al. 1992, Aeschlimann and Paulsson 1994).

At present, little is known about the physiologic role of hTG$_P$. Many more studies have been performed on its analogue in the rat, ratTG$_P$ (rTG$_P$), or dorsal protein 1 (DP1). DP1 is a secretory glycoprotein (Seitz et al. 1991a, Ho et al. 1992, Esposito et al. 1996) that is responsible for the rapid cross-linking of the copulatory plug after mating (Williams-Ashman 1984). DP1 activity might further lead to seminal fluid and sperm modifications resulting in a reduced immunogenicity of semen (Paonessa et al. 1984, Porta et al. 1993, Peluso et al. 1994). The enzyme is abundantly expressed in epithelial cells of the dorsal prostate, the coagulating gland (anterior prostate), and the lateral type 1 prostate and is also expressed in minor amounts by the lateral type 2 prostate, but not in the ventral prostate (Wilson and French 1980, Romijn 1990, Seitz et al. 1990, Hayashi et al. 1991, Kinbara and Cunha 1996). DP1 immunoreactivity has also been detected in myoepithelial and stromal cells, but not in acinar cells of salivary glands (Aumuller et al. 1995). Expression of DP1 in rat urogenital tissues is androgen-regulated and can be influenced by mesenchymal components (Kinbara et al. 1996). DP1 levels are reduced by castration and restored by subsequent androgen replacement (Wilson and French 1980, Seitz et al. 1990, Ho et al. 1992). In explant cultures of rat prostatic tissue, DP1 expression could only be demonstrated when grown in the presence of androgen (Lopes et al. 1996).

In human seminal plasma, a calcium-dependent TGase activity was detected (Porta et al. 1986, Enderle-Schmitt et al. 1989, Ablin et al. 1990), which might correspond to hTG$_P$. TGase/hTG$_P$ does not seem to play a role in the formation of the soft gel, which is quickly formed upon ejaculation, because this does not contain TGase-specific bounds (Lilja and Laurell 1985). However, hTG$_P$ may be involved in sperm maturation and suppression of semen antigenicity by the introduction of covalent modifications (James and Hargreave 1984, Porta et al. 1986, Ablin et al. 1990). Several candidate substrates have been described through which hTG$_P$ may exert its function including polyamines that are present in high concentrations in semen (Porta et al. 1986) and the major gel-forming proteins in human semen semenogelin I and II (Peter et al. 1998).

Until recently, studies on hTG$_P$ have been hampered by the fact that the protein could not be isolated and immunologically detected. The recent identification of the hTG$_P$ cDNA (Grant et al. 1994, Dubbink et al. 1996) made it possible to study its expression and regulation as well as to resolve the genomic structure of the corresponding *TGM4* gene (Dubbink et al. 1996, 1998). The 35-kb *TGM4* gene encompasses 13 exons and 12 introns and is transcribed into a 3.5-kb mRNA encoding a 77-kDa protein. hTG$_P$ expression was up-regulated by androgens in the androgen-responsive human prostate cancer cell line PC346C (Dubbink et al. 1996). By screening of a large series of normal tissues hTG$_P$ mRNA expression was found entirely restricted to the prostate (Dubbink et al. 1996, 1998).

To increase our knowledge of hTG$_P$, we raised antisera against the C-terminal region of the protein so that we could study the presence and localization of hTG$_P$ in more detail. We examined hTG$_P$ expression in benign and malignant prostate tissues and in fluids from the prostate and seminal vesicles. An extended large series of normal human tissues was investigated immunohistochemically for the expression of hTG$_P$. The results from these studies further substantiate the prostate-specific expression of hTG$_P$ and revealed an up to now unique expression pattern in the prostate.

Materials and Methods

Tissues and fluids

hTG_P expression was studied in benign prostate tissue, BPH, prostate cancer, and in a variety of human tissues of other origin (Table 1). Standard sextant needle biopsy specimens from the apical, intermediate, and basal part of the prostate were obtained from participants of a screening program for prostate cancer (Hoedemaeker et al. 1997). To study the hTG_P distribution within the normal prostate, entirely benign biopsy sets were selected from participants with a serum PSA value below 4.0 ng/ml. By these criteria, biopsy sets were considered to be obtained from benign prostates. BPH tissues were obtained from transurethral resections. Prostate cancer tissues were obtained from radical prostatectomy specimens from the European Randomized Study for Prostate Cancer [ERSPC (Hoedemaeker et al. 1997)]. To avoid fixation artefacts, radical prostatectomy specimens of men with a diagnosis of prostate cancer were entirely fixed by formalin injections (Hoedemaeker et al. 1998). Every radical prostatectomy specimen was systematically sectioned and totally embedded in paraffin. Human tissues of other origin (Table 1) were obtained as formalin-fixed paraffin-embedded archival material from the Department of Pathology.

Prostatic fluids and seminal vesicle fluids were obtained from radical prostatectomy specimens by means of gentle massage. Semen samples were obtained from men visiting the Andrology Clinic. The samples were allowed to liquefy at room temperature. Sperm cells, debris, or both, were removed by centrifugation at 10,000 x *g* at room temperature. For most experiments these seminal plasmas were freshly used. The remainder was stored at -20°C until further use.

Preparation of fusion constructs

The hTG_P-GST and TG_C-GST fusion constructs were obtained by in-frame subcloning of a 1.3 kb *Sau*3AI-*Sau*3AI fragment of hTG_P cDNA (Dubbink et al. 1996) and a 350 bp *Bam*HI-*Bam*HI fragment of hTG_C cDNA (Gentile et al. 1991), respectively, into the *Bam*HI site of the bacterial expression vector pGEX-3X (Pharmacia, LKB Biotech, Uppsala, Sweden). The hTG_P-GST and TG_C-GST fusion constructs encode the C-terminal amino acids 531-684 of hTG_P (Dubbink et al. 1996) and 476-593 of hTG_C (Gentile et al. 1991), respectively. hTG_C cDNA was kindly provided by Dr. P.J.A. Davies (Department of Pharmacology, Houston, TX) and pGEX-3X by Dr. A. Hoogeveen (Department of Clinical Genetics, Rotterdam, The Netherlands).

Induction and purification of fusion proteins

Fusion proteins were isolated from cultures of *Escherichia coli* strain JM101 transformed with the fusion constructs. Overnight cultures were diluted 1:10 in 500 ml of 2xYT medium containing 100 µg/ml ampicillin and grown to an OD_{600} of 1.2. IPTG was then added to a final concentration of 0.1 mM and bacteria were grown for another 4 h. Bacteria were pelleted, resuspended in 25 ml ice-cold PBS, and sonicated 10 times 1 minute on ice. After the addition of Triton X-100 to a final concentration of 1% and an incubation of 30 minutes

at 4°C, the bacterial lysates were centrifuged at 3000 x g for 15 minutes at 4°C. The pellet fractions, containing inclusion bodies consisting largely of fusion protein but presumably contaminated with bacterial proteins, were washed three times with 25 ml of buffer composed of 25 mM Tris (pH 7.5), 1 mM EDTA, 100 mM NaCl, and 1% Triton X-100. Fusion proteins were further purified by 7 % Prep Cell SDS-PAGE (Bio-Rad, Munich, Germany). Collected fractions were dialyzed against 10 mM ammonium hydrogen carbonate (NH_4HCO_3), concentrated by a Centriprep 10 concentrator (Amicon, Beverly, MA), and used for immunizations.

Immunizations

Two polyclonal antisera (1919.2 and 1920.2) were obtained by repeated intradermal injections of 150 µg of the hTG$_P$-GST fusion protein into New Zealand White rabbits. For primary injections the concentrated fusion protein was mixed with an equal volume of complete Freunds adjuvants (Difco laboratories, Detroit, MI). Booster injections were performed with incomplete in stead of complete Freunds adjuvants 3 and 9 weeks after the first injections. Blood samples were allowed to clot for 1 hour at 4°C. The clots were removed by centrifugation at 3000 x *g*. Sera were stored in aliquots at -20°C.

Western blotting

Protein preparations for Western blot analysis of prostatic tissue specimens were obtained by homogenization of the tissues in RIPA buffer [150 mM NaCl, 1.0% Triton X-100, 1.0% deoxycholate, 0.1% SDS, 5 mM EDTA and 10 mM Tris (pH 7.4)], containing a protease inhibitor cocktail (Complete, Boehringer, Mannheim, Germany). Debris was removed by centrifugation at 350,000 x *g* in a tabletop centrifuge (Optima® TLX; Beckman Instruments, Inc., Fullerton, CA) for 10 minutes at 4°C. Protein concentrations in the supernatant were measured with the Bio-Rad protein assay by using BSA as a standard.

Samples of prostatic tissue, prostatic fluid, seminal vesicle fluid and seminal plasma were subjected to 10% SDS-PAGE. Prestained markers (Novex, San Diego, CA) were used as size standards. The separated proteins were electrophoretically transferred to nitrocellulose filters. The filters were blocked with PBS containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h. Polyclonal antisera and preimmune sera were diluted 1:5000 in blocking solution and incubations were performed overnight at 4°C. Subsequent incubations with a 1:3000 dilution of the secondary alkaline phosphatase (AP)-conjugated goat anti-rabbit antibody (DAKO, Glostrup, Denmark) were carried out at room temperature for 1 h. The blots were developed by using a chromogenic AP substrate (GIBCO, Grand Island, NY) or a chemiluminescence detection reagent (DuPont NEN, Boston, MA). Competition experiments were performed by the addition of 1 µg of the hTG$_P$-GST or TG$_C$-GST fusion protein during the incubation with the primary antisera.

Immunohistochemical staining

Five micrometers formalin-fixed paraffin-embedded sections of human tissues were deparaffinized, and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 20 minutes. The sections were preincubated with PBS containing 5%

BSA (PBS-BSA) and 10% normal goat serum (DAKO) for 15 minutes. Preimmune or immune sera were then applied at a 1:1000 dilution in PBS-BSA at room temperature for 30 minutes. After rinsing three times with PBS containing 0.1% Tween 20 (PBS-Tween), the sections were incubated with biotinylated anti-immunoglobulins (1:50, Multilink, Biogenex, San Ramon, CA) in PBS-BSA at room temperature for 30 minutes. After rinsing three times with PBS-Tween, the sections were incubated with horseradish peroxidase-labeled streptavidin (1:50, Biogenex) in PBS-BSA containing 2% normal goat serum (DAKO) and 2% normal human serum for 30 minutes at room temperature and washed three times with PBS. Immunoreactivity was visualized with 150 mg of diaminobenzidine hydrochloride (DAB, Fluka, Neu-Ulm, Germany) in 200 ml of PBS supplemented with 0.08% H_2O_2. The sections were counterstained with Mayer's hematoxylin. Competition experiments were performed by incubation of the immune sera with 3 µg (in a 100 µl final volume) of either the hTG_P-GST or the TG_C-GST fusion protein, 1 hour before and during the incubation with the primary antisera. Serial sections of all tissues listed in Table 1 were tested in parallel with preimmune serum of the same rabbit that had been used for the preparation of antiserum 1920.2.

Results

Specificity of hTG_P antisera

Polyclonal antisera were raised against the C-terminal region of hTG_P, because this part has the lowest similarity with other TGases. Reactivity and specificity of the antisera were checked by Western blot analysis of prostatic fluid, considering that this secretory product is likely to contain relatively high concentrations of hTG_P. Initial screening results indicated that lot 1920.2 was the most reactive and specific antiserum. By using this antiserum, it was shown that prostatic fluid contained two immunoreactive bands, a major band of 77 kDa and a minor band of approximately 72 kDa (Figure 1A, lane 2). The bands were specific because preimmune serum showed no reactive proteins (Figure 1A, lane 1) and both bands disappeared by competition with the hTG_P-GST fusion protein, but not with a control fusion protein, TG_C-GST, containing the C-terminal region of human cellular or tissue-type TGase (Figure 1A, lanes 6 and 5, respectively). Antiserum dilutions up to 80,000 times still showed immunoreactivity (Figure 1A, lanes 3 and 4).

By using the same strategy, immunohistochemical reactivity and specificity of antiserum 1920.2 was determined (Figs. 1, B to E). In benign prostatic glands, several luminal epithelial cells were stained with the antiserum (Figure 1C). Immunostaining could be competed for only by the hTG_P-GST fusion protein (Figure 1E) but not by the TG_C-GST (Figure 1D) fusion protein. The background staining, which can be observed in the sections incubated with the fusion proteins, is likely to be due to immunoreactivity against the GST-portion of nonspecifically bound fusion protein. The control with preimmune serum was entirely negative (Figure 1B).

Comparable results for both Western blotting and immunohistochemistry were obtained with the other antiserum 1919.2. Antigen retrieval by microwave irradiation of the sections

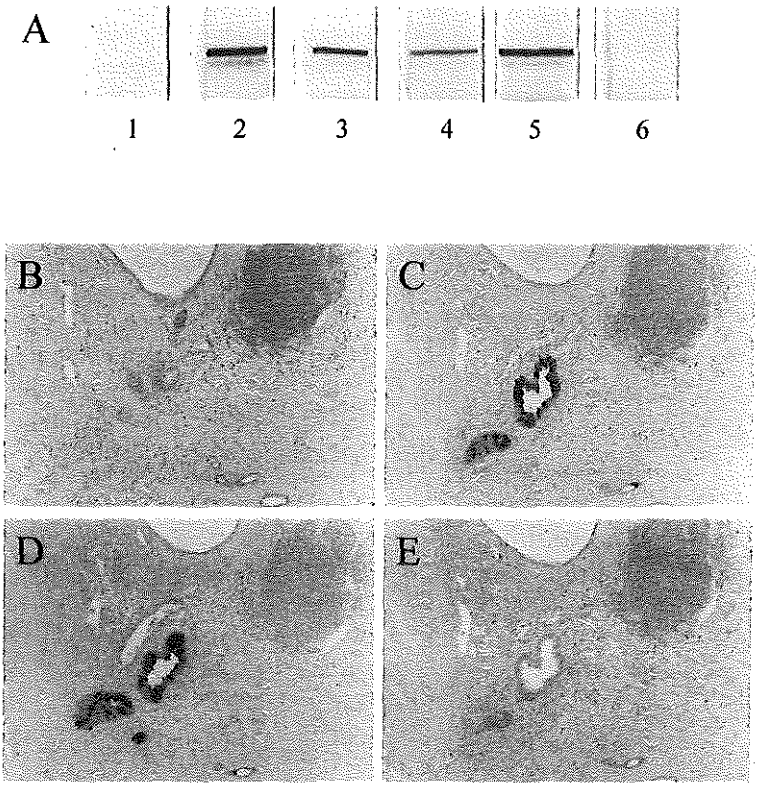


Figure 1. Specificity of polyclonal antiserum 1920.2 as determined by Western blot analysis of prostatic fluid (A) and immunohistochemical staining of serial sections of formalin-fixed paraffin-embedded benign prostatic hypertrophy (BPH) tissue (B-E). Similar results were obtained with antiserum 1919.2. (A) Western blot analysis. Each lane contains 1 µl of prostatic fluid. Lane 1: preimmune serum (1:5000). Lanes 2-4: immune serum diluted 1:5000 (lane 2), 1:20,000 (lane 3), and 1:80,000 (lane 4). Lanes 5 and 6 are competition experiments with the fusion proteins TG$_C$-GST (containing the C-terminal region of human cellular or tissue-type TGase) and hTG$_P$-GST, respectively, and demonstrate specificity for both the 77-kDa band and the 72-kDa band. (B-E) Immunohistochemical staining. Preimmune and immune serum dilutions were 1:1000. (B) Preimmune serum; (C) immune serum; (D) immune serum in competition with fusion protein TG$_C$-GST; and (E) immune serum in competition with fusion protein hTG$_P$-GST. The background staining in D and E seemed to be due to immunoreactivity against the GST-portion of fusion proteins that nonspecifically attached to the sections. Original magnifications, x88.

did not improve the staining results, suggesting that the antisera recognized easily available epitopes. Preliminary experiments with these antisera using frozen sections showed a high background.

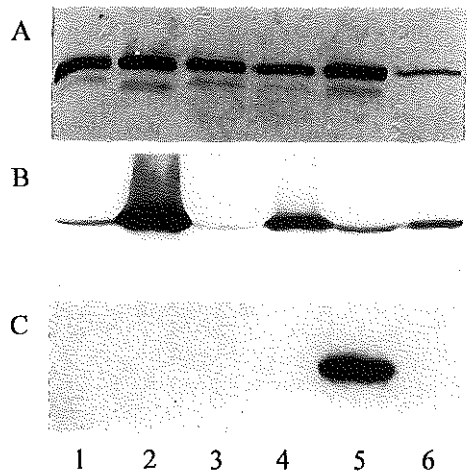


Figure 2. Western blot analysis of seminal plasmas, prostatic fluids, and normal prostatic tissues. Antiserum dilutions were 1:5000. (A) Prostatic fluid. Each lane contains 1 µl of fluid. Note the variation in size of the minor band (70 -to 72 kDa). (B) Semina plasma. Each lane contains 10 µl of seminal plasma of different individuals. Note the variation i expression level of hTG_P. (C) Prostate tissue extracts. Each lane contains 20 µg of protein. Lanes 1-3, BPH tissues; Lanes 4-6, normal prostate tissue obtained from radical cystoprostatectomy specimens Note that only in one of six individuals could clear hTG_P expression be demonstrated. After long exposure also in lane 2 and 6, a low expression o hTG_P became apparent.

Western blot analysis of hTG_P expression in prostatic fluids, seminal plasmas, seminal vesicle fluids and prostatic tissues

We examined eight prostatic fluids and 30 seminal plasmas for hTG_P expression with antiserum 1920.2. Figure 2, A and B, show representative examples of this study. The 77-kDa protein could be demonstrated in all prostatic fluids and in all but one (not shown) seminal plasmas. In most prostatic fluids, the minor band was also present, but its exact size varied between individuals (70 -to 72 kDa). Both prostatic fluid and seminal plasma sometimes additionally contained specific bands of approximately 33 kDa as well as high molecular mass bands (>200 kDa). No expression was observed in seminal vesicle fluids (data not shown).

The hTG_P expression levels in seminal plasmas strongly varied among individuals (Figure 2B). Also in a series of prostatic tissues hTG_P expression was highly variable. Only in one of six examined prostatic tissues could a clear expression be demonstated (Figure 2C). Also in two other tissue lysates, after long exposure, very low expression could be detected.

Prostate-specific expression of hTG_P

To confirm the prostate-specific expression of hTG_P indicated in our previous studies at the mRNA level (Dubbink et al. 1996, 1998), a large panel of normal human tissues was examined by immunohistochemistry. No specific immunoreactivity could be detected in any tissue other than the prostate. In Table 1, the tissues tested in this immunohistochemical screening are summarized together with the earlier obtained results.

Table 1. Tissue distribution of hTG$_P$ [a].

Tissue	Immunohistochemical	mRNA
Adrenal gland	-	-
Appendix	nd	-
Aorta	nd	-
Bladder	-	-
Bone marrow	nd	-
Brain	-	-
Breast	-	-
Cartilage	-	nd
Cervix	-	nd
Colon	-	-
Epididymus	-	nd
Esophagus	-	nd
Fat tissue	-	nd
Heart	-	-
Kidney	-	-
Liver	-	-
Lung	-	-
Lymph node	-	-
Ovary	-	-
Pancreas	-	-
Parathyroid gland	-	nd
Peripheral leukocyte	nd	-
Pituitary gland	nd	-
Placenta	-	-
Prostate	+	+
Salivary gland	-	-
Seminal vesicles	-	nd
Skeletal muscle	-	-
Skin	-	nd
Small intestine	-	-
Spleen	-	-
Stomach	-	-
Tongue	-	nd
Testis	-	-
Thymus	-	-
Thyroid gland	-	-
Tonsil	-	nd
Trachea	-	-
Ureter	-	nd
Uterus	-	-

[a] Expression of hTG$_P$ was determined by immunohistochemistry with antiserum 1920.2. In parallel, serial sections were incubated with preimmune serum. Antiserum and preimmune serum dilutions were 1:1000. The results obtained in previous studies, in which the tissue specificity was investigated by Northern blot analysis (Dubbink et al. 1996) and by spot blot analysis using a human RNA master blot (Dubbink et al. 1998), are also summarized in this table. In addition to the tissues presented in this table, no expression of hTG$_P$ could be demonstrated in several tissues of fetal origin (Dubbink et al. 1998). ND, not determined.

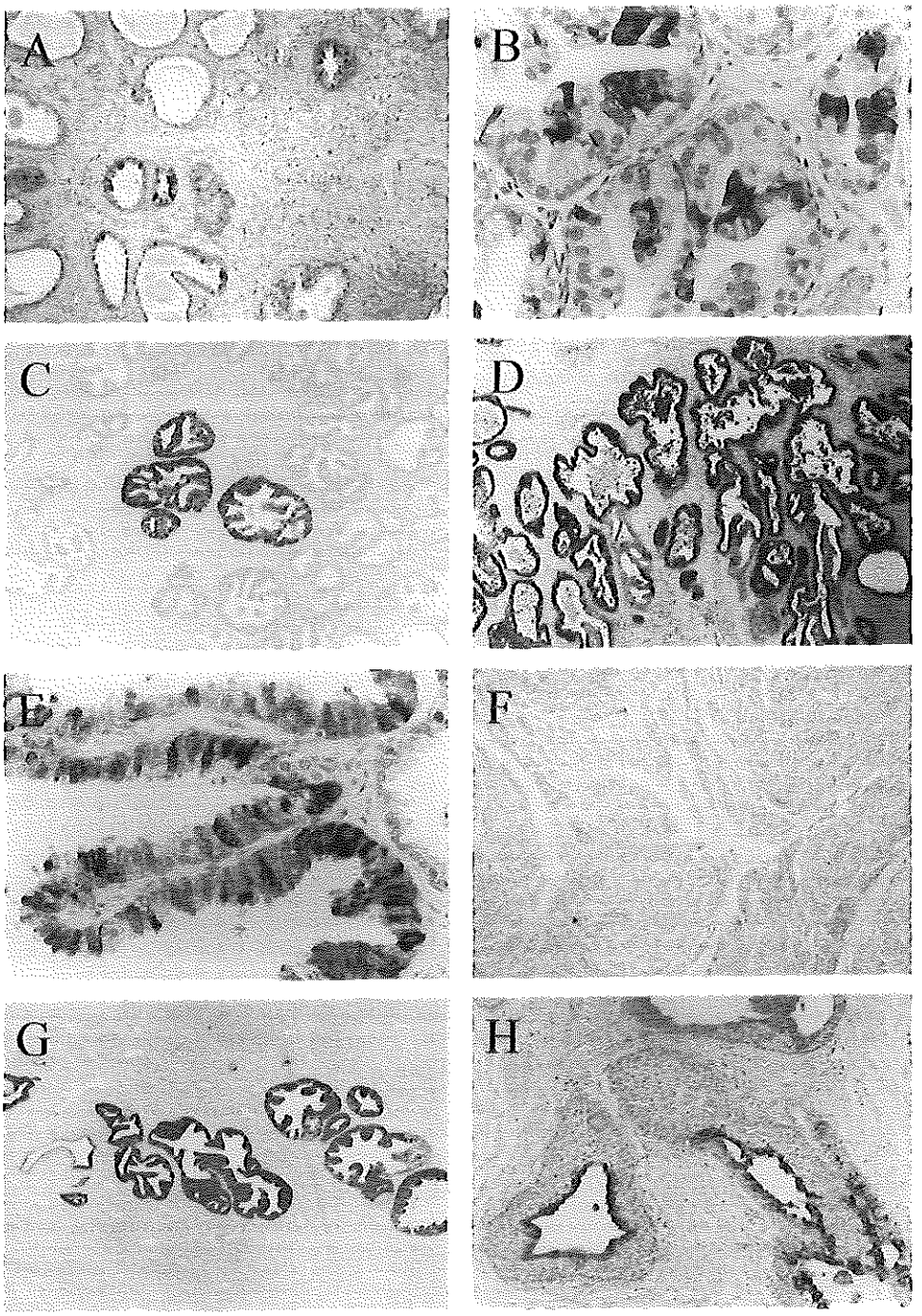
Immunohistochemical analysis of hTG_P expression in prostatic tissues

Positive staining was observed only in the cytoplasm of the secretory prostatic epithelial cells. No basal epithelial cells or stromal cells were found to stain positive (see eg, Figure 3, B and E). Immunopositive cells were usually heavily stained and could be clearly distinguished from the surrounding negative cells. Positive epithelial cells can have a tall columnar or cuboidal appearance, but also cells with a typical atrophic morphology can be intensively positive. Sometimes the luminal content of prostatic ducts showed immunoreactivity (data not shown).

To investigate the distribution of hTG_P expression throughout the prostatic gland and to address its expression in prostate carcinoma tissue, we stained a large number of sections (Table 2) obtained from entirely paraffin-embedded radical prostatectomy specimens. Four different staining patterns could be classified: (1) negative, ie, large entirely hTG_P negative areas; (2) focal staining, ie, regions with only few single, but intensively stained cells (Figure 3A); (3) moderate staining, ie, regions with several positive cells with a scattered distribution pattern (Figure 3B) and/or single or only few glands in which all luminal epithelial cells were stained (Figure 3C) and (4) extensive staining, ie, regions with many stained cells in multiple glands in which all luminal epithelial cells expressed large amounts of hTG_P (Figure 3D) and/or in which the positive cells were alternated with negative cells (Figure 3E).

We studied whether a particular staining pattern could be associated with the prostate basis or apex, or with the peripheral or transition zone. The extensive and moderate hTG_P staining patterns were relatively more common at the basal side, whereas the focal staining pattern was dominant in the apex of the prostate (data not shown). Further evaluation of staining distribution showed that the moderate and extensive staining patterns occurred most frequently, but not exclusively, in the peripheral zone, whereas the focal staining pattern was observed more frequently in the transition zone (Table 2). Most benign prostatic hyperplasia (BPH) tissues obtained by transurethral resection, which generally consist of transition zone tissue (McNeal 1988), showed little or no hTG_P-expressing cells, a finding that agrees with the absence of expression in most of the BPH tissues analyzed by Western blotting (Figure 2C). No hTG_P expression was demonstrated in any of the prostate carcinoma cells (Figure 3F). In contrast to prostate carcinoma cells, prostatic intraepithelial neoplasia (PIN) lesions were occasionally positive for hTG_P (Figure 3G).

Figure 3. Distribution patterns of hTG_P in prostate tissues. The tissues were formalin-fixed and paraffin-embedded. Antiserum 1920.2 was used in a 1:1000 dilution. (A) Focal staining pattern; (B) moderate staining pattern, scattered distribution; (C) moderate staining pattern, positive glands with all luminal epithelial cells being stained; (D) extensive staining pattern, many entirely positive glands; (E) extensive staining pattern, glands with positive cells alternated with negative cells; (F) prostate carcinoma tissue, entirely negative; (G) strongly positive prostatic intraepithelial neoplasia lesions surrounded by negative tumor tissue; (H) basal cell hyperplasia of prostate glands with one row of hTG_P-positive cells. Original magnifications: F, x11; C, D and G, x44; A, x88; H, x176; B and E, x361.


A
B
C
D
E
F
G
H

To examine the correlation between immunostaining and Western blot analysis results, sections were made from tissues that were found strongly positive (Figure 2C, lane 5) or almost negative (Figure 2C, lane 2) by Western blot analysis. The latter tissue specimen contained only some scattered positive cells whereas the other tissue showed an area with many immunopositive glands. Strikingly, these positive glands contained one single row of stained luminal cells that were positioned on top of a multilayered cell population with the characteristics of a basal cell hyperplasia (Figure 3H). This example clearly demonstrated that hTG_P expression is restricted to the secretory cells of the prostate.

Distribution of hTG_P in the normal prostate as approached by a needle biopsy study
To perform a more systematic analysis of the hTG_P distribution through the prostate, we investigated needle biopsy sets of 11 prostates, which are considered to be without abnormalities, with antiserum 1920.2. Because the biopsy specimens were small and contain relatively few glands, only three groups of staining patterns were discriminated: entirely negative, focal expression, and extensive staining. The results indeed suggest that immunopositive cells tend to occur at a higher frequency at the basal side of the prostate (data not shown).

Table 2. Expression patterns and distribution of hTG_P within the prostate*.

Staining pattern	Transitional zone	Peripheral zone	Total
Extensive	4 (11)	12 (23)	16 (18)
Moderate	6 (17)	26 (50)	32 (36)
Focal	22 (61)	11 (21)	33 (38)
Negative	4 (11)	3 (6)	7 (8)
Total	36 (100)	52 (100)	88 (100)

hTG_P distribution was assessed by screening 20 tumor-containing prostates. From each prostate, at least four sections were stained of which at least two contained adenocarcinoma. A total number of 88 sections was examined. Four staining patterns were distinguished: entirely negative sections; focal expression (only few separate positive cells); moderate expression (many positive cells with scattered distribution occasionally in combination with some entirely positive glands); and extensive staining (many stained cells in multiple glands in which all luminal epithelial cells expressed large amounts of hTG_P and/or in which the positive cells were alternated with negative cells). If more than one staining pattern was present, sections were grouped into the pattern with the most positive cells. Tumor-containing tissue sections always contained surrounding benign prostatic glands, in which immunopositive cells could occassionally be detected. The staining distibution seemed not to be dependent on the presence of tumor. Numbers refer to the frequency at which the specified staining patterns were observed at the indicated site; percentages are given in parentheses.

Discussion

In this study, we describe the generation of a polyclonal antiserum, 1920.2, directed against the C-terminal region of hTG$_P$, which enabled us to visualize the enzyme by immunoblotting and immunohistochemistry of formalin-fixed paraffin-embedded tissue sections. The 1920.2 hTG$_P$ antiserum seems to be highly specific, because no cross-reactivity was observed with other TGase types expressed in the prostate such as FXIIIa and tissue-type TGase (Friedrichs et al. 1995, Muszbek et al. 1996). No immunoreactivity was found in blood plasma samples (FXIIIa) and in lysates from the human prostate cancer cell line PC-3, known to contain high amounts of tissue-type TGase [own unpublished results (Friedrichs et al. 1995)]. The antiserum was suitable to detect hTG$_P$ expression in archival material, indicating that hTG$_P$ epitopes are well conserved during storage and, therefore, allowed retrospective studies.

The size of the most abundant immunoreactive band with antiserum 1920.2, both in prostate tissue, prostatic fluid, and seminal plasma, is 77 kDa. A protein of similar size has been found to be cross-reactive with an antiserum against rat DP1 (Aumuller and Seitz 1990, Friedrichs et al. 1995) and could be translated from hTG$_P$ cDNA (Dubbink et al. 1996). The latter suggests that the protein does not undergo large modifications, before and after being secreted into semen. In prostatic fluid, a slightly smaller band could also be observed. This varied in size between individuals from about 70 to 72 kDa. We have also found this band in a prostate tissue extract (data not shown), but never in seminal plasmas. The functional significance of two different molecular forms of hTG$_P$ remains to be resolved. It is possible that only one of the two forms represents the active protein. Cleavage of the largest band could perhaps result in an enzymatically active smaller protein or could represent a first step in the degradation of active hTG$_P$ protein. Rat DP1 is known to be a glycoprotein and experiments suggested that DP1 in its glycosylated state remains protected against its own cross-linking activity (Seitz et al. 1991a, Esposito et al. 1996). Biochemical experiments are necessary to solve the glycosylation status of hTG$_P$ and the mechanisms by which hTG$_P$ is guarded against autoaggregation.

Prostatic fluids and seminal plasmas may both contain specific high molecular mass bands, suggesting that hTG$_P$ can incorporate itself into larger protein complexes. In addition, we demonstrated specific bands of approximately 33 kDa band and smaller in both fluids. These bands appeared to represent breakdown products, because incubation of fresh seminal plasmas at 37 °C showed the appearance of the 33-kDa band and disappearance of the 77-kDa band in time (data not shown). Some studies indicated that a semipurified protein fraction of 20 -to 40 kDa from seminal plasma still contained TGase activity (Porta et al. 1986, Ablin et al. 1990), whereas others stated that proteins of that size have lost their enzymatic activity (Enderle-Schmitt et al. 1989).

We could identify hTG$_P$ in all but 1 of the 30 seminal plasma samples examined. In contrast to our results, Aumüller and Seitz (1990) could only show expression in about 30% of the investigated seminal plasmas (300 samples). An explanation for their lower detection rate might be the very low hTG$_P$ expression levels present in some individuals together with the use of rat DP1 antiserum, which is less specific for hTG$_P$. The large variations in expression

levels we observed by immunoblotting correspond very well with the large fluctuations that Porta et al. (1986) found for TGase enzymatic activity in normal human seminal plasmas. In 30% of the cases (6 of 18 cases) no activity was measurable, suggesting that immunoblotting is a more sensitive method for hTG_P detection. Together, these results strongly suggest that previously measured TGase activity was entirely due to hTG_P and that there is a natural variation in hTG_P expression in seminal plasma. Whether there is a direct correlation between TGase activity and immunoblot data is presently under investigation. A separate study is necessary to investigate the natural expression of hTG_P in seminal plasma and whether the enzyme might be involved in certain fertility disorders.

hTG_P could exclusively be demonstrated in fluids derived from the prostate and in prostate tissue sections, but not in other human tissues. These results confirmed an earlier study in which we could detect hTG_P mRNA only in the prostate [Table 1 (Dubbink et al. 1996, 1998)]. It further extends these data and shows that also no expression in individual cells could be detected in any human tissue or cell type other than prostate epithelium. This differs from its rat analogue DP1, which is also present in salivary glands (Aumuller et al. 1995).

We immunohistochemically evaluated hTG_P expression in benign and malignant prostate tissues. Like other secreted prostate markers, eg, prostate specific antigen (PSA), prostatic acid phosphatase (PAP), and prostatic secretory protein94 (PSP94; β-microseminoprotein; β-inhibin) (Lilja and Abrahamsson 1988), hTG_P expression was confined strictly to the cytoplasm of the secretory epithelial cells and could not be detected in the stromal compartment of the prostate. To our knowledge, an expression and distribution pattern similar to what we have found for hTG_P has not been described previously for any other prostate marker. In the normal prostate, a uniform staining for PSA, PAP, and PSP94 of most if not all epithelial cells can be found with only slight variations in staining intensity from cell to cell (Brar et al. 1988, Lilja and Abrahamsson 1988). The striking difference in expression patterns between these prostate markers and hTG_P suggests that they are differentially regulated. The significance of the differences in hTG_P distribution patterns between the transition and the peripheral zones is unclear. It might be interesting to know whether the heterogeneic expression pattern of hTG_P reflects the presence of functionally distinct cell populations within the human prostate.

Of interest is a comparison with DP1 expression, which is often used as a marker for functional differentiation of the epithelium of the rat dorsolateral prostate and coagulating gland. The number of individual DP1-positive cells increases with age and under the influence of androgen (Lopes et al. 1996), and DP1 expression is controlled by stromal components (Hayashi et al. 1993, Kinbara et al. 1996). It would be interesting to know which factors could induce hTG_P expression. Androgen-sensitivity of hTG_P expression *in vitro* has already been found (Dubbink et al. 1996) but obviously cannot alone explain the observed *in vivo* expression patterns with local variations.

PIN lesions are considered to represent precursor lesions of prostate adenocarcinoma (Bostwick 1995). In this respect, it is remarkable that some PIN lesions were found to be hTG_P positive, whereas associated prostatic adenocarcinoma was entirely negative. This may suggest that PIN lesions loose their ability to express hTG_P during further progression

toward tumor tissue. At least two explanations can be given for the lack of hTG$_P$ staining of adenocarcinoma cells: carcinoma cells either loose the capacity to express hTG$_P$ or arise exclusively from hTG$_P$-negative prostate cells. Poorly differentiated tumors have lower PSA, PAP, and PSP94 expression compared with well-differentiated tumors (Abrahamsson et al. 1988). For these markers, however, a decline in expression is easily detectable because all normal prostate cells stain for these markers.

An important conclusion that can be drawn from this study is that hTG$_P$ represents a new highly specific prostate marker. The data suggest that the enzyme may not be very useful as a diagnostic and prognostic tool in prostate cancer, but the question remains how tissue-specificity is regulated and whether regulatory DNA elements and factors involved in directing tissue-specific expression can be identified. At present *TGM4* promoter studies are under way as a first step toward the elucidation of the regulatory mechanisms underlying hTG$_P$ expression (Dubbink et al. 1998).

Acknowledgements

We thank Dr. P.J.A. Davies for supplying us with hTG$_C$ cDNA, Dr. A. Hoogeveen for providing the pGEX-3X vector, and Dr. J.T.M. Vreeburg for arranging the availability of semen samples from the Andrology Clinic. We are indebted to Rien van Haperen and Rick Janssens for their assistance during the purification of the fusion proteins, Frieda van der Ham and Nicole Verkaik for initial help with the immunohistochemical staining procedure, Michael den Bakker for providing some of the normal human tissue sections and help with the interpretation of the results, and Frank van der Panne for photographic assistance.

Chapter 6

General Discussion

General discussion and future aspects

This thesis describes the characterization of the TGase hTG_P and its corresponding gene. hTG_P expression was found to be strictly confined to prostate tissues, the prostate cancer cell line PC346C and fluids containing secretory products of the prostate, prostatic fluid and seminal plasma. Within the prostate, expression was exclusively found in a subset of luminal epithelial cells. hTG_P expressing cells were sometimes found to be distributed in a scattered pattern or concentrated in single or multiple glands, but also large areas without hTG_P positive cells were present. Possible functions which can be attributed to seminal plasma components, including hTG_P, have been discussed in the Introduction and will not be further addressed.

hTG_P expression in seminal plasma

hTG_P expression in seminal plasma was found to be highly variable among individuals. In about one third of the samples expression appeared to be very low. This observation agrees well with a report of Porta et al. (1986), who demonstrated TGase activity in 60% of normal seminal plasmas. A causal relationship between aberrant hTG_P expression and fertility problems of some of the donors of our study cannot be excluded, but it seems more likely that most if not all observed expression levels represent natural variation. Like hTG_P, PSA expression is also variable in seminal plasmas of different individuals (Rittenhouse et al. 1998). Part of these fluctuations might be due to normal differences in the relative contributions of prostate, seminal vesicle and urethral gland secretions to seminal plasma (Mann and Lutwak-Mann 1981). In case of hTG_P, it may additionally result from the heterogeneous expression within the prostate (Chapter 5) combined with a lack of coordinated secretion. In this respect, it would be of interest to study whether there is a correlation between seminal plasma expression levels of hTG_P and other prostate secretory proteins, such as PSA and PAP. In a small series of 4 seminal plasma samples, variation in hTG_P and PSA levels did not correlate, but a systematic study with larger numbers of seminal plasmas would be required to answer this question more definitely.

The question remains how hTG_P could efficiently function in immunomodulation of spermatozoa or as sperm cell surface modifier (Chapter 1), thus helping in optimizing the fertilization process, if it is present at such different levels. This may imply that only small amounts of this enzyme are adequate or that the enzyme is redundant in the sense that other proteins have complementary functions in seminal plasma. Alternatively, hTG_P might have its major function within the prostate rather than in the semen.

hTG_P expression in the prostate

The heterogeneous expression patterns of hTG_P, described in Chapter 5, distinguish this enzyme from other prostate secretory proteins, PSA, PAP, and PSP94, which are abundantly expressed throughout the entire prostate (Lilja and Abrahamsson 1988). It is not clear how the specific expression of hTG_P can be explained. No other characteristic features of hTG_P expressing cells could be identified so far. To achieve that, it would be necessary

to get detailed immunohistochemical expression data of whole prostates. Regional differences in expression patterns may reflect microheterogeneity present in the prostate in e.g. hormone or growth factor environment and the influence of the underlying stroma on the normal function of the secretory epithelium (Cunha et al. 1987, Tenniswood 1997). Possibly, hTG_P is involved in local tissue remodeling processes, like those that have been attributed to other TGase types, such as apoptosis or ECM formation. Resolving the mechanisms underlying this differential expression may yield information on normal physiological processes occurring in the prostate.

Study of differential expression in the prostate can be done by comparison of gene expression profiles of entirely hTG_P-positive and hTG_P-negative glands. An experimental approach to perform this could be by large-scale sequencing of cDNA libraries to generate Expressed Sequence Tags (EST) databases of the selected tissue areas (He et al. 1997, Nelson et al. 1998). Another useful high-throughput sequencing method may be Serial Analysis of Gene Expression or SAGE (Velculescu et al. 1995, Zhang et al. 1997), which allows analysis of relatively small amounts of mRNA (Datson et al. 1999). Gene expression profiles may also be obtained via micro-array technology (Schena et al. 1995).

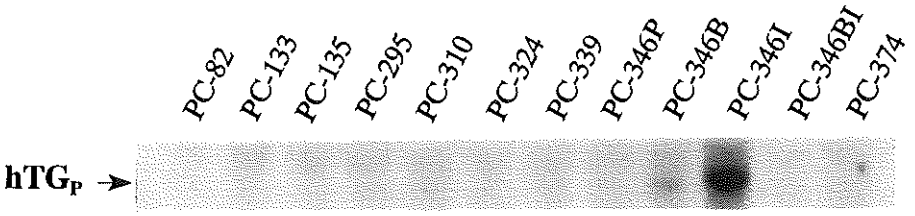


Figure 1. Northern blot analysis of a panel of human prostate tumor xenografts (van Weerden et al. 1996) for the expression of hTG_P.

Prostate-specific expression of hTG_P

A major issue in this thesis is the prostate-specific expression of hTG_P as demonstrated by Northern (spot) blotting (Chapters 2 and 3) and immunohistochemical staining (Chapter 5). In addition, the enzyme was shown to be usually absent in PIN lesions and malignant prostate tissue and thus not suitable as a tumor marker. Northern blot analysis of a xenograft panel of human prostate cancer tissues (van Weerden et al. 1996) largely confirms this observation, because, with the exception of two xenografts of the PC346 lineage, all other xenografts do not express hTG_P (Figure 1). Similarly, only the PC346C prostate cancer cell line, which has been established from a PC346 xenograft (Romijn et al. 1995), expresses hTG_P, whereas others do not (Chapter 2).

Further support for the prostate-specificity and preference of hTG_P for normal prostate epithelium comes from large-scale sequencing projects of cDNA libraries of many different tissues as part of the ongoing Cancer Genome Anatomy Project (CGAP). Of the 440 cDNA

libraries, 27 were prepared from prostate tissues, including 13 from normal prostate tissue, 5 from microdissected PIN lesions, and 8 from cancer tissue. Table 1 shows the frequency of hTG_P, PSA, PAP, and PSP94 ESTs in distinct libraries of prostate and non-prostate origin. In contrast to PSA, PAP and PSP94, expression of hTG_P is absent in non-prostate tissue and PIN lesions and only one EST was found in cDNA from tumor tissue (see also Huang et al. 1999). Of interest is further that hTG_P expression was relatively high in a prostate library subtracted with brain, liver and placental tissue, leading to enrichment of prostate-specific cDNAs (Nelson et al. 1999). Additionally, the data in Table 1 illustrate that the overall expression of hTG_P in prostate tissues is much lower in comparison with PSA, PAP and PSP94, possibly reflecting the heterogeneous distribution of hTG_P-positive cells in the prostate (Chapter 5). However, the CGAP and Genbank database do not completely overlap. A search in the GenBank database with the hTG_P cDNA sequence additionally showed ESTs in cDNA libraries of non-prostate tissue, i.e., Ewing sarcoma (1), placenta (1), uterus carcinoma (1), colon tumor (1), a colon cell line (1), fetal lung (2), lung tumor (1), and pooled libraries of fetal, adult and tumor origin (3).

Table 1. Frequency of hTG_P, PAP, PSA and PSP94 expression in cDNA libraries [a].

				Prostate tissues		
	Total ESTs [b]	Non-Prostate	Prostate	Normal	PIN	Tumor
hTG_P	26	0	26	25	0	1
PAP	231	7	224	91	78	55
PSA	539	80	459	172	163	124
PSP94	359	24	335	182	85	68
Total ESTs [c]		1.335.367	61.172	35.771	9.785	14.941

[a] Data obtained from http://www.ncbi.nlm.nih.gov/ncicgap/cgapba.cgi.

[b] Total number of ESTs in the CGAP database of the corresponding proteins at time of analysis.

[c] Total number of ESTs sequenced at time of analysis.

TGM4 promoter research and prostate cancer gene-therapy

Because the expression of hTG_P is largely confined to prostate epithelium, basic research on the regulation of its prostate-specific expression may be useful for future gene-therapeutic protocols. A major problem in the treatment of non-organ confined inoperable prostate cancer is that after an initial response to endocrine therapy (androgen withdrawal), metastasized prostate cancer often becomes androgen independent leading to therapeutic failure (Stearns and McGarvey 1992, Koivisto et al. 1998). No other effective treatment schedules leading to prolonged survival are presently available. Therefore, novel therapeutic approaches need to be developed to circumvent or treat hormone-refractory disease (Carducci et al. 1996). In this respect, gene-therapeutic strategies based upon

cytotoxic-gene targeting by prostate-specific gene promoters might be of value. The ultimate goal will be the development of highly prostate-specific promoter constructs which are capable of high levels of effector gene expression in all prostatic tumor cells independent of localization, androgen dependence and other properties. Insight into the regulatory elements and factors underlying prostate-specific expression of prostate-specific genes, including the *TGM4* gene, may provide the basis for the construction of such therapeutic vectors.

So far, the molecular mechanisms underlying prostate-specific expression of hTG_P are unclear. The promoter studies presented in Chapter 4 have shown that this is not directed via the 2.1 kb of the proximal *TGM4* promoter. The regulatory regions may be present downstream the transcriptional start site or more upstream in the promoter, like has been described for the *PSA* promoter. Both *in vitro* and in transgenic mice, prostate-specific PSA expression appears to be mediated by a complex upstream enhancer region at -4.3 to -3.9 kb, (Schuur et al. 1996, Cleutjens et al. 1997a, 1997b, Pang et al. 1997). Furthermore, studies with *PSA* promoter constructs hooked to effector genes have shown their ability to direct selective cytotoxicity towards PSA-producing cells both *in vitro* and *in vivo* in xenograft models (Rodriguez et al. 1997, Gotoh et al. 1998, Segawa et al. 1998). These results suggest that prostate-specific promoter-based gene therapy may be a promising approach in the treatment of advanced prostate cancer.

The TGM4 promoter and the development of prostate cancer models

Regulatory elements in the *TGM4* promoter or elsewhere in the gene that are able to drive prostate-specific expression may also be useful for the generation of transgenic mice prone to develop prostate cancer. The advantage of such models above available xenograft models (van Weerden et al. 1996) is that these models may become suitable for studying the early stages, development and progression of prostate cancer against a standardized genetic background. Mice transgenes prepared with the probasin promoter linked to transforming SV40 large T antigen have shown that this is an attractive approach, because these mice display different steps of prostate carcinogenesis (Kasper et al. 1998). Because rodent $DP1/rTG_P$ is abundantly expressed in several prostate lobes, the mouse *TGM4* promoter may be of additional value for the development of transgenic prostate cancer models, using a similar or variant approach e.g. tissue-specific knockout of putatively essential genes in development of prostate cancer. Studies to implement *TGM4* promoter research in programs meant to develop prostate tumor models are now underway.

References

Ablin, R.J., Bartkus, J.M., and Polgar, J. (1990). Effect of human seminal plasma on the lytic activity of natural killer cells and presumptive identification of participant macromolecules. *Am J Reprod Immunol* **24**: 15-21.

Ablin, R.J. and Whyard, T.C. (1991). Identification and possible biological relevance of spermatozoal transglutaminase. *Experientia* **47**: 277-279.

Abrahamsson, P.A., Lilja, H., Falkmer, S., and Wadstrom, L.B. (1988). Immunohistochemical distribution of the three predominant secretory proteins in the parenchyma of hyperplastic and neoplastic prostate glands. *Prostate* **12**: 39-46.

Achyuthan, K.E. and Greenberg, C.S. (1987). Identification of a guanosine triphosphate-binding site on guinea pig liver transglutaminase. Role of GTP and calcium ions in modulating activity. *J Biol Chem* **262**: 1901-1906.

Adany, R. (1996). Intracellular factor XIII: cellular distribution of factor XIII subunit a in humans. *Semin Thromb Hemost* **22**: 399-408.

Adany, R. and Antal, M. (1996). Three different cell types can synthesize factor XIII subunit A in the human liver. *Thromb Haemost* **76**: 74-9.

Aeschlimann, D., Wetterwald, A., Fleisch, H., and Paulsson, M. (1993). Expression of tissue transglutaminase in skeletal tissues correlates with events of terminal differentiation of chondrocytes. *J Cell Biol* **120**: 1461-1470.

Aeschlimann, D. and Paulsson, M. (1994). Transglutaminases: protein cross-linking enzymes in tissues and body fluids. *Thromb Haemost* **71**: 402-415.

Aeschlimann, D., Kaupp, O., and Paulsson, M. (1995). Transglutaminase-catalyzed matrix cross-linking in differentiating cartilage: identification of osteonectin as a major glutaminyl substrate. *J Cell Biol* **129**: 881-92.

Aeschlimann, D., Mosher, D., and Paulsson, M. (1996). Tissue transglutaminase and factor XIII in cartilage and bone remodeling. *Semin Thromb Hemost* **22**: 437-443.

Aeschlimann, D., Koeller, M.K., Allen-Hoffmann, B.L., and Mosher, D.F. (1998). Isolation of a cDNA encoding a novel member of the transglutaminase gene family from human keratinocytes. Detection and identification of transglutaminase gene products based on reverse transcription- polymerase chain reaction with degenerate primers. *J Biol Chem* **273**: 3452-3460.

Aitken, R.J. (1997). The cell biology of fertilization. *Adv Exp Med Biol* **424**: 291-299.

Alexander, N.J. and Anderson, D.J. (1987). Immunology of semen. *Fertil Steril* **47**: 192-205.

Allsbrook, W.C. and Pfeifer, E.A. (1997). Histochemistry of the prostate. In *Pathology of the prostate* (ed. C.S. Foster and D.G. Bostwick), pp. 282-303. W.B. Saunders Company, Philadelphia London Toronto Montreal Sydney Tokyo.

Amelar, R.D. (1962). Coagulation, liquefaction and viscosity of human semen. *J Urol* **87**: 187-190.

Auffray, C. and Rougeon, F. (1980). Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem* **107**: 303-314.

Aumuller, G. (1983). Morphologic and endocrine aspects of prostatic function. *Prostate* **4**: 195-214.

Aumuller, G. and Seitz, J. (1990). Protein secretion and secretory processes in male accessory sex glands. *Int Rev Cytol* **121**: 127-231.

Aumuller, G., Seitz, J., Lilja, H., Abrahamsson, P.A., von der Kammer, H., and Scheit, K.H. (1990). Species- and organ-specificity of secretory proteins derived from human prostate and seminal vesicles. *Prostate* **17**: 31-40.

Aumuller, G., Arce, E.A., Heyns, W., Vercaeren, I., Dammshauser, I., and Seitz, J. (1995). Immunocytochemical localization of seminal proteins in salivary and lacrimal glands of the rat. *Cell Tissue Res* **280**: 171-181.

Aumuller, G., Renneberg, H., and Hasilik, A. (1997a). Distribution and subcellular localization of a lysosome-associated protein in human genital organs. *Cell Tissue Res* **287**: 335-342.

Aumuller, G., Renneberg, H., Schiemann, P.J., Wilhelm, B., Seitz, J., Konrad, L., and Wennemuth, G. (1997b). The role of apocrine released proteins in the post-testicular regulation of human sperm function. *Adv Exp Med Biol* **424**: 193-219.

Baek, K.J., Kwon, N.S., Lee, H.S., Kim, M.S., Muralidhar, P., and Im, M.J. (1996). Oxytocin receptor couples to the 80 kDa Gh alpha family protein in human myometrium. *Biochem J* **315**: 739-44.

Ballestar, E., Abad, C., and Franco, L. (1996). Core histones are glutaminyl substrates for tissue transglutaminase. *J Biol Chem* **271**: 18817-24.

Banas, B., Blaschke, D., Fittler, F., and Horz, W. (1994). Analysis of the promoter of the human prostatic acid phosphatase gene. *Biochim Biophys Acta* **1217**: 188-194.

Barsigian, C., Fellin, F.M., Jain, A., and Martinez, J. (1988). Dissociation of fibrinogen and fibronectin binding from transglutaminase-mediated cross-linking at the hepatocyte surface. *J Biol Chem* **263**: 14015-14022.

Barsigian, C., Stern, A.M., and Martinez, J. (1991). Tissue (type II) transglutaminase covalently incorporates itself, fibrinogen, or fibronectin into high molecular weight complexes on the extracellular surface of isolated hepatocytes. Use of 2-[(2-oxopropyl)thio] imidazolium derivatives as cellular transglutaminase inactivators. *J Biol Chem* **266**: 22501-22509.

Bartlett, R.J., French, F.S., and Wilson, E.M. (1984). In vitro synthesis and glycosylation of androgen-dependent secretory proteins of rat dorsal prostate and coagulating gland. *Prostate* **5**: 75-91.

Bergamini, C.M. and Signorini, M. (1993). Studies on tissue transglutaminases: interaction of erythrocyte type-2 transglutaminase with GTP. *Biochem J* **291**: 37-39.

Bhatia-Gaur, R., Donjacour, A.A., Sciavolino, P.J., Kim, M., Desai, N., Young, P., Norton, C.R., Gridley, T., Cardiff, R.D., Cunha, G.R., Abate-Shen, C., and Shen, M.M. (1999). Roles for nkx3.1 in prostate development and cancer. *Genes Dev* **13**: 966-77.

Board, P.G., Webb, G.C., McKee, J., and Ichinose, A. (1988). Localization of the coagulation factor XIII A subunit gene (F13A) to chromosome bands 6p24----p25. *Cytogenet Cell Genet* **48**: 25-27.

Board, P.G., Losowsky, M.S., and Miloszewski, K.J. (1993). Factor XIII: inherited and acquired deficiency. *Blood Rev* **7**: 229-42.

Bonkhoff, H. and Remberger, K. (1996). Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: a stem cell model. *Prostate* **28**: 98-106.

Bostwick, D.G. (1995). High grade prostatic intraepithelial neoplasia. *Cancer* **75**: 1823-1836.

Bostwick, D.G. and Devaraj, L.T. (1997). Basal cell proliferations and tumors of the prostate. In *Pathology of the prostate* (ed. C.S. Foster and D.G. Bostwick), pp. 157-171. W.B. Saunders Company, Philadelphia London Toronto Montreal Sydney Tokyo.

Bostwick, D.G., Pacelli, A., Blute, M., Roche, P., and Murphy, G.P. (1998). Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 184 cases. *Cancer* **82**: 2256-2261.

Brar, A., Mbikay, M., Sirois, F., Fournier, S., Seidah, N.G., and Chretien, M. (1988). Localization of the human prostatic secretory protein PSP94 and its mRNA in the epithelial cells of the prostate. *J Androl* **9**: 253-260.

Byrd, W.J., Jacobs, D.M., and Amoss, M.S. (1977). Synthetic polyamines added to cultures containing bovine sera reversibly inhibit in vitro parameters of immunity. *Nature* **267**: 621-3.

Candi, E., Melino, G., Mei, G., Tarcsa, E., Chung, S.I., Marekov, L.N., and Steinert, P.M. (1995). Biochemical, structural, and transglutaminase substrate properties of human loricrin, the major epidermal cornified cell envelope protein. *J Biol Chem* **270**: 26382-26390.

Candi, E., Melino, G., Lahm, A., Ceci, R., Rossi, A., Kim, I.G., Ciani, B., and Steinert, P.M. (1998a). Transglutaminase 1 mutations in lamellar ichthyosis. Loss of activity due to failure of activation by proteolytic processing. *J Biol Chem* **273**: 13693-702.

Candi, E., Tarcsa, E., Digiovanna, J.J., Compton, J.G., Elias, P.M., Marekov, L.N., and Steinert, P.M. (1998b). A highly conserved lysine residue on the head domain of type II keratins is essential for the attachment of keratin intermediate filaments to the cornified cell envelope through isopeptide crosslinking by transglutaminases. *Proc Natl Acad Sci U S A* **95**: 2067-2072.

Candi, E., Tarcsa, E., Idler, W.W., Kartasova, T., Marekov, L.N., and Steinert, P.M. (1999). Transglutaminase cross-linking properties of the small proline-rich 1 family of cornified cell envelope proteins. Integration with loricrin. *J Biol Chem* **274**: 7226-37.

Carducci, M.A., DeWeese, T.L., Nelson, W.G., Simons, J.W., Sinibaldi, V., and Eisenberger, M.A. (1996). Prostate cancer treatment strategies based on tumor-specific biological principles: future directions. *Semin Oncol* **23**: 56-62.

Carter, H.B. and Coffey, D.S. (1990). The prostate: an increasing medical problem. *Prostate* **16**: 39-48.

Carter, R.E., Feldman, A.R., and Coyle, J.T. (1996). Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. *Proc Natl Acad Sci U S A* **93**: 749-753.

Chaistitvanich, N. and Boonsaeng, V. (1983). Molecular structure of human seminal coagulum: the role of disulfide bonds. *Andrologia* **15**: 446-451.

Chakravarty, R. and Rice, R.H. (1989). Acylation of keratinocyte transglutaminase by palmitic and myristic acids in the membrane Anchorage region. *J Biol Chem* **264**: 625-629.

Chen, C. and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7**: 2745-2752.

Chen, H.M., Pahl, H.L., Scheibe, R.J., Zhang, D.E., and Tenen, D.G. (1993). The Sp1 transcription factor binds the CD11b promoter specifically in myeloid cells in vivo and is essential for myeloid-specific promoter activity. *J Biol Chem* **268**: 8230-8239.

Chen, S., Lin, F., Iismaa, S., Lee, K.N., Birckbichler, P.J., and Graham, R.M. (1996). Alpha1-adrenergic receptor signaling via Gh is subtype specific and independent of its transglutaminase activity. *J Biol Chem* **271**: 32385-91.

Chiocca, E.A., Davies, P.J., and Stein, J.P. (1988). The molecular basis of retinoic acid action. Transcriptional regulation of tissue transglutaminase gene expression in macrophages. *J Biol Chem* **263**: 11584-11589.

Chung, L.W., Gleave, M.E., Hsieh, J.T., Hong, S.J., and Zhau, H.E. (1991). Reciprocal mesenchymal-epithelial interaction affecting prostate tumour growth and hormonal responsiveness. *Cancer Surv* **11**: 91-121.

Cleary, K.R., Choi, H.Y., and Ayala, A.G. (1983). Basal cell hyperplasia of the prostate. *Am J Clin Pathol* **80**: 850-854.

Cleutjens, K.B., van Eekelen, C.C., van der Korput, H.A., Brinkman, A.O., and Trapman, J. (1996). Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *J Biol Chem* **271**: 6379-6388.

Cleutjens, K.B., van der Korput, H.A., Ehren-van Eekelen, C.C., Sikes, R.A., Fasciana, C., Chung, L.W., and Trapman, J. (1997a). A 6-kb promoter fragment mimics in transgenic mice the prostate-specific and androgen-regulated expression of the endogenous prostate- specific antigen gene in humans. *Mol Endocrinol* **11**: 1256-1265.

Cleutjens, K.B., van der Korput, H.A., van Eekelen, C.C., van Rooij, H.C., Faber, P.W., and Trapman, J. (1997b). An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol Endocrinol* **11**: 148-161.

Cohen, C.M., Dotimas, E., and Korsgren, C. (1993). Human erythrocyte membrane protein band 4.2 (pallidin). *Semin Hematol* **30**: 119-137.

Cooper, A.J., Sheu, K.F., Burke, J.R., Strittmatter, W.J., Gentile, V., Peluso, G., and Blass, J.P. (1999). Pathogenesis of inclusion bodies in (CAG)n/Qn-expansion diseases with special reference to the role of tissue transglutaminase and to selective vulnerability. *J Neurochem* **72**: 889-99.

Cooper, T.G. and Yeung, C.-H. (1997). Physiology of sperm maturation and fertilization. In *Andrology. Male reproductive health and dysfunction* (ed. E. Nieschlag and H.M. Behre), pp. 60-78. Springer, Berlin.

Credo, R.B., Curtis, C.G., and Lorand, L. (1978). Ca2+-related regulatory function of fibrinogen. *Proc Natl Acad Sci U S A* **75**: 4234-7.

Cunha, G.R., Donjacour, A.A., Cooke, P.S., Mee, S., Bigsby, R.M., Higgins, S.J., and Sugimura, Y. (1987). The endocrinology and developmental biology of the prostate. *Endocr Rev* **8**: 338-362.

Cunha, G.R., Alarid, E.T., Turner, T., Donjacour, A.A., Boutin, E.L., and Foster, B.A. (1992). Normal and abnormal development of the male urogenital tract. Role of androgens, mesenchymal-epithelial interactions, and growth factors. *J Androl* **13**: 465-75.

Datson, N.A., van der Perk-de Jong, J., van den Berg, M.P., de Kloet, E.R., and Vreugdenhil, E. (1999). MicroSAGE: a modified procedure for serial analysis of gene expression in limited amounts of tissue. *Nucleic Acids Res* **27**: 1300-7.

Deperthes, D., Frenette, G., Brillard-Bourdet, M., Bourgeois, L., Gauthier, F., Tremblay, R.R., and Dube, J.Y. (1996). Potential involvement of kallikrein hK2 in the hydrolysis of the human seminal vesicle proteins after ejaculation. *J Androl* **17**: 659-665.

Di Sant'Agnese, P.A. (1992). Neuroendocrine differentiation in human prostatic carcinoma. *Hum Pathol* **23**: 287-296.

Di Sant'Agnese, P.A. (1997). Identification and pathologic significance of neuroendocrine differentiation in human prostate carcinoma. In *Pathology of the prostate* (ed. C.S. Foster and D.G. Bostwick), pp. 352-363. W.B. Saunders Company, Philadelphia London Toronto Montreal Sydney Tokyo.

Di Sant'Agnese, P.A. (1998). Neuroendocrine differentiation in prostatic carcinoma: an update. *Prostate Suppl* **8**: 74-9.

Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E.O., and Schuppan, D. (1997). Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* **3**: 797-801.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11**: 1475-1489.

Dubbink, H.J., Verkaik, N.S., Faber, P.W., Trapman, J., Schroder, F.H., and Romijn, J.C. (1996). Tissue-specific and androgen-regulated expression of human prostate-specific transglutaminase. *Biochem J* **315**: 901-908.

Dubbink, H.J., de Waal, L., van Haperen, R., Verkaik, N.S., Trapman, J., and Romijn, J.C. (1998). The human prostate-specific transglutaminase gene (TGM4): genomic organization, tissue-specific expression and promoter characterization. *Genomics* **51**: 434-444.

Dubbink, H.J., Cleutjens, K.B.J.M., Van der Korput, H.A.G.M., Trapman, J., and Romijn, J.C. (1999a). An Sp1 binding site is essential for basal activity of the human prostate-specific transglutaminase gene (TGM4) promoter. *Gene* **in press**.

Dubbink, H.J., Hoedemaeker, R.F., Van der Kwast, T.H., Schroder, F.H., and Romijn, J.C. (1999b). Human prostate-specific transglutaminase: a new prostatic marker with a unique distribution pattern. *Lab Invest* **79**: 141-150.

Dube, J.Y. and Tremblay, R.R. (1997). Biochemistry and potential roles of prostatic kallikrein hK2. *Mol Urol* **1**: 279-285.

Dupe, V., Ghyselinck, N.B., Thomazy, V., Nagy, L., Davies, P.J.A., Chambon, P., and Mark, M. (1999). Essential roles of retinoic acid signaling in interdigital apoptosis and control of BMP-7 expression in mouse autopods. *Dev Biol* **208**: 30-43.

Eckert, R.L., Crish, J.F., and Robinson, N.A. (1997). The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiol Rev* **77**: 397-424.

Egbring, R., Kroniger, A., and Seitz, R. (1996). Factor XIII deficiency: pathogenic mechanisms and clinical significance. *Semin Thromb Hemost* **22**: 419-425.

Eitan, S. and Schwartz, M. (1993). A transglutaminase that converts interleukin-2 into a factor cytotoxic to oligodendrocytes. *Science* **261**: 106-108.

Eitan, S., Solomon, A., Lavie, V., Yoles, E., Hirschberg, D.L., Belkin, M., and Schwartz, M. (1994). Recovery of visual response of injured adult rat optic nerves treated with transglutaminase. *Science* **264**: 1764-1768.

Eizenberg, O., Faber-Elman, A., Gottlieb, E., Oren, M., Rotter, V., and Schwartz, M. (1995). Direct involvement of p53 in programmed cell death of oligodendrocytes. *Embo J* **14**: 1136-44.

el Alaoui, S., Legastelois, S., Roch, A.M., Chantepie, J., and Quash, G. (1991). Transglutaminase activity and N epsilon (gamma glutamyl) lysine isopeptide levels during cell growth: an enzymic and immunological study. *Int J Cancer* **48**: 221-226.

el Alaoui, S., Mian, S., Lawry, J., Quash, G., and Griffin, M. (1992). Cell cycle kinetics, tissue transglutaminase and programmed cell death (apoptosis). *FEBS Lett* **311**: 174-178.

Enderle-Schmitt, U., Erkel, J., Goebel, H.W., Heils, A., Rausch, U., Seitz, J., and Aumuller, G. 1989. Specific protein of the prostate. In *New aspects in the regulation of prostatic function* (ed. G. Aumuller, M. Krieg, and T. Senge), pp. 82-96. Zuckschwerdt-Verlag, Munchen, Bern, Wien, San Francisco.

Englund, P.T. (1993). The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Annu Rev Biochem* **62**: 121-138.

Esposito, C., Pucci, P., Amoresano, A., Marino, G., Cozzolino, A., and Porta, R. (1996). Transglutaminase from rat coagulating gland secretion. Post-translational modifications and activation by phosphatidic acids. *J Biol Chem* **271**: 27416-27423.

Fawell, S.E., Pappin, D.J., McDonald, C.J., and Higgins, S.J. (1986). Androgen-regulated proteins of rat seminal vesicle secretion constitute a structurally related family present in the copulatory plug. *Mol Cell Endocrinol* **45**: 205-13.

Fawell, S.E. and Higgins, S.J. (1987). Formation of rat copulatory plug: purified seminal vesicle secretory proteins serve as transglutaminase substrates. *Mol Cell Endocrinol* **53**: 149-52.

Feng, J.F., Rhee, S.G., and Im, M.J. (1996). Evidence that phospholipase delta1 is the effector in the Gh (transglutaminase II)-mediated signaling. *J Biol Chem* **271**: 16451-4.

Feng, J.F., Gray, C.D., and Im, M.J. (1999). Alpha 1B-adrenoceptor interacts with multiple sites of transglutaminase II: characteristics of the interaction in binding and activation. *Biochemistry* **38**: 2224-32.

Fesus, L., Falus, A., Erdei, A., and Laki, K. (1981). Human beta 2-microglobulin is a substrate of tissue transglutaminase: polymerization in solution and on the cell surface. *J Cell Biol* **89**: 706-710.

Fesus, L., Thomazy, V., and Falus, A. (1987). Induction and activation of tissue transglutaminase during programmed cell death. *FEBS Lett* **224**: 104-8.

Fesus, L., Thomazy, V., Autuori, F., Ceru, M.P., Tarcsa, E., and Piacentini, M. (1989). Apoptotic hepatocytes become insoluble in detergents and chaotropic agents as a result of transglutaminase action. *FEBS Lett* **245**: 150-4.

Fesus, L., Davies, P.J., and Piacentini, M. (1991). Apoptosis: molecular mechanisms in programmed cell death. *Eur J Cell Biol* **56**: 170-177.

Fesus, L. (1993). Biochemical events in naturally occurring forms of cell death. *FEBS Lett* **328**: 1-5.

Finlay, J.A., Evans, C.L., Day, J.R., Payne, J.K., Mikolajczyk, S.D., Millar, L.S., Kuus-Reichel, K., Wolfert, R.L., and Rittenhouse, H.G. (1998). Development of monoclonal antibodies specific for human glandular kallikrein (hK2): development of a dual antibody immunoassay for hK2 with negligible prostate-specific antigen cross-reactivity. *Urology* **51**: 804-809.

Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F.X. (1989). Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature* **337**: 476-478.

Fisher, S., Rikover, M., and Naor, S. (1966). Factor 13 deficiency with severe hemorrhagic diathesis. *Blood* **28**: 34-39.

Floyd, E.E. and Jetten, A.M. (1989). Regulation of type I (epidermal) transglutaminase mRNA levels during squamous differentiation: down regulation by retinoids. *Mol Cell Biol* **9**: 4846-4851.

Folk, J.E. (1980). Transglutaminases. *Annu Rev Biochem* **49**: 517-531.

Folk, J.E., Park, M.H., Chung, S.I., Schrode, J., Lester, E.P., and Cooper, H.L. (1980). Polyamines as physiological substrates for transglutaminases. *J Biol Chem* **255**: 3695-3700.

Folk, J.E. (1983). Mechanism and basis for specificity of transglutaminase-catalyzed epsilon-(gamma-glutamyl) lysine bond formation. *Adv Enzymol Relat Areas Mol Biol* **54**: 1-56.

Fraij, B.M. and Gonzales, R.A. (1997). Organization and structure of the human tissue transglutaminase gene. *Biochim Biophys Acta* **1354**: 65-71.

Fraser, L.R. (1995). Cellular biology of capacitation and the acrosome reaction. *Hum Reprod* **10**: 22-30.

Fraser, L.R., Hanyaloglu, A., and Cockle, S.M. (1997). A fertilization promoting peptide (FPP)-related tripeptide competitively inhibits responses to FPP: a cause of male subfertility? *Mol Reprod Dev* **48**: 529-35.

Fraser, L.R. (1998a). Fertilization promoting peptide: an important regulator of sperm function in vivo? *Rev Reprod* **3**: 151-4.

Fraser, L.R. (1998b). Modulation of mammalian sperm function by fertilization promoting peptide (FPP). *Andrologia* **30**: 241-7.

Friedrichs, B., Riedmiller, H., Goebel, H.W., Rausch, U., and Aumuller, G. (1995). Immunological characterization and activity of transglutaminases in human normal and malignant prostate and in prostate cancer cell lines. *Urol Res* **23**: 301-310.

Gala, J.L., Heusterspreute, M., Loric, S., Hanon, F., Tombal, B., Van Cangh, P., De Nayer, P., and Philippe, M. (1998). Expression of prostate-specific antigen and prostate-specific membrane antigen transcripts in blood cells: implications for the detection of hematogenous prostate cells and standardization. *Clin Chem* **44**: 472-481.

Gallee, M.P., Visser-de Jong, E., van der Korput, J.A., van der Kwast, T.H., ten Kate, F.J., Schroeder, F.H., and Trapman, J. (1990). Variation of prostate-specific antigen expression in different tumour growth patterns present in prostatectomy specimens. *Urol Res* **18**: 181-7.

Gentile, V., Saydak, M., Chiocca, E.A., Akande, O., Birckbichler, P.J., Lee, K.N., Stein, J.P., and Davies, P.J. (1991). Isolation and characterization of cDNA clones to mouse macrophage and human endothelial cell tissue transglutaminases. *J Biol Chem* **266**: 478-483.

Gentile, V., Thomazy, V., Piacentini, M., Fesus, L., and Davies, P.J. (1992). Expression of tissue transglutaminase in Balb-C 3T3 fibroblasts: effects on cellular morphology and adhesion. *J Cell Biol* **119**: 463-474.

Gentile, V., Davies, P.J., and Baldini, A. (1994). The human tissue transglutaminase gene maps on chromosome 20q12 by in situ fluorescence hybridization. *Genomics* **20**: 295-297.

Gentile, V., Grant, F.J., Porta, R., and Baldini, A. (1995). Localization of the human prostate transglutaminase (type IV) gene (TGM4) to chromosome 3p21.33-p22 by fluorescence in situ hybridization. *Genomics* **27**: 219-220.

Giovannucci, E. (1995). Epidemiologic characteristics of prostate cancer. *Cancer Suppl* **75**: 1766-1777.

Glander, H.J., Herrmann, K., and Haustein, U.F. (1987). The equatorial fibronectin band (EFB) on human spermatozoa - a diagnostic help for male fertility? *Andrologia* **19**: 456-459.

Golan, D.E., Corbett, J.D., Korsgren, C., Thatte, H.S., Hayette, S., Yawata, Y., and Cohen, C.M. (1996). Control of band 3 lateral and rotational mobility by band 4.2 in intact erythrocytes: release of band 3 oligomers from low-affinity binding sites. *Biophys J* **70**: 1534-42.

Gotoh, A., Ko, S.C., Shirakawa, T., Cheon, J., Kao, C., Miyamoto, T., Gardner, T.A., Ho, L.J., Cleutjens, C.B., Trapman, J., Graham, F.L., and Chung, L.W. (1998). Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer [In Process Citation]. *J Urol* **160**: 220-229.

Grant, F.J., Taylor, D.A., Sheppard, P.O., Mathewes, S.L., Lint, W., Vanaja, E., Bishop, P.D., and O'Hara, P.J. (1994). Molecular cloning and characterization of a novel transglutaminase cDNA from a human prostate cDNA library. *Biochem Biophys Res Commun* **203**: 1117-1123.

Greenberg, C.S., Enghild, J.J., Mary, A., Dobson, J.V., and Achyuthan, K.E. (1988). Isolation of a fibrin-binding fragment from blood coagulation factor XIII capable of cross-linking fibrin(ogen). *Biochem J* **256**: 1013-9.

Greenberg, C.S., Birckbichler, P.J., and Rice, R.H. (1991). Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *Faseb J* **5**: 3071-3077.

Groos, S., Wilhelm, B., Renneberg, H., Riva, A., Reichelt, R., Seitz, J., and Aumuller, G. (1999). Simultaneous apocrine and merocrine secretion in the rat coagulating gland. *Cell Tissue Res* **295**: 495-504.

Grootjans, J.J., Groenen, P.J., and de Jong, W.W. (1995). Substrate requirements for transglutaminases. Influence of the amino acid residue preceding the amine donor lysine in a native protein. *J Biol Chem* **270**: 22855-22858.

Grundmann, U., Amann, E., Zettlmeissl, G., and Kupper, H.A. (1986). Characterization of cDNA coding for human factor XIIIa. *Proc Natl Acad Sci U S A* **83**: 8024-8.

Guenette, R.S., Corbeil, H.B., Leger, J., Wong, K., Mezl, V., Mooibroek, M., and Tenniswood, M. (1994a). Induction of gene expression during involution of the lactating mammary gland of the rat. *J Mol Endocrinol* **12**: 47-60.

Guenette, R.S., Daehlin, L., Mooibroek, M., Wong, K., and Tenniswood, M. (1994b). Thanatogen expression during involution of the rat ventral prostate after castration. *J Androl* **15**: 200-211.

Haendler, B., Hofer-Warbinek, R., and Hofer, E. (1987). Complementary DNA for human T-cell cyclophilin. *Embo J* **6**: 947-950.

Haendler, B. and Hofer, E. (1990). Characterization of the human cyclophilin gene and of related processed pseudogenes. *Eur J Biochem* **190**: 477-482.

Hagen, G., Muller, S., Beato, M., and Suske, G. (1992). Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes. *Nucleic Acids Res* **20**: 5519-5525.

Hagen, G., Muller, S., Beato, M., and Suske, G. (1994). Sp1-mediated transcriptional activation is repressed by Sp3. *Embo J* **13**: 3843-3851.

Hayashi, N., Sugimura, Y., Kawamura, J., Donjacour, A.A., and Cunha, G.R. (1991). Morphological and functional heterogeneity in the rat prostatic gland. *Biol Reprod* **45**: 308-321.

Hayashi, N., Cunha, G.R., and Parker, M. (1993). Permissive and instructive induction of adult rodent prostatic epithelium by heterotypic urogenital sinus mesenchyme. *Epithelial Cell Biol* **2**: 66-78.

He, W.W., Sciavolino, P.J., Wing, J., Augustus, M., Hudson, P., Meissner, P.S., Curtis, R.T., Shell, B.K., Bostwick, D.G., Tindall, D.J., Gelmann, E.P., Abate-Shen, C., and Carter, K.C. (1997). A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. *Genomics* **43**: 69-77.

Henttu, P., Liao, S.S., and Vihko, P. (1992). Androgens up-regulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. *Endocrinology* **130**: 766-72.

Hettasch, J.M. and Greenberg, C.S. (1994). Analysis of the catalytic activity of human factor XIIIa by site-directed mutagenesis. *J Biol Chem* **269**: 28309-28313.

Hettasch, J.M., Bandarenko, N., Burchette, J.L., Lai, T.S., Marks, J.R., Haroon, Z.A., Peters, K., Dewhirst, M.W., Iglehart, J.D., and Greenberg, C.S. (1996). Tissue transglutaminase expression in human breast cancer. *Lab Invest* **75**: 637-645.

Ho, K.C., Quarmby, V.E., French, F.S., and Wilson, E.M. (1992). Molecular cloning of rat prostate transglutaminase complementary DNA. The major androgen-regulated protein DP1 of rat dorsal prostate and coagulating gland. *J Biol Chem* **267**: 12660-12667.

Hoedemaeker, R.F., Rietbergen, J.B., Kranse, R., van der Kwast, T.H., and Schroder, F.H. (1997). Comparison of pathologic characteristics of T1c and non-T1c cancers detected in a population-based screening study, the European Randomized Study of Screening for Prostate Cancer. *World J Urol* **15**: 339-345.

Hoedemaeker, R.F., Ruijter, E., Ruizeveld de Winter, J.A., van de Kaa, C., the, B.-I.I.M.P.C.s.g., and van der Kwast, T.H. (1998). Processing radical prostatectomy specimens: a comprehensive and standardized protocol. *J Urol Pathol* **9**: 211-222.

Hornyak, T.J. and Shafer, J.A. (1991). Role of calcium ion in the generation of factor XIII activity. *Biochemistry* **30**: 6175-82.

Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Chu, T.M., Mirand, E.A., and Murphy, G.P. (1983). LNCaP model of human prostatic carcinoma. *Cancer Res* **43**: 1809-1818.

Huang, G.M., Ng, W., Farkas, J., He, L., Liang, H.A., Gordon, D., Yu, J., and Hood, L. (1999). Prostate cancer expression profiling by cDNA sequencing analysis. *Genomics* **59**: 178-86.

Huber, M., Rettler, I., Bernasconi, K., Frenk, E., Lavrijsen, S.P., Ponec, M., Bon, A., Lautenschlager, S., Schorderet, D.F., and Hohl, D. (1995). Mutations of keratinocyte transglutaminase in lamellar ichthyosis. *Science* **267**: 525-528.

Hwang, K.C., Gray, C.D., Sivasubramanian, N., and Im, M.J. (1995). Interaction site of GTP binding Gh (transglutaminase II) with phospholipase C. *J Biol Chem* **270**: 27058-27062.

Ichinose, A., Hendrickson, L.E., Fujikawa, K., and Davie, E.W. (1986). Amino acid sequence of the a subunit of human factor XIII. *Biochemistry* **25**: 6900-6906.

Ichinose, A. and Davie, E.W. (1988). Characterization of the gene for the a subunit of human factor XIII (plasma transglutaminase), a blood coagulation factor. *Proc Natl Acad Sci U S A* **85**: 5829-5833.

Ichinose, A., Bottenus, R.E., and Davie, E.W. (1990). Structure of transglutaminases. *J Biol Chem* **265**: 13411-13414.

Iismaa, S.E., Chung, L., Wu, M.J., Teller, D.C., Yee, V.C., and Graham, R.M. (1997). The core domain of the tissue transglutaminase Gh hydrolyzes GTP and ATP. *Biochemistry* **36**: 11655-11664.

Im, M.J., Russell, M.A., and Feng, J.F. (1997). Transglutaminase II: a new class of GTP-binding protein with new biological functions. *Cell Signal* **9**: 477-482.

Imade, G.E., Baker, H.W., de Kretser, D.M., and Hedger, M.P. (1997). Immunosuppressive activities in the seminal plasma of infertile men: relationship to sperm antibodies and autoimmunity. *Hum Reprod* **12**: 256-62.

Isaacs, J.T. (1997). Molecular markers for prostate cancer metastasis. Developing diagnostic methods for predicting the aggressiveness of prostate cancer. *Am J Pathol* **150**: 1511-1521.

Israeli, R.S., Powell, C.T., Corr, J.G., Fair, W.R., and Heston, W.D. (1994). Expression of the prostate-specific membrane antigen. *Cancer Res* **54**: 1807-1811.

Israeli, R.S., Grob, M., and Fair, W.R. (1997). Prostate-specific membrane antigen and other prostatic tumor markers on the horizon. *Urol Clin North Am* **24**: 439-450.

James, K. and Hargreave, T.B. (1984). Immunosuppression by seminal plasma and its possible clinical significance. *Immunol Today* **5**: 357-363.

Janus, T.J., Lewis, S.D., Lorand, L., and Shafer, J.A. (1983). Promotion of thrombin-catalyzed activation of factor XIII by fibrinogen. *Biochemistry* **22**: 6269-72.

Jeon, S., Djian, P., and Green, H. (1998). Inability of keratinocytes lacking their specific transglutaminase to form cross-linked envelopes: absence of envelopes as a simple diagnostic test for lamellar ichthyosis. *Proc Natl Acad Sci U S A* **95**: 687-90.

Jeong, J.M., Murthy, S.N., Radek, J.T., and Lorand, L. (1995). The fibronectin-binding domain of transglutaminase. *J Biol Chem* **270**: 5654-5658.

Johnson, T.S., Knight, C.R., el-Alaoui, S., Mian, S., Rees, R.C., Gentile, V., Davies, P.J., and Griffin, M. (1994). Transfection of tissue transglutaminase into a highly malignant hamster fibrosarcoma leads to a reduced incidence of primary tumour growth. *Oncogene* **9**: 2935-2942.

Johnson, T.S., Griffin, M., Thomas, G.L., Skill, J., Cox, A., Yang, B., Nicholas, B., Birckbichler, P.J., Muchaneta-Kubara, C., and Meguid El Nahas, A. (1997). The role of transglutaminase in the rat subtotal nephrectomy model of renal fibrosis. *J Clin Invest* **99**: 2950-60.

Jones, R.A., Nicholas, B., Mian, S., Davies, P.J., and Griffin, M. (1997). Reduced expression of tissue transglutaminase in a human endothelial cell line leads to changes in cell spreading, cell adhesion and reduced polymerisation of fibronectin. *J Cell Sci* **110**: 2461-72.

Joseph, B., Lefebvre, O., Mereau-Richard, C., Danze, P.M., Belin-Plancot, M.T., and Formstecher, P. (1998). Evidence for the involvement of both retinoic acid receptor- and retinoic X receptor-dependent signaling pathways in the induction of tissue transglutaminase and apoptosis in the human myeloma cell line RPMI 8226. *Blood* **91**: 2423-32.

Jurka, J. and Smith, T. (1988). A fundamental division in the Alu family of repeated sequences. *Proc Natl Acad Sci U S A* **85**: 4775-4778.

Kaartinen, M.T., Pirhonen, A., Linnala-Kankkunen, A., and Maenpaa, P.H. (1997). Transglutaminase-catalyzed cross-linking of osteopontin is inhibited by osteocalcin. *J Biol Chem* **272**: 22736-41.

Kaartinen, M.T., Pirhonen, A., Linnala-Kankkunen, A., and Maenpaa, P.H. (1999). Cross-linking of osteopontin by tissue transglutaminase increases its collagen binding properties. *J Biol Chem* **274**: 1729-35.

Kadonaga, J.T., Jones, K.A., and Tjian, R. (1986). Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem Sci* **11**: 20-23.

Kahlem, P., Terre, C., Green, H., and Djian, P. (1996). Peptides containing glutamine repeats as substrates for transglutaminase-catalyzed cross-linking: relevance to diseases of the nervous system. *Proc Natl Acad Sci U S A* **93**: 14580-5.

Kahlem, P., Green, H., and Djian, P. (1998). Transglutaminase action imitates Huntington's disease: selective polymerization of Huntingtin containing expanded polyglutamine. *Mol Cell* **1**: 595-601.

Kaighn, M.E., Narayan, K.S., Ohnuki, Y., Lechner, J.F., and Jones, L.W. (1979). Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* **17**: 16-23.

Kanaji, T., Ozaki, H., Takao, T., Kawajiri, H., Ide, H., Motoki, M., and Shimonishi, Y. (1993). Primary structure of microbial transglutaminase from Streptoverticillium sp. strain s-8112. *J Biol Chem* **268**: 11565-11572.

Kasper, S., Sheppard, P.C., Yan, Y., Pettigrew, N., Borowsky, A.D., Prins, G.S., Dodd, J.G., Duckworth, M.L., and Matusik, R.J. (1998). Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer. *Lab Invest* **78**: 319-333.

Katz, A.E., Devries, G.M., Begg, M.D., Raffo, A.J., Cama, C., Otoole, K., Buttyan, R., Benson, M.C., and Olsson, C.A. (1995). Enhanced reverse transcriptase polymerase chain reaction for prostate specific antigen as an indicator of true pathologic stage in patients with prostate cancer. *Cancer* **75**: 1642-1648.

Kawakami, M. and Nakayama, J. (1997). Enhanced expression of prostate-specific membrane antigen gene in prostate cancer as revealed by in situ hybridization. *Cancer Res* **57**: 2321-2324.

Kelly, R.W. (1995). Immunosuppressive mechanisms in semen: implications for contraception. *Hum Reprod* **10**: 1686-1693.

Kelly, R.W. and Critchley, H.O.D. (1997). Immunomodulation by human seminal plasma: a benefit for spermatozoon and pathogen? *Hum Reprod* **12**: 2200-2207.

Key, T. (1995). Risk factors for prostate cancer. *Cancer Surv* **23**: 63-77.

Kim, H.C., Lewis, M.S., Gorman, J.J., Park, S.C., Girard, J.E., Folk, J.E., and Chung, S.I. (1990). Protransglutaminase E from guinea pig skin. Isolation and partial characterization. *J Biol Chem* **265**: 21971-8.

Kim, H.C., Idler, W.W., Kim, I.G., Han, J.H., Chung, S.I., and Steinert, P.M. (1991). The complete amino acid sequence of the human transglutaminase K enzyme deduced from the nucleic acid sequences of cDNA clones. *J Biol Chem* **266**: 536-539.

Kim, I.G., McBride, O.W., Wang, M., Kim, S.Y., Idler, W.W., and Steinert, P.M. (1992). Structure and organization of the human transglutaminase 1 gene. *J Biol Chem* **267**: 7710-7717.

Kim, I.G., Gorman, J.J., Park, S.C., Chung, S.I., and Steinert, P.M. (1993). The deduced sequence of the novel protransglutaminase E (TGase3) of human and mouse. *J Biol Chem* **268**: 12682-12690.

Kim, I.G., Lee, S.C., Lee, J.H., Yang, J.M., Chung, S.I., and Steinert, P.M. (1994a). Structure and organization of the human transglutaminase 3 gene: evolutionary relationship to the transglutaminase family. *J Invest Dermatol* **103**: 137-142.

Kim, S.Y., Kim, I.G., Chung, S.I., and Steinert, P.M. (1994b). The structure of the transglutaminase 1 enzyme. Deletion cloning reveals domains that regulate its specific activity and substrate specificity. *J Biol Chem* **269**: 27979-27986.

Kim, S.Y., Chung, S.I., and Steinert, P.M. (1995a). Highly active soluble processed forms of the transglutaminase 1 enzyme in epidermal keratinocytes. *J Biol Chem* **270**: 18026-18035.

Kim, S.Y., Chung, S.I., Yoneda, K., and Steinert, P.M. (1995b). Expression of transglutaminase 1 in human epidermis. *J Invest Dermatol* **104**: 211-217.

Kinbara, H. and Cunha, G.R. (1996). Ductal heterogeneity in rat dorsal-lateral prostate. *Prostate* **28**: 58-64.

Kinbara, H., Cunha, G.R., Boutin, E., Hayashi, N., and Kawamura, J. (1996). Evidence of stem cells in the adult prostatic epithelium based upon responsiveness to mesenchymal inductors. *Prostate* **29**: 107-116.

Kingsley, C. and Winoto, A. (1992). Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. *Mol Cell Biol* **12**: 4251-4261.

Kirchhoff, C., Pera, I., Derr, P., Yeung, C.-H., and Cooper, T. (1997). The molecular biology of the sperm surface. Post-testicular membrane remodelling. *Adv Exp Med Biol* **424**: 221-232.

Kitchens, C.S. and Newcomb, T.F. (1979). Factor XIII. *Medicine (Baltimore)* **58**: 413-429.

Kleman, J.P., Aeschlimann, D., Paulsson, M., and van der Rest, M. (1995). Transglutaminase-catalyzed cross-linking of fibrils of collagen V/XI in A204 rhabdomyosarcoma cells. *Biochemistry* **34**: 13768-13775.

Knebelmann, B., Forestier, L., Drouot, L., Quinones, S., Chuet, C., Benessy, F., Saus, J., and Antignac, C. (1995). Splice-mediated insertion of an Alu sequence in the COL4A3 mRNA causing autosomal recessive Alport syndrome. *Hum Mol Genet* **4**: 675-679.

Knight, C.R., Rees, R.C., Elliott, B.M., and Griffin, M. (1990). The existence of an inactive form of transglutaminase within metastasising tumours. *Biochim Biophys Acta* **1053**: 13-20.

Knight, C.R., Rees, R.C., and Griffin, M. (1991). Apoptosis: a potential role for cytosolic transglutaminase and its importance in tumour progression. *Biochim Biophys Acta* **1096**: 312-318.

Koivisto, P., Kolmer, M., Visakorpi, T., and Kallioniemi, O.P. (1998). Androgen receptor gene and hormonal therapy failure of prostate cancer. *Am J Pathol* **152**: 1-9.

Kojima, S., Nara, K., and Rifkin, D.B. (1993). Requirement for transglutaminase in the activation of latent transforming growth factor-beta in bovine endothelial cells. *J Cell Biol* **121**: 439-448.

Kojima, S., Inui, T., Muramatsu, H., Suzuki, Y., Kadomatsu, K., Yoshizawa, M., Hirose, S., Kimura, T., Sakakibara, S., and Muramatsu, T. (1997). Dimerization of midkine by tissue transglutaminase and its functional implication. *J Biol Chem* **272**: 9410-6.

Korsgren, C., Lawler, J., Lambert, S., Speicher, D., and Cohen, C.M. (1990). Complete amino acid sequence and homologies of human erythrocyte membrane protein band 4.2. *Proc Natl Acad Sci U S A* **87**: 613-617.

Korsgren, C. and Cohen, C.M. (1991). Organization of the gene for human erythrocyte membrane protein 4.2: structural similarities with the gene for the a subunit of factor XIII. *Proc Natl Acad Sci U S A* **88**: 4840-4844.

Kozak, M. (1994). Determinants of translational fidelity and efficiency in vertebrate mRNAs. *Biochimie* **76**: 815-821.

Kumar, A., Mikolajczyk, S.D., Goel, A.S., Millar, L.S., and Saedi, M.S. (1997). Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2. *Cancer Res* **57**: 3111-4.

Lai, T.S., Achyuthan, K.E., Santiago, M.A., and Greenberg, G.S. (1994). Carboxyl-terminal truncation of recombinant factor XIII A-chains. Characterization of minimum structural requirement for transglutaminase activity. *J Biol Chem* **269**: 24596-24601.

Lai, T.S., Slaughter, T.F., Koropchak, C.M., Haroon, Z.A., and Greenberg, C.S. (1996). C-terminal deletion of human tissue transglutaminase enhances magnesium-dependent GTP/ATPase activity. *J Biol Chem* **271**: 31191-31195.

Lai, T.S., Slaughter, T.F., Peoples, K.A., Hettasch, J.M., and Greenberg, C.S. (1998). Regulation of human tissue transglutaminase function by magnesium- nucleotide complexes. Identification of distinct binding sites for Mg- GTP and Mg-ATP. *J Biol Chem* **273**: 1776-1781.

Lee, K.N., Birckbichler, P.J., and Patterson, M.K., Jr. (1989). GTP hydrolysis by guinea pig liver transglutaminase. *Biochem Biophys Res Commun* **162**: 1370-5.

Lee, K.N., Arnold, S.A., Birckbichler, P.J., Patterson, M.K., Jr., Fraij, B.M., Takeuchi, Y., and Carter, H.A. (1993). Site-directed mutagenesis of human tissue transglutaminase: Cys-277 is essential for transglutaminase activity but not for GTPase activity. *Biochim Biophys Acta* **1202**: 1-6.

Lesort, M., Attanavanich, K., Zhang, J., and Johnson, G.V. (1998). Distinct nuclear localization and activity of tissue transglutaminase. *J Biol Chem* **273**: 11991-11994.

Lilja, H. and Laurell, C.B. (1984). Liquefaction of coagulated human semen. *Scand J Clin Lab Invest* **44**: 447-452.

Lilja, H. (1985). A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. *J Clin Invest* **76**: 1899-1903.

Lilja, H. and Laurell, C.B. (1985). The predominant protein in human seminal coagulate. *Scand J Clin Lab Invest* **45**: 635-641.

Lilja, H., Oldbring, J., Rannevik, G., and Laurell, C.B. (1987). Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. *J Clin Invest* **80**: 281-285.

Lilja, H. and Abrahamsson, P.A. (1988). Three predominant proteins secreted by the human prostate gland. *Prostate* **12**: 29-38.

Lilja, H., Abrahamsson, P.A., and Lundwall, A. (1989). Semenogelin, the predominant protein in human semen. Primary structure and identification of closely related proteins in the male accessory sex glands and on the spermatozoa. *J Biol Chem* **264**: 1894-1900.

Lilja, H. (1990). Cell biology of semenogelin. *Andrologia* **22**: 132-141.

Limon, J., Lundgren, R., Elfving, P., Heim, S., Kristoffersson, U., Mandahl, N., and Mitelman, F. (1990). An improved technique for short-term culturing of human prostatic adenocarcinoma tissue for cytogenetic analysis. *Cancer Genet Cytogenet* **46**: 191-199.

Liu, H., Moy, P., Kim, S., Xia, Y., Rajasekaran, A., Navarro, V., Knudsen, B., and Bander, N.H. (1997). Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. *Cancer Res* **57**: 3629-3634.

Liu, H., Rajasekaran, A.K., Moy, P., Xia, Y., Kim, S., Navarro, V., Rahmati, R., and Bander, N.H. (1998). Constitutive and antibody-induced internalization of prostate-specific membrane antigen. *Cancer Res* **58**: 4055-60.

Lopes, E.S., Foster, B.A., Donjacour, A.A., and Cunha, G.R. (1996). Initiation of secretory activity of rat prostatic epithelium in organ culture. *Endocrinology* **137**: 4225-4234.

Lorand, L. and Conrad, S.M. (1984). Transglutaminases. *Mol Cell Biochem* **58**: 9-35.

Lorand, L. (1996). Neurodegenerative diseases and transglutaminase. *Proc Natl Acad Sci U S A* **93**: 14310-3.

Lovgren, J., Rajakoski, K., Karp, M., Lundwall, A., and Lilja, H. (1997). Activation of the zymogen form of prostate-specific antigen by human glandular kallikrein 2. *Biochem Biophys Res Commun* **238**: 549-555.

Mahony, M.C. and Alexander, N.J. (1991). Sites of antisperm antibody action. *Hum Reprod* **6**: 1426-30.

Mahoney, S.A., Perry, M., Seddon, A., Bohlen, P., and Haynes, L. (1996). Transglutaminase forms midkine homodimers in cerebellar neurons and modulates the neurite-outgrowth response. *Biochem Biophys Res Commun* **224**: 147-52.

Majello, B., De Luca, P., and Lania, L. (1997). Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains. *J Biol Chem* **272**: 4021-4026.

Makalowski, W., Mitchell, G.A., and Labuda, D. (1994). Alu sequences in the coding regions of mRNA: a source of protein variability. *Trends Genet* **10**: 188-193.

Malm, J., Hellman, J., Magnusson, H., Laurell, C.B., and Lilja, H. (1996). Isolation and characterization of the major gel proteins in human semen, semenogelin I and semenogelin II. *Eur J Biochem* **238**: 48-53.

Manjunath, R., Chung, S.I., and Mukherjee, A.B. (1984). Crosslinking of uteroglobin by transglutaminase. *Biochem Biophys Res Commun* **121**: 400-407.

Mann, T. and Lutwak-Mann, C. 1981. Male reproductive function and semen. Themes and trends in physiology, biochemistry and investigative andrology. Springer-Verlag, Berlin.

Mantile, G., Miele, L., Cordella-Miele, E., Singh, G., Katyal, S.L., and Mukherjee, A.B. (1993). Human Clara cell 10-kDa protein is the counterpart of rabbit uteroglobin. *J Biol Chem* **268**: 20343-20351.

Marsh, M.N. (1997). Transglutaminase, gluten and celiac disease: food for thought. Transglutaminase is identified as the autoantigen of celiac disease. *Nat Med* **3**: 725-6.

Marshburn, P.B. and Kutteh, W.H. (1994). The role of antisperm antibodies in infertility. *Fertil Steril* **61**: 799-811.

Martinez, J., Chalupowicz, D.G., Roush, R.K., Sheth, A., and Barsigian, C. (1994). Transglutaminase-mediated processing of fibronectin by endothelial cell monolayers. *Biochemistry* **33**: 2538-2545.

Matsuki, M., Yamashita, F., Ishida-Yamamoto, A., Yamada, K., Kinoshita, C., Fushiki, S., Ueda, E., Morishima, Y., Tabata, K., Yasuno, H., Hashida, M., Iizuka, H., Ikawa, M., Okabe, M., Kondoh, G., Kinoshita, T., Takeda, J., and Yamanishi, K. (1998). Defective stratum corneum and early neonatal death in mice lacking the gene for transglutaminase 1 (keratinocyte transglutaminase). *Proc Natl Acad Sci U S A* **95**: 1044-1049.

McDonagh, J. and Fukue, H. (1996). Determinants of substrate specificity for factor XIII. *Semin Thromb Hemost* **22**: 369-376.

McNeal, J.E. (1988). Normal histology of the prostate. *Am J Surg Pathol* **12**: 619-633.

McNeal, J.E. (1992). Cancer volume and site of origin of adenocarcinoma in the prostate: relationship to local and distant spread. *Hum Pathol* **23**: 258-266.

McNeal, J.E. (1997). Anatomy and normal histology of the human prostate. In *Pathology of the prostate* (ed. C.S. Foster and D.G. Bostwick), pp. 19-34. W.B. Saunders Company, Philadelphia London Toronto Montreal Sydney Tokyo.

Melino, G., Annicchiarico-Petruzzelli, M., Piredda, L., Candi, E., Gentile, V., Davies, P.J., and Piacentini, M. (1994). Tissue transglutaminase and apoptosis: sense and antisense transfection studies with human neuroblastoma cells. *Mol Cell Biol* **14**: 6584-6596.

Melino, G., Draoui, M., Bellincampi, L., Bernassola, F., Bernardini, S., Piacentini, M., Reichert, U., and Cohen, P. (1997). Retinoic acid receptors alpha and gamma mediate the induction of "tissue" transglutaminase activity and apoptosis in human neuroblastoma cells. *Exp Cell Res* **235**: 55-61.

Melino, G. and Piacentini, M. (1998). 'Tissue' transglutaminase in cell death: a downstream or a multifunctional upstream effector? *FEBS Lett* **430**: 59-63.

Melino, G., De Laurenzi, V., Catani, M.V., Terrinoni, A., Ciani, B., Candi, E., Marekov, L., and Steinert, P.M. (1998). The cornified envelope: a model of cell death in the skin. *Results Probl Cell Differ* **24**: 175-212.

Menge, A.C. and Naz, R.K. (1988). Immunologic reactions involving sperm cells and preimplantation embryos. *Am J Reprod Immunol Microbiol* **18**: 17-20.

Miele, L., Cordella-Miele, E., Mantile, G., Peri, A., and Mukherjee, A.B. (1994). Uteroglobin and uteroglobin-like proteins: the uteroglobin family of proteins. *J Endocrinol Invest* **17**: 679-692.

Mikolajczyk, S.D., Millar, L.S., Marker, K.M., Grauer, L.S., Goel, A., Cass, M.M.J., Kumar, A., and Saedi, M.S. (1997). Ala217 is important for the catalytic function and autoactivation of prostate-specific human kallikrein 2. *Eur J Biochem* **246**: 440-446.

Mikolajczyk, S.D., Millar, L.S., Kumar, A., and Saedi, M.S. (1998). Human glandular kallikrein, hK2, shows arginine-restricted specificity and forms complexes with plasma protease inhibitors. *Prostate* **34**: 44-50.

Mirza, A., Liu, S.L., Frizell, E., Zhu, J., Maddukuri, S., Martinez, J., Davies, P., Schwarting, R., Norton, P., and Zern, M.A. (1997). A role for tissue transglutaminase in hepatic injury and fibrogenesis, and its regulation by NF-kappaB. *Am J Physiol* **272**: G281-G288.

Mitchell, G.A., Labuda, D., Fontaine, G., Saudubray, J.M., Bonnefont, J.P., Lyonnet, S., Brody, L.C., Steel, G., Obie, C., and Valle, D. (1991). Splice-mediated insertion of an Alu sequence inactivates ornithine delta-aminotransferase: a role for Alu elements in human mutation. *Proc Natl Acad Sci U S A* **88**: 815-819.

Molberg, O., McAdam, S.N., Korner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Noren, O., Roepstorff, P., Lundin, K.E., Sjostrom, H., and Sollid, L.M. (1998). Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat Med* **4**: 713-7.

Monsonego, A., Friedmann, I., Shani, Y., Eisenstein, M., and Schwartz, M. (1998). GTP-dependent Conformational Changes Associated with the Functional Switch between Galpha and Cross-linking Activities in Brain-derived Tissue Transglutaminase. *J Mol Biol* **282**: 713-720.

Montie, J.E. and Meyers, S.E. (1997). Defining the ideal tumor marker for prostate cancer. *Urol Clin North Am* **24**: 247-252.

Mortimer, S.T., Swan, M.A., and Mortimer, D. (1998). Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. *Hum Reprod* **13**: 2139-2146.

Mosesson, M.W. (1992). The roles of fibrinogen and fibrin in hemostasis and thrombosis. *Semin Hematol* **29**: 177-88.

Mukherjee, D.C., Agrawal, A.K., Manjunath, R., and Mukherjee, A.B. (1983). Suppression of epididymal sperm antigenicity in the rabbit by uteroglobin and transglutaminase in vitro. *Science* **219**: 989-991.

Murphy, G.P., Kenny, G.M., Ragde, H., Wolfert, R.L., Boynton, A.L., Holmes, E.H., Misrock, S.L., Bartsch, G., Klocker, H., Pointner, J., Reissigl, A., McLeod, D.G., Douglas, T., Morgan, T., and Gilbaugh, J., Jr. (1998). Measurement of serum prostate-specific membrane antigen, a new prognostic marker for prostate cancer. *Urology* **51**: 89-97.

Murtha, P., Tindall, D.J., and Young, C.Y. (1993). Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5' promoter region of the gene. *Biochemistry* **32**: 6459-6464.

Murthy, S.N., Wilson, J.H., Lukas, T.J., Kuret, J., and Lorand, L. (1998). Cross-linking sites of the human tau protein, probed by reactions with human transglutaminase. *J Neurochem* **71**: 2607-14.

Muszbek, L., Haramura, G., and Polgar, J. (1995). Transformation of cellular factor XIII into an active zymogen transglutaminase in thrombin-stimulated platelets. *Thromb Haemost* **73**: 702-705.

Muszbek, L., Adany, R., and Mikkola, H. (1996). Novel aspects of blood coagulation factor XIII. I. Structure, distribution, activation, and function. *Crit Rev Clin Lab Sci* **33**: 357-421.

Nagy, L., Saydak, M., Shipley, N., Lu, S., Basilion, J.P., Yan, Z.H., Syka, P., Chandraratna, R.A., Stein, J.P., Heyman, R.A., and Davies, P.J. (1996). Identification and characterization of a versatile retinoid response element (retinoic acid receptor response element-retinoid X receptor response element) in the mouse tissue transglutaminase gene promoter. *J Biol Chem* **271**: 4355-4365.

Nagy, L., Thomazy, V.A., Saydak, M.M., Stein, J.P., and Davies, P.J.A. (1997). The promoter of the mouse tissue transglutaminase gene directs tissue-specific, retinoid-regulated and apoptosis-linked expression. *Cell Death Differ* **4**: 435-547.

Nakaoka, H., Perez, D.M., Baek, K.J., Das, T., Husain, A., Misono, K., Im, M.J., and Graham, R.M. (1994). Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. *Science* **264**: 1593-1596.

Naz, R.K. and Menge, A.C. (1994). Antisperm antibodies: origin, regulation, and sperm reactivity in human infertility. *Fertil Steril* **61**: 1001-1013.

Nelson, P.S., Ng, W.L., Schummer, M., True, L.D., Liu, A.Y., Bumgarner, R.E., Ferguson, C., Dimak, A., and Hood, L. (1998). An expressed-sequence-tag database of the human prostate: sequence analysis of 1168 cDNA clones. *Genomics* **47**: 12-25.

Nelson, P.S., Gan, L., Ferguson, C., Moss, P., Gelinas, R., Hood, L., and Wang, K. (1999). Molecular cloning and characterization of prostase, an androgen- regulated serine protease with prostate-restricted expression. *Proc Natl Acad Sci U S A* **96**: 3114-3119.

Nemes, Z., Jr., Friis, R.R., Aeschlimann, D., Saurer, S., Paulsson, M., and Fesus, L. (1996). Expression and activation of tissue transglutaminase in apoptotic cells of involuting rodent mammary tissue. *Eur J Cell Biol* **70**: 125-33.

Nemes, Z., Jr., Adany, R., Balazs, M., Boross, P., and Fesus, L. (1997). Identification of cytoplasmic actin as an abundant glutaminyl substrate for tissue transglutaminase in HL-60 and U937 cells undergoing apoptosis. *J Biol Chem* **272**: 20577-83.

Noordzij, M.A., van Steenbrugge, G.J., van der Kwast, T.H., and Schroder, F.H. (1995). Neuroendocrine cells in the normal, hyperplastic and neoplastic prostate. *Urol Res* **22**: 333-341.

Noti, J.D., Reinemann, B.C., and Petrus, M.N. (1996). Sp1 binds two sites in the CD11c promoter in vivo specifically in myeloid cells and cooperates with AP1 to activate transcription. *Mol Cell Biol* **16**: 2940-2950.

Nunes, I., Shapiro, R.L., and Rifkin, D.B. (1995). Characterization of latent TGF-beta activation by murine peritoneal macrophages. *J Immunol* **155**: 1450-9.

Nunes, I., Gleizes, P.E., Metz, C.N., and Rifkin, D.B. (1997). Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. *J Cell Biol* **136**: 1151-63.

Nurminskaya, M., Magee, C., Nurminsky, D., and Linsenmayer, T.F. (1998). Plasma transglutaminase in hypertrophic chondrocytes: expression and cell-specific intracellular activation produce cell death and externalization. *J Cell Biol* **142**: 1135-44.

Oesterling, J.E. (1991). Prostate specific antigen: a critical assessment of the most useful tumor marker for adenocarcinoma of the prostate. *J Urol* **145**: 907-923.

Ohtaka-Maruyama, C., Wang, X., Ge, H., and Chepelinsky, A.B. (1998). Overlapping Sp1 and AP2 binding sites in a promoter element of the lens- specific MIP gene. *Nucleic Acids Res* **26**: 407-414.

Oliverio, S., Amendola, A., Di Sano, F., Farrace, M.G., Fesus, L., Nemes, Z., Piredda, L., Spinedi, A., and Piacentini, M. (1997). Tissue transglutaminase-dependent posttranslational modification of the retinoblastoma gene product in promonocytic cells undergoing apoptosis. *Mol Cell Biol* **17**: 6040-8.

Olsson, C.A., de Vries, G.M., Buttyan, R., and Katz, A.E. (1997). Reverse transcriptase-polymerase chain reaction assays for prostate cancer. *Urol Clin North Am* **24**: 367-378.

Pan, H.Q., Wang, Y.P., Chissoe, S.L., Bodenteich, A., Wang, Z., Iyer, K., Clifton, S.W., Crabtree, J.S., and Roe, B.A. (1994). The complete nucleotide sequences of the SacBII Kan domain of the P1 pAD10-SacBII cloning vector and three cosmid cloning vectors: pTCF, svPHEP, and LAWRIST16. *Genet Anal Tech Appl* **11**: 181-186.

Pang, S., Dannull, J., Kaboo, R., Xie, Y., Tso, C.L., Michel, K., Dekernion, J.B., and Belldegrun, A.S. (1997). Identification of a positive regulatory element responsible for tissue-specific expression of prostate-specific antigen. *Cancer Res* **57**: 495-499.

Paonessa, G., Metafora, S., Tajana, G., Abrescia, P., De Santis, A., Gentile, V., and Porta, R. (1984). Transglutaminase-mediated modifications of the rat sperm surface in vitro. *Science* **226**: 852-855.

Parameswaran, K.N., Cheng, X.F., Chen, E.C., Velasco, P.T., Wilson, J.H., and Lorand, L. (1997). Hydrolysis of gamma:epsilon isopeptides by cytosolic transglutaminases and by coagulation factor XIIIa. *J Biol Chem* **272**: 10311-7.

Park, E.S., Won, J.H., Han, K.J., Suh, P.G., Ryu, S.H., Lee, H.S., Yun, H.Y., Kwon, N.S., and Baek, K.J. (1998). Phospholipase C-delta1 and oxytocin receptor signalling: evidence of its role as an effector. *Biochem J* **331**: 283-9.

Parker, S.L., Tong, T., Bolden, S., and Wingo, P.A. (1996). Cancer statistics. *CA Cancer J Clin* **46**: 5-27.

Parmentier, L., Blanchet-Bardon, C., Nguyen, S., Prud'homme, J.F., Dubertret, L., and Weissenbach, J. (1995). Autosomal recessive lamellar ichthyosis: identification of a new mutation in transglutaminase 1 and evidence for genetic heterogeneity. *Hum Mol Genet* **4**: 1391-1395.

Partin, A.W. and Coffey, D.S. (1998). The molecular biology, endocrinology, and physiology of the prostate and seminal vesicles. In *Campbell's Urology* (ed. P.C. Walsh, A.B. Retik, J. Vaughan, E. D.,

and A.J. Wein), pp. 1381-1428. W. B. Saunders Company, Philadelphia London Toronto Montreal Sydney Tokyo.

Peluso, G., Porta, R., Esposito, C., Tufano, M.A., Toraldo, R., Vuotto, M.L., Ravagnan, G., and Metafora, S. (1994). Suppression of rat epididymal sperm immunogenicity by a seminal vesicle secretory protein and transglutaminase both in vivo and in vitro. *Biol Reprod* **50**: 593-602.

Peter, A., Lilja, H., Lundwall, A., and Malm, J. (1998). Semenogelin I and semenogelin II, the major gel-forming proteins in human semen, are substrates for transglutaminase. *Eur J Biochem* **252**: 216-221.

Phillips, M.A., Stewart, B.E., Qin, Q., Chakravarty, R., Floyd, E.E., Jetten, A.M., and Rice, R.H. (1990). Primary structure of keratinocyte transglutaminase. *Proc Natl Acad Sci U S A* **87**: 9333-9337.

Phillips, M.A., Stewart, B.E., and Rice, R.H. (1992). Genomic structure of keratinocyte transglutaminase. Recruitment of new exon for modified function. *J Biol Chem* **267**: 2282-2286.

Piacentini, M., Annicchiarico Petruzzelli, M., Oliverio, S., Piredda, L., Biedler, J.L., and Melino, E. (1992). Phenotype-specific "tissue" transglutaminase regulation in human neuroblastoma cells in response to retinoic acid: correlation with cell death by apoptosis. *Int J Cancer* **52**: 271-278.

Piacentini, M. and Autuori, F. (1994). Immunohistochemical localization of tissue transglutaminase and Bcl-2 in rat uterine tissues during embryo implantation and post-partum involution. *Differentiation* **57**: 51-61.

Piacentini, M. (1995). Tissue transglutaminase: a candidate effector element of physiological cell death. *Curr Top Microbiol Immunol* **200**: 163-175.

Piredda, L., Amendola, A., Colizzi, V., Davies, P.J., Farrace, M.G., Fraziano, M., Gentile, V., Uray, I., Piacentini, M., and Fesus, L. (1997). Lack of 'tissue' transglutaminase protein cross-linking leads to leakage of macromolecules from dying cells: relationship to development of autoimmunity in MRLlpr/lpr mice. *Cell Death Differ* **4**: 463-472.

Piredda, L., Farrace, M.G., Lo Bello, M., Malorni, W., Melino, G., Petruzzelli, R., and Piacentini, M. (1999). Identification of 'tissue' transglutaminase binding proteins in neural cells committed to apoptosis. *Faseb J* **13**: 355-64.

Polakowska, R.R., Piacentini, M., Bartlett, R., Goldsmith, L.A., and Haake, A.R. (1994). Apoptosis in human skin development: morphogenesis, periderm, and stem cells. *Dev Dyn* **199**: 176-188.

Porta, R., Esposito, C., De Santis, A., Fusco, A., Iannone, M., and Metafora, S. (1986). Sperm maturation in human semen: role of transglutaminase-mediated reactions. *Biol Reprod* **35**: 965-970.

Porta, R., Esposito, C., Metafora, S., Malorni, A., Pucci, P., Siciliano, R., and Marino, G. (1991). Mass spectrometric identification of the amino donor and acceptor sites in a transglutaminase protein substrate secreted from rat seminal vesicles. *Biochemistry* **30**: 3114-20.

Porta, R., Metafora, S., Esposito, C., Mariniello, L., Persico, P., Mancuso, F., and Peluso, G. (1993). Biological activities of a major protein secreted from the rat seminal vesicles after structural modification catalyzed by transglutaminase in vitro. *Immunopharmacology* **25**: 179-188.

Prescott, J.L., Blok, L., and Tindall, D.J. (1998). Isolation and androgen regulation of the human homeobox cDNA, NKX3.1. *Prostate* **35**: 71-80.

Quan, C.P., Roux, C., Pillot, J., and Bouvet, J.P. (1990). Delineation between T and B suppressive molecules from human seminal plasma: II. Spermine is the major suppressor of T-lymphocytes in vitro. *Am J Reprod Immunol* **22**: 64-9.

Raghunath, M., Hopfner, B., Aeschlimann, D., Luthi, U., Meuli, M., Altermatt, S., Gobet, R., Bruckner-Tuderman, L., and Steinmann, B. (1996). Cross-linking of the dermo-epidermal junction of skin regenerating from keratinocyte autografts. Anchoring fibrils are a target for tissue transglutaminase. *J Clin Invest* **98**: 1174-84.

Reiter, R.E., Gu, Z., Watabe, T., Thomas, G., Szigeti, K., Davis, E., Wahl, M., Nisitani, S., Yamashiro, J., Le Beau, M.M., Loda, M., and Witte, O.N. (1998). Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. *Proc Natl Acad Sci U S A* **95**: 1735-1740.

Rice, R.H., Mehrpouyan, M., O'Callahan, W., Parenteau, N.L., and Rubin, A.L. (1992). Keratinocyte transglutaminase: differentiation marker and member of an extended family. *Epithelial Cell Biol* **1**: 128-137.

Riegman, P.H., Vlietstra, R.J., van der Korput, H.A., Romijn, J.C., and Trapman, J. (1991a). Identification and androgen-regulated expression of two major human glandular kallikrein-1 (hGK-1) mRNA species. *Mol Cell Endocrinol* **76**: 181-190.

Riegman, P.H., Vlietstra, R.J., van der Korput, J.A., Brinkmann, A.O., and Trapman, J. (1991b). The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. *Mol Endocrinol* **5**: 1921-1930.

Risinger, M.A., Dotimas, E.M., and Cohen, C.M. (1992). Human erythrocyte protein 4.2, a high copy number membrane protein, is N-myristylated. *J Biol Chem* **267**: 5680-5.

Rittenhouse, H.G., Finlay, J.A., Mikolajczyk, S.D., and Partin, A.W. (1998). Human kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. *Crit Rev Clin Lab Sci* **35**: 275-368.

Ritter, S.J. and Davies, P.J. (1998). Identification of a transforming growth factor-beta1/Bone morphogenetic protein 4 (TGF-beta1/BMP4) response element within the mouse tissue transglutaminase gene promoter. *J Biol Chem* **273**: 12798-12806.

Rittmaster, R.S., Manning, A.P., Wright, A.S., Thomas, L.N., Whitefield, S., Norman, R.W., Lazier, C.B., and Rowden, G. (1995). Evidence for atrophy and apoptosis in the ventral prostate of rats given the 5 alpha-reductase inhibitor finasteride. *Endocrinology* **136**: 741-748.

Robert, M. and Gagnon, C. (1995). Sperm motility inhibitor from human seminal plasma: association with semen coagulum. *Hum Reprod* **10**: 2192-2197.

Robert, M. and Gagnon, C. (1996). Purification and characterization of the active precursor of a human sperm motility inhibitor secreted by the seminal vesicles: identity with semenogelin. *Biol Reprod* **55**: 813-21.

Rodriguez, R., Schuur, E.R., Lim, H.Y., Henderson, G.A., Simons, J.W., and Henderson, D.R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res* **57**: 2559-2563.

Romijn, J.C. (1990). Polyamines and transglutaminase actions. *Andrologia* **22**: 83-91.

Romijn, J.C., Erkens-Schulze, S., and Schroder, F.H. (1995). Perspectives of the use of tissue culture methods as an alternative to human prostate cancer xenografts in nude mice. In *Contributions to oncology* (ed. W. Arnold). **51**, pp 209-213. Karger, Basel.

Roop, D. (1995). Defects in the barrier. *Science* **267**: 474-5.

Russell, L.J., Digiovanna, J.J., Rogers, G.R., Steinert, P.M., Hashem, N., Compton, J.G., and Bale, S.K. (1995). Mutations in the gene for transglutaminase 1 in autosomal recessive lamellar ichthyosis. *Nature Genet* **9**: 279-283.

Rybicki, A.C., Musto, S., and Schwartz, R.S. (1995). Identification of a band-3 binding site near the N-terminus of erythrocyte membrane protein 4.2. *Biochem J* **309**: 677-81.

Rybicki, A.C., Schwartz, R.S., Hustedt, E.J., and Cobb, C.E. (1996). Increased rotational mobility and extractability of band 3 from protein 4.2-deficient erythrocyte membranes: evidence of a role for protein 4.2 in strengthening the band 3-cytoskeleton linkage. *Blood* **88**: 2745-53.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sartorelli, V., Webster, K.A., and Kedes, L. (1990). Muscle-specific expression of the cardiac alpha-actin gene requires MyoD1, CArG-box binding factor, and Sp1. *Genes Dev* 4: 1811-1822.

Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**: 467-70.

Schittny, J.C., Paulsson, M., Vallan, C., Burri, P.H., Kedei, N., and Aeschlimann, D. (1997). Protein cross-linking mediated by tissue transglutaminase correlates with the maturation of extracellular matrices during lung development. *Am J Respir Cell Mol Biol* **17**: 334-43.

Schuur, E.R., Henderson, G.A., Kmetec, L.A., Miller, J.D., Lamparski, H.G., and Henderson, D.R. (1996). Prostate-specific antigen expression is regulated by an upstream enhancer. *J Biol Chem* **271**: 7043-7051.

Sciavolino, P.J., Abrams, E.W., Yang, L., Austenberg, L.P., Shen, M.M., and Abate-Shen, C. (1997). Tissue-specific expression of murine Nkx3.1 in the male urogenital system. *Dev Dyn* **209**: 127-38.

Segawa, T., Takebayashi, H., Kakehi, Y., Yoshida, O., Narumiya, S., and Kakizuka, A. (1998). Prostate-specific amplification of expanded polyglutamine expression: a novel approach for cancer gene therapy. *Cancer Res* **58**: 2282-2287.

Seitz, J. and Aumuller, G. (1990). Biochemical properties of secretory proteins from rat seminal vesicles. *Andrologia* **22**: 25-32.

Seitz, J., Keppler, C., Rausch, U., and Aumuller, G. (1990). Immunohistochemistry of secretory transglutaminase from rodent prostate. *Histochemistry* **93**: 525-530.

Seitz, J., Keppler, C., Huntemann, S., Rausch, U., and Aumuller, G. (1991a). Purification and molecular characterization of a secretory transglutaminase from coagulating gland of the rat. *Biochim Biophys Acta* **1078**: 139-146.

Seitz, J., Keppler, C., and Huntemann, S.B. (1991b). Purification and characterization of transglutaminases from the genital tract of the male rat. *J Chromatogr* **587**: 55-60.

Serafini-Fracassini, D., Del Duca, S., and Beninati, S. (1995). Plant transglutaminases. *Phytochemistry* **40**: 355-65.

Shan, J.D., Porvari, K., Ruokonen, M., Karhu, A., Launonen, V., Hedberg, P., Oikarinen, J., and Vihko, P. (1997). Steroid-involved transcriptional regulation of human genes encoding prostatic acid phosphatase, prostate-specific antigen, and prostate- specific glandular kallikrein. *Endocrinology* **138**: 3764-3770.

Shapiro, M.B. and Senapathy, P. (1987). RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res* **15**: 7155-7174.

Sharp, P.A. (1981). Speculations on RNA splicing. *Cell* **23**: 643-646.

Singer, M.A., Hortsch, M., Goodman, C.S., and Bentley, D. (1992). Annulin, a protein expressed at limb segment boundaries in the grasshopper embryo, is homologous to protein cross-linking transglutaminases. *Dev Biol* **154**: 143-159.

Singh, U.S., Erickson, J.W., and Cerione, R.A. (1995). Identification and biochemical characterization of an 80 kilodalton GTP-binding/transglutaminase from rabbit liver nuclei. *Biochemistry* **34**: 15863-15871.

Singh, U.S. and Cerione, R.A. (1996). Biochemical effects of retinoic acid on GTP-binding Protein/Transglutaminases in HeLa cells. Stimulation of GTP-binding and transglutaminase activity, membrane association, and phosphatidylinositol lipid turnover. *J Biol Chem* **271**: 27292-27298.

Smethurst, P.A. and Griffin, M. (1996). Measurement of tissue transglutaminase activity in a permeabilized cell system: its regulation by Ca2+ and nucleotides. *Biochem J* **313**: 803-808.

Smit, A.F., Toth, G., Riggs, A.D., and Jurka, J. (1995). Ancestral, mammalian-wide subfamilies of LINE-1 repetitive sequences. *J Mol Biol* **246**: 401-417.

Sogawa, K., Imataka, H., Yamasaki, Y., Kusume, H., Abe, H., and Fujii-Kuriyama, Y. (1993). cDNA cloning and transcriptional properties of a novel GC box-binding protein, BTEB2. *Nucleic Acids Res* **21**: 1527-1532.

Solin, T., Kontturi, M., Pohlmann, R., and Vihko, P. (1990). Gene expression and prostate specificity of human prostatic acid phosphatase (PAP): evaluation by RNA blot analyses. *Biochim Biophys Acta* **1048**: 72-77.

Stearns, M.E. and McGarvey, T. (1992). Prostate cancer: therapeutic, diagnostic, and basic studies. *Lab Invest* **67**: 540-552.

Steinert, P.M. and Marekov, L.N. (1995). The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope. *J Biol Chem* **270**: 17702-17711.

Steinert, P.M., Chung, S.I., and Kim, S.Y. (1996a). Inactive zymogen and highly active proteolytically processed membrane- bound forms of the transglutaminase 1 enzyme in human epidermal keratinocytes. *Biochem Biophys Res Commun* **221**: 101-6.

Steinert, P.M., Kim, S.Y., Chung, S.I., and Marekov, L.N. (1996b). The transglutaminase 1 enzyme is variably acylated by myristate and palmitate during differentiation in epidermal keratinocytes. *J Biol Chem* **271**: 26242-50.

Steinert, P.M., Candi, E., Kartasova, T., and Marekov, L. (1998). Small proline-rich proteins are cross-bridging proteins in the cornified cell envelopes of stratified squamous epithelia. *J Struct Biol* **122**: 76-85.

Steinhoff, M., Eicheler, W., Holterhus, P.M., Rausch, U., Seitz, J., and Aumuller, G. (1994). Hormonally induced changes in apocrine secretion of transglutaminase in the rat dorsal prostate and coagulating gland. *Eur J Cell Biol* **65**: 49-59.

Sung, L.A., Chien, S., Chang, L.S., Lambert, K., Bliss, S.A., Bouhassira, E.E., Nagel, R.L., Schwartz, R.S., and Rybicki, A.C. (1990). Molecular cloning of human protein 4.2: a major component of the erythrocyte membrane. *Proc Natl Acad Sci U S A* **87**: 955-959.

Sung, L.A., Chien, S., Fan, Y.S., Lin, C.C., Lambert, K., Zhu, L., Lam, J.S., and Chang, L.S. (1992). Human erythrocyte protein 4.2: isoform expression, differential splicing, and chromosomal assignment. *Blood* **79**: 2763-2770.

Szecsi, P.B. and Lilja, H. (1993). Gastricsin-mediated proteolytic degradation of human seminal fluid proteins at pH levels found in the human vagina. *J Androl* **14**: 351-358.

Taipale, J., Miyazono, K., Heldin, C.H., and Keski-Oja, J. (1994). Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. *J Cell Biol* **124**: 171-81.

Takahashi, N., Hayano, T., and Suzuki, M. (1989). Peptidyl-prolyl cis-trans isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **337**: 473-475.

Takayama, T.K., Fujikawa, K., and Davie, E.W. (1997). Characterization of the precursor of prostate-specific antigen. Activation by trypsin and by human glandular kallikrein. *J Biol Chem* **272**: 21582-21588.

Tarcsa, E., Candi, E., Kartasova, T., Idler, W.W., Marekov, L.N., and Steinert, P.M. (1998). Structural and transglutaminase substrate properties of the small proline-rich 2 family of cornified cell envelope proteins. *J Biol Chem* **273**: 23297-303.

Tauber, P.F., Zaneveld, L.J., Propping, D., and Schumacher, G.F. (1976). Components of human split ejaculates. II. Enzymes and proteinase inhibitors. *J Reprod Fertil* **46**: 165-171.

Tauber, P.F., Propping, D., Schumacher, G.F.B., and Zaneveld, L.J.D. (1980). Biochemical aspects of the coagulation and liquefaction of human semen. *J Androl* **1**: 280-288.

Tenniswood, M. (1997). Apoptosis, tumour invasion and prostate cancer. *Br J Urol* **79**: 27-34.

Thacher, S.M. and Rice, R.H. (1985). Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. *Cell* **40**: 685-95.

Thomazy, V. and Fesus, L. (1989). Differential expression of tissue transglutaminase in human cells. An immunohistochemical study. *Cell Tissue Res* **255**: 215-224.

Thomazy, V.A. and Davies, P.J. (1999). Expression of tissue transglutaminase in the developing chicken limb is associated both with apoptosis and endochondral ossification. *Cell Death Differ* 6: 146-54.

Thompson, J.F., Lira, M.E., Lloyd, D.B., Hayes, L.S., Williams, S., and Elsenboss, L. (1993). Cholesterol 7 alpha hydroxylase promoter separated from cyclophilin pseudogene by Alu sequence. *Biochim Biophys Acta* **1168**: 239-242.

Tokunaga, F., Yamada, M., Miyata, T., Ding, Y.L., Hiranaga Kawabata, M., Muta, T., Iwanaga, S., Ichinose, A., and Davie, E.W. (1993). Limulus hemocyte transglutaminase. Its purification and characterization, and identification of the intracellular substrates. *J Biol Chem* **268**: 252-261.

Trapman, J. and Cleutjens, K.B.J.M. (1998). Androgen regulated gene expression in prostate cancer. *Sem Cancer Biol* **8**: 29-36.

Troyer, J.K., Beckett, M.L., and Wright, G.L., Jr. (1995). Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int J Cancer* **62**: 552-558.

Upchurch, H.F., Conway, E., Patterson, M.K., Jr., and Maxwell, M.D. (1991). Localization of cellular transglutaminase on the extracellular matrix after wounding: characteristics of the matrix bound enzyme. *J Cell Physiol* **149**: 375-82.

van de Wal, Y., Kooy, Y., van Veelen, P., Pena, S., Mearin, L., Papadopoulos, G., and Koning, F. (1998). Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. *J Immunol* **161**: 1585-8.

van Weerden, W.M., de Ridder, C.M., Verdaasdonk, C.L., Romijn, J.C., van der Kwast, T.H., Schroder, F.H., and van Steenbrugge, G.J. (1996). Development of seven new human prostate tumor xenograft models and their histopathological characterization. *Am J Pathol* **149**: 1055-1062.

Vasmatzis, G., Essand, M., Brinkmann, U., Lee, B., and Pastan, I. (1998). Discovery of three genes specifically expressed in human prostate by expressed sequence tag database analysis. *Proc Natl Acad Sci U S A* **95**: 300-304.

Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. (1995). Serial analysis of gene expression. *Science* **270**: 484-7.

Verderio, E., Nicholas, B., Gross, S., and Griffin, M. (1998). Regulated expression of tissue transglutaminase in Swiss 3T3 fibroblasts: effects on the processing of fibronectin, cell attachment, and cell death. *Exp Cell Res* **239**: 119-38.

Verkaik, N.S., Schroder, F.H., and Romijn, J.C. (1997). Clinical usefulness of RT-PCR detection of hematogenous prostate cancer spread [editorial]. *Urol Res* **25**: 373-384.

Vessella, R.L. and Lange, P.H. (1997). Issues in the assessment of prostate-specific antigen immunoassays. An update. *Urol Clin North Am* **24**: 261-268.

Vezza, R., Habib, A., and FitzGerald, G.A. (1999). Differential signaling by the thromboxane receptor isoforms via the novel GTP-binding protein, Gh. *J Biol Chem* **274**: 12774-9.

Villard, E., Tiret, L., Visvikis, S., Rakotovao, R., Cambien, F., and Soubrier, F. (1996). Identification of new polymorphisms of the angiotensin I-converting enzyme (ACE) gene, and study of their relationship to plasma ACE levels by two-QTL segregation-linkage analysis. *Am J Hum Genet* **58**: 1268-1278.

Wang, M., Kim, I.G., Steinert, P.M., and McBride, O.W. (1994). Assignment of the human transglutaminase 2 (TGM2) and transglutaminase 3 (TGM3) genes to chromosome 20q11.2. *Genomics* **23**: 721-722.

Wei, C., Willis, R.A., Tilton, B.R., Looney, R.J., Lord, E.M., Barth, R.K., and Frelinger, J.G. (1997). Tissue-specific expression of the human prostate-specific antigen gene in transgenic mice: implications for tolerance and immunotherapy. *Proc Natl Acad Sci U S A* **94**: 6369-6374.

White, R.A., Peters, L.L., Adkison, L.R., Korsgren, C., Cohen, C.M., and Lux, S.E. (1992). The murine pallid mutation is a platelet storage pool disease associated with the protein 4.2 (pallidin) gene. *Nat Genet* **2**: 80-3.

Wickens, M. (1990). How the messenger got its tail: addition of poly(A) in the nucleus. *Trends Biochem Sci* **15**: 277-281.

Willenbrink, W., Halaschek, J., Schuffenhauer, S., Kunz, J., and Steinkasserer, A. (1995). Cyclophilin A, the major intracellular receptor for the immunosuppressant cyclosporin A, maps to chromosome 7p11.2-p13: four pseudogenes map to chromosomes 3, 10, 14, and 18. *Genomics* **28**: 101-104.

Williams-Ashman, H.G. and Lockwood, D.H. (1970). Role of polyamines in reproductive physiology and sex hormone action. *Ann NY Acad Sci* **171**: 882-894.

Williams-Ashman, H.G., Notides, A.C., Pabalan, S.S., and Lorand, L. (1972). Transamidase reactions involved in the enzymic coagulation of semen: isolation of -glutamyl- -lysine dipeptide from clotted secretion protein of guinea pig seminal vesicle. *Proc Natl Acad Sci U S A* **69**: 2322-2325.

Williams-Ashman, H.G. (1984). Transglutaminases and the clotting of mammalian seminal fluids. *Mol Cell Biochem* **58**: 51-61.

Wilson, E.M. and French, F.S. (1980). Biochemical homology between rat dorsal prostate and coagulating gland. Purification of a major androgen-induced protein. *J Biol Chem* **255**: 10946-10953.

Wilson, M.J. (1995). Proteases in prostate development, function, and pathology. *Microsc Res Tech* **30**: 305-18.

Wright, G.L., Haley, C., Beckett, M.L., and Schellhammer, P.F. (1995). Expression of prostate-specific membrane antigen in normal, benign, and malignant prostate tissues. *Urol Oncol* **1**: 18-28.

Xue, Y., Smedts, F., Verhofstad, A., Debruyne, F., de la Rosette, J., and Schalken, J. (1998). Cell kinetics of prostate exocrine and neuroendocrine epithelium and their differential interrelationship: new perspectives. *Prostate Suppl* **8**: 62-73.

Yamanishi, K., Inazawa, J., Liew, F.M., Nonomura, K., Ariyama, T., Yasuno, H., Abe, T., Doi, H., Hirano, J., and Fukushima, S. (1992). Structure of the gene for human transglutaminase 1. *J Biol Chem* **267**: 17858-17863.

Yawata, Y. (1994a). Band 4.2 abnormalities in human red cells. *Am J Med Sci* **307**: 190-203.

Yawata, Y. (1994b). Red cell membrane protein band 4.2: phenotypic, genetic and electron microscopic aspects. *Biochim Biophys Acta* **1204**: 131-48.

Yee, V.C., Pedersen, L.C., Le Trong, I., Bishop, P.D., Stenkamp, R.E., and Teller, D.C. (1994). Three-dimensional structure of a transglutaminase: human blood coagulation factor XIII. *Proc Natl Acad Sci U S A* **91**: 7296-300.

Yee, V.C., Le Trong, I., Bishop, P.D., Pedersen, L.C., Stenkamp, R.E., and Teller, D.C. (1996). Structure and function studies of factor XIIIa by x-ray crystallography. *Semin Thromb Hemost* **22**: 377-84.

Young, C.Y., Andrews, P.E., Montgomery, B.T., and Tindall, D.J. (1992). Tissue-specific and hormonal regulation of human prostate-specific glandular kallikrein. *Biochemistry* **31**: 818-824.

Young, C.Y., Seay, T., Hogen, K., Charlesworth, M.C., Roche, P.C., Klee, G.G., and Tindall, D.J. (1996). Prostate-specific human kallikrein (hK2) as a novel marker for prostate cancer. *Prostate Suppl* **7**: 17-24.

Yu, D.C., Sakamoto, G.T., and Henderson, D.R. (1999). Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Res* **59**: 1498-504.

Yulug, I.G., Yulug, A., and Fisher, E.M. (1995). The frequency and position of Alu repeats in cDNAs, as determined by database searching. *Genomics* **27**: 544-548.

Zaneveld, L.J., Tauber, P.F., Port, C., Propping, D., and Schumacher, G.F. (1974). Scanning electron microscopy of the human, guinea-pig and rhesus monkey seminal coagulum. *J Reprod Fertil* **40**: 223-5.

Zeeuwen, P.L., Hendriks, W., de Jong, W.W., and Schalkwijk, J. (1997). Identification and sequence analysis of two new members of the SKALP/elafin and SPAI-2 gene family. Biochemical properties of the transglutaminase substrate motif and suggestions for a new nomenclature. *J Biol Chem* **272**: 20471-20478.

Zhang, J., Lesort, M., Guttmann, R.P., and Johnson, G.V. (1998). Modulation of the in situ activity of tissue transglutaminase by calcium and GTP. *J Biol Chem* **273**: 2288-2295.

Zhang, L., Zhou, W., Velculescu, V.E., Kern, S.E., Hruban, R.H., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. (1997). Gene expression profiles in normal and cancer cells. *Science* **276**: 1268-1272.

Zhang, L.X., Mills, K.J., Dawson, M.I., Collins, S.J., and Jetten, A.M. (1995). Evidence for the involvement of retinoic acid receptor RAR alpha-dependent signaling pathway in the induction of tissue transglutaminase and apoptosis by retinoids. *J Biol Chem* **270**: 6022-6029.

Zhu, L., Kahwash, S.B., and Chang, L.S. (1998). Developmental expression of mouse erythrocyte protein 4.2 mRNA: evidence for specific expression in erythroid cells. *Blood* **91**: 695-705.

Zincke, H., Bergstralh, E.J., Blute, M.L., Myers, R.P., Barrett, D.M., Lieber, M.M., Martin, S.K., and Oesterling, J.E. (1994). Radical prostatectomy for clinically localized prostate cancer: long-term results of 1,143 patients from a single institution. *J Clin Oncol* **12**: 2254-2263.

Summary

The human prostate is a secretory organ that is characterized by the abundant production and secretion of several proteins. Many prostate-specific proteins are excreted into semen by the luminal epithelium of the prostate where they are supposed to function in optimizing the reproductive process (reviewed in Chapter 1). One of these secretory proteins is the TGase hTG_P of which the role in semen is so far unclear. This thesis deals with the evaluation of hTG_P at the genomic and expressional level with the purpose to ultimately increase the knowledge of prostate physiology.

Based upon the expected similarity with other members of the TGase family (reviewed in Chapter 1), the complete hTG_P cDNA was isolated from a human prostate cDNA library (Chapter 2). The cDNA, with a length of 2983 bp, encodes for a protein of 684 amino acids with a predicted molecular mass of 77 kDa and a pI of 6.94. *In vitro* transcription-translation confirmed the size of the protein. The deduced amino acid sequence contains the active-site cysteine embedded in the GQCWVFA sequence, which is characteristic for functional TGases (Chapter 1). Northern blotting analysis of prostate cancer cell lines showed neither hTG_P expression in the androgen-responsive LNCaP cells nor in the androgen-independent PC3 cells. hTG_P expression was only demonstrated in the androgen-sensitive PC346C cell line revealing a 3.5 kb mRNA. The level of hTG_P mRNA expression was stimulated up to 3-fold by the presence of (synthetic) androgen. The gene encoding hTG_P, *TGM4*, was found to be localized on chromosome 3.

Chapter 3 describes the genomic organization of the *TGM4* gene. The *TGM4* gene spans 35 kb of genomic DNA, consists of 13 exons and 12 introns and has striking structural similarity with the other TGase genes. RT-PCR analysis showed a complex splicing pattern of intron 1. It was found that an anti-sense *Alu* element was involved which is located approximately 3.5 kb upstream of exon II. A major transcriptional initiation site was determined to be located at 52 bp upstream of the translational start codon. The *TGM4* gene promoter was analysed by sequencing and transfection experiments. The most prominent structural features of the *TGM4* promoter were a CATAA motif at positions -48 to -44, an Sp1 binding motif at position -96 to -87 and a *PPIP* pseudogene at positions -1276 to -563. Deletion mapping of 2.1 kb of the *TGM4* promoter in transiently transfected PC346C cells showed comparable activity of 2.1-, 1.5-, and 0.5-kb promoter fragments, demonstrating that the proximal promoter is responsible for basal activity.

By different methods, including Northern (spot) blotting and immunohistochemical staining, the expression of hTG_P was shown to be strictly confined to the prostate (Chapters 2, 3 and 5). Because of its potential value in a gene-therapeutic setting, it is important to know which mechanisms underly the prostate-specific expression of hTG_P at the molecular level. Therefore, the 2.1 kb *TGM4* promoter region was further studied in the prostate cell lines, PC346C and LNCaP, and the hepatic cancer cell line, Hep3B (Chapter 4). The data showed, however, that the 2.1 kb proximal promoter was equally active in all cell lines, suggesting that other sequence determinants are necessary for tissue-specificity. Extensive deletion mapping of the *TGM4* promoter showed that the most proximal promoter between

positions -113 and -61 was responsible for basal promoter activity. This region contained the sequence 5'-ACCCCGCCCC-3' at positions -96 to -87, which perfectly matches a medium affinity binding site of the Sp1 multigene family. The functional importance of this binding motif was determined by mutational analysis. By EMSAs of LNCaP nuclear extracts, it was demonstrated that the Sp1 binding site binds to the ubiquitous transcription factors Sp1 and Sp3.

In Chapter 5 the generation of a specific polyclonal antiserum against the C-terminal region of hTG_P is described. The antiserum was suitable for Western blotting and immunohistochemical staining of formalin-fixed paraffin-embedded tissues. hTG_P expression in prostatic tissues, prostatic fluids and seminal plasmas was addressed by Western blotting. The expression was found to be highly variable, in particular in the tissue extracts and in seminal plasmas. In few prostatic tissues, in most seminal plasmas and in all prostatic fluids hTG_P expression could be detected as a 77 kDa protein by Western blotting. Additionally, in prostatic tissues and in prostatic fluids a protein band of 72 kDa was observed. Furthermore, breakdown products of approximately 33 kDa and smaller and high molecular mass bands could be demonstrated in both fluids. The latter may represent protein complexes containing hTG_P possibly formed by its own cross-linking activity.

Immunohistochemical analysis of several formalin-fixed paraffin-embedded human tissues showed no expression in any other tissue or cell type than prostate epithelium, confirming the results obtained by Northern (spot) blotting (Chapters 2 and 3). Extensive immunohistochemical analysis of prostate tissue sections and needle biopsies containing prostate adenocarcinoma and/or normal tissue showed a remarkable expression pattern of hTG_P within the prostate. hTG_P expression was entirely restricted to the luminal epithelium and no expression was found in basal epithelial and stromal cells. Within the prostate, large areas without any hTG_P positive cells were seen. Immunopositive cells were present either in a scattered pattern or concentrated in single or multiple glands in which all glandular cells expressed hTG_P. The latter staining pattern occurred frequently, but not exclusively, in the peripheral zone, whereas scattered expression was most often observed in the transition zone. hTG_P protein expression could occasionally be observed in high grade prostatic intraepithelial neoplasia, but was not detected in prostate carcinoma cells. The expression pattern as observed for hTG_P has not been found thus far for any other prostate-specific marker.

Although it still remains difficult to asses the physiological role of hTG_P, several data have been obtained and tools are generated which may help to elucidate the precise biological importance of this enzyme. Because of its prostate-specificity and its typical expression pattern within the prostate, studying hTG_P may also help in understanding physiological processes in the prostate. hTG_P may turn out not to be useful as a diagnostic or prognostic tool in prostate cancer, because of its heterogeneous cellular expression pattern and down-regulated expression in tumor tissue, but it is discussed that the identification of DNA modules and other regulatory factors involved in prostate-specific expression of hTG_P might be helpful in the development of gene-therapeutic protocols to specifically eliminate metastasized prostate cancer cells

Samenvatting

In de menselijke prostaat worden verschillende eiwitten geproduceerd, waarvan vele gesecreteerd worden in het semen. Deze eiwitten worden verondersteld te functioneren bij het optimaliseren van het voortplantingsproces (een overzicht hiervan is gegeven in hoofdstuk 1). Eén van deze eiwitten is prostaat-specifiek transglutaminase, hTG_P, waarvan de functie tot nu onduidelijk is. Het in dit proefschrift beschreven onderzoek had tot doel de eigenschappen van hTG_P vast te stellen teneinde inzicht te verkrijgen in de (veronderstelde) rol van hTG_P in door de prostaat gereguleerde fysiologische processen.
Door gebruik te maken van de verwachte overeenkomst met andere leden van de TGase familie (voor overzicht zie hoofdstuk 1), kon het complete hTG_P cDNA geïsoleerd worden uit een humane prostaat cDNA bank (hoofstuk 2). Het cDNA, met een lengte van 2983 bp, codeert voor een eiwit van 684 aminozuren met een te verwachten molecuulmassa van 77 kDa en een pI van 6.94. *In vitro* transcriptie-translatie experimenten bevestigden dit voor wat betreft de grootte. De afgeleide aminozuursequentie bevat een actief-centrum cysteine dat is ingebed in de GQCWVFA sequentie, die karakteristiek is voor functionele TGases (hoofdstuk 1). Met behulp van Northern blot analyse van prostaatkankercellijnen kon geen hTG_P expressie aangetoond worden in de androgeen-responsieve cellijn LNCaP en de androgeen-onafhankelijke cellijn PC3, maar wel in de androgeen-gevoelige cellijn PC346C. Hierin werd een hTG_P mRNA van 3.5 kb gevonden, waarvan het expressie niveau drievoudig werd verhoogd onder invloed van (synthetisch) androgeen. Het gen voor hTG_P, *TGM4*, werd gelokaliseerd op chromosoom 3.
Hoofdstuk 3 beschrijft de genomische organisatie van het *TGM4* gen. Het *TGM4* gen beslaat 35 kb genomisch DNA en bevat 13 exonen en 12 intronen en heeft een opvallende structurele overeenkomst met andere TGase genen. RT-PCR analyse toonde een complex splicingspatroon aan van intron 1. Hierbij was een 3.5 kb stroomopwaarts van exon II gelegen anti-sense *Alu* element betrokken. Een belangrijke transcriptiestartplaats werd bepaald op 52 bp stroomopwaarts van het translatiestartcodon. De *TGM4* promoter werd gekarakteriseerd door sequentieanalyse en transfectiestudies. De meest opvallende structurele kenmerken van de *TGM4* promoter waren een CATAA motief op positie –48 tot –44, een Sp1 bindingsplaats op positie –96 tot –87 en een *PIPP* pseudo-gen op positie –1276 tot –563. Deletieconstructen met 2.1-, 1.5- of 0.5 kb *TGM4* promoter fragmenten, getest in de PC346C cellijn, waren vergelijkbaar actief, waarmee aangetoond werd dat de proximale promoter verantwoordelijk is voor de basale activiteit. Door middel van verschillende methoden, waaronder Northern (spot) blotten en immunohistochemische aankleuring, werd aangetoond dat de expressie van hTG_P volledig beperkt is tot de prostaat (hoofdstuk 2, 3 en 5).
In verband met mogelijke toekomstige gen-therapeutische toepassingen zou het van belang kunnen zijn de moleculaire mechanismen welke ten grondslag liggen aan de prostaat-specifieke expressie van hTG_P te identificeren. Daarom werd de 2.1 kb *TGM4* promoter regio verder onderzocht in de prostaatkankercellijnen, PC346C en LNCaP, en de levercellijn, Hep3B (hoofdstuk 4). De 2.1 kb proximale promoter was echter vergelijkbaar

actief in alle cellijnen wat suggereert dat elders gelegen DNA elementen nodig zijn voor weefselspecificiteit. Uitgebreide deletie analyse van de *TGM4* promoter toonden aan dat de proximale promoter tussen positie –113 en –61 verantwoordelijk is voor de basale promoter activiteit. Deze regio bevat de sequentie 5'-ACCCCGCCCC-3' op positie –96 en –87, welke identiek is aan een bindingsplaats met een gemiddelde affiniteit voor de Sp1 multigen familie. Het functionele belang van deze bindingsplaats werd bepaald door mutatie analyse. Door middel van EMSAs met kernextracten van LNCaP cellen werd aangetoond dat de algemeen voorkomende transcriptiefactoren Sp1 and Sp3 binden aan de Sp1 bindingsplaats.

In hoofdstuk 5 wordt de ontwikkeling van een specifiek polyclonaal antiserum tegen de C-terminus van hTG_P beschreven. Het antiserum bleek geschikt voor Western blot analyse en immunohistochemische aankleuring van formaline-gefixeerd en paraffine-ingebed weefsel. hTG_P expressie in prostaatweefsels, prostaatvloeistof en seminaal plasma werd onderzocht door middel van Western blot analyse. De expressie vertoonde grote variaties, in het bijzonder in de weefselextracten en in seminale plasma's. In weinig prostaatweefsels, in bijna alle seminale plasma's en in alle prostaatvloeistofmonsters kon hTG_P expressie worden aangetoond als een eiwit van 77 kDa. Verder werden afbraakproducten van 33 kDa en kleiner en wellicht door autokatalyse gevormde hoog moleculaire banden aangetoond in beide typen vloeistofmonsters.

Immunohistochemische analyse van verschillende formaline-gefixeerde en paraffine-ingebedde humane weefsels kon geen expressie aantonen in andere weefsels of celtypes dan prostaatepitheel, hetgeen de door Northern (spot) blotten verkregen resultaten (hoofdstuk 2 en 3) bevestigt. Uitgebreide immunohistochemische analyse van prostaatweefsels en naaldbiopten die adenocarcinoma en/of normaal prostaatweefsel bevatten toonden een opmerkelijke distributie aan van hTG_P in de prostaat. hTG_P expressie was volledig beperkt tot de luminale epitheliale cellen. In basale epitheliale en stromale cellen werd geen expressie gevonden. Grote gebieden zonder enige hTG_P positieve cel waren aanwezig. Immunopositieve cellen lagen verspreid of waren geconcentreerd in een of meerdere klierbuisjes waarin alle kliercellen hTG_P tot expressie brachten. Het laatstgenoemde expressiepatroon kwam vaak, maar niet uitsluitend, voor in de perifere zone, terwijl verspreid liggende cellen het meest werden gezien in de transitionele zone. hTG_P expressie werd een enkele keer gevonden in hoog-gradige preneoplastische intraepitheliale neoplasie van de prostaat, maar niet in prostaatcarcinomacellen. Het expressiepatroon zoals waargenomen voor hTG_P is tot nu toe niet gevonden voor andere prostaat-specifieke markers.

Hoewel het moeilijk blijft om de fysiologische rol van hTG_P in te schatten zijn er veel gegevens verkregen en experimentele hulpmiddelen gegenereerd die er mogelijk aan kunnen bijdragen hierin meer inzicht te verkrijgen. Vanwege zijn prostaat-specificiteit en typische distributie kan verder onderzoek aan hTG_P mogelijk helpen bij het ophelderen van de fysiologische processen die zich afspelen in de prostaat. hTG_P is hoogst waarschijnlijk niet geschikt als diagnostische of prognostische marker bij prostaatkanker, vanwege zijn heterogene expressiepatroon en afwezigheid in tumorweefsel. De identificatie van DNA modules en andere regulerende factoren die betrokken zijn bij de prostaat-specifieke

expressie van hTG_P zou kunnen helpen bij het ontwikkelen van gen-therapeutische protocollen om specifiek metastaserende prostaatkankercellen te elimineren.

Curriculum vitae

De schrijver van dit proefschrift werd op 7 maart 1966 in Utrecht geboren. In 1984 behaalde hij het atheneum-B diploma aan de Gereformeerde Scholengemeenschap te Amersfoort, waarna werd begonnen met de studie Medische Biologie aan de Rijksuniversiteit te Utrecht. In 1992 werd het doctoraalexamen behaald, met als hoofdvak Medische Enzymologie (Prof. Dr. G.E.J. Staal, Dr. E.C. Heesbeen) en als bijvakken Experimentele Pathologie (Prof. Dr. W. den Otter, Dr. H.F.J. Dullens) en Moleculaire Biologie (Prof. L.A. Grivell, Dr. H.J. Pel). Dit laatste bijvak werd gedaan aan de Universiteit van Amsterdam. Tijdens de doctoraalstudie assisteerde hij bij diverse practica. In 1992 en 1993 deed hij onderzoek bij de afdeling Bacteriologie (Prof. Dr. B.A.M. van der Zeijst) van de Rijksuniversiteit te Utrecht. Het in dit proefschrift beschreven onderzoek werd in juli 1993 aangevangen bij de afdeling Urologie van de Erasmus Universiteit Rotterdam. Sinds 1 november 1998 werkt hij bij de afdeling Pathologie van dezelfde universiteit op een door de Nederlandse Kankerbestrijding gesubsidieerd onderzoeksprojekt.

List of publications

Roholl, P.J.M., Dullens, H.F.J., Kleijne, J., Dubbink, E.J., and den Otter, W. (1991). Acid ethanol fixation and polyester wax embedding combines preservation of antigenic determinants with good morphology and enables simultaneous bromodeoxyuridine (BrdU) labeling. *Biotech Histochem* 1: 55-62.

Pel, H.J., Rep, M., Dubbink, H.J., and Grivell, L.A. (1993). Single point mutations in domain II of the yeast mitochondrial release factor mRF-1 affect ribosome binding. *Nucleic Acids Res* **21**: 5308-5315.

Dubbink, H.J., Verkaik, N.S., Faber, P.W., Trapman, J., Schroder, F.H., and Romijn, J.C. (1996). Tissue-specific and androgen-regulated expression of human prostate-specific transglutaminase. *Biochem J* **315**: 901-908.

Wosten, M., Dubbink, H.J., and Van der Zeijst, B.A.M. (1996). The aroA gene of Campylobacter jejuni. *Gene* **181**: 109-112.

Dubbink, H.J., de Waal, L., van Haperen, R., Verkaik, N.S., Trapman, J., and Romijn, J.C. (1998). The human prostate-specific transglutaminase gene (TGM4): genomic organization, tissue-specific expression and promoter characterization. *Genomics* **51**: 434-444.

Weijerman, P.C., Zhang, Y., Shen, J., Dubbink, H.J., Romijn, J.C., Peehl, D.M., and Schroder, F.H. (1998). Expression of prostatic factors measured by reverse transcription polymerase chain reaction in human papillomavirus type 18 deoxyribonucleic acid immortalized prostate cell lines. *Urology* **51**: 657-662.

Dubbink, H.J., Hoedemaeker, R.F., Van der Kwast, T.H., Schroder, F.H., and Romijn, J.C. (1999). Human prostate-specific transglutaminase: a new prostatic marker with a unique distribution pattern. *Lab Invest* **79**: 141-150.

Dubbink, H.J., Cleutjens, K.B.J.M., Van der Korput, H.A.G.M., Trapman, J., and Romijn, J.C. (1999). An Sp1 binding site is essential for basal activity of the human prostate-specific transglutaminase gene (TGM4) promoter. *Gene* **in press**.

Dankwoord

Er waren heel veel mensen op een of andere manier betrokken bij mijn onderzoek. Een onmogelijkheid dus om iedereen persoonlijk te bedanken. Zonder iemand te kort te willen doen, wil ik toch een aantal namen noemen.
Prof. Schröder, ik wil u bedanken voor de geboden kans om aan het oncologisch onderzoek van de afdeling deel te nemen. Hans, bedankt voor de vele uren die je gestoken hebt in het luisteren naar en helpen oplossen van de problemen waar ik tegen aan liep. Op geplande én ongeplande tijden kon ik bij je terecht voor raad. Bedankt ook voor de vrijheid die je me gegeven hebt om deze studie te doen vanuit een moleculair biologisch aanpak ook al zal jouw voorkeur misschien meer uitgegaan zijn naar de biochemische kant van transglutaminases. Jan, door jouw betrokkenheid bij de moleculair biologische aspecten van mijn onderzoek heb je een essentiële bijdrage geleverd aan mijn proefschrift. De werkbesprekingen waren altijd erg stimulerend en je wist door jouw inzicht en commentaar vaak een nieuwe impuls aan het onderzoek te geven. Bedankt ook voor de tijd en ruimte die ik heb gekregen om het schrijven af te ronden. Ik verheug me op de komende jaren AR werk! Peter, bedankt voor de goede adviezen die je hebt gegeven gedurende de werkbesprekingen in het eerste jaar.
Nicole en Leon, jullie experimentele steun en gedrevenheid om het onderzoek zo goed mogelijk te doen heeft mij veel werk uit handen genomen. Nicole, het was heel plezierig om nog lang na je stage te blijven profiteren van de door jou opgebouwde kennis en experimentele ervaring onder het motto 'TGases stick together'.
Corrina, Nicole, Carl en Wytske, het kan haast niet anders of kamer Ee 1006 was het gezelligste kamertje van de 10^{e} etage, met zowel een hoog wel en wee gehalte als veel interessante discussies. Dit alles heeft bijgedragen aan de goede tijd die ik heb gehad bij de Urologie. En Carl, de werkbesprekingen die wij hebben gehad in en aan het zwembad zal wel nooit iemand willen geloven. Monique, vanaf de eerste dag was je mijn labgenoot en dat heb je geweten ook. Bedankt voor het inwerken in allerlei technieken. Sigrun, bedankt voor de kweken die je hebt verricht om de androgeen regulatie van hTG_P aan te tonen. Verder wil ik ook Burt, Jerome, Johan, Josée, Robert, Arjen, Hans S., Gert Jan, Wilma, Marja, Hans V., Cristel en Constant bedanken voor de gezelligheid, de gesprekken, het overleg, de wijze raad en nog heel veel andere dingen. Wilma, succes met het vervolg onderzoek aan hTG_P.
Heel veel van het onderzoek berust op contacten met collega's van de Pathologie. Al die mensen waar ik voor raad en daad terecht kon, bedankt. Jullie zijn nog niet van me af. De volgende mensen zou ik graag in het bijzonder willen bedanken voor hun bijdragen. Kitty en Hetty, het was voor mij een openbaring dat een experiment zo snel tot een zo mooi resultaat kan leiden. De bandshifts hebben hoofdstuk 4 publiceerbaar gemaakt. Theo, bedankt voor je enthousiaste belangstelling voor en het meedenken over de immunohistochemische studie. Robert H., de kennis die ik nu heb van de histologie van de prostaat (hoe beperkt ook nog) heb ik voornamelijk bij jou opgedaan, en het ordenen van de immunohistochemische gegevens is aan jou te danken. Frieda, ik ben je heel dankbaar voor

de eerste antisera tests op coupes. Daardoor is de aanzet gegeven tot de studie van hoofdstuk 5. Frank, bedankt voor al het fotowerk dat je gedaan hebt.
Rien en Rick van Celbiologie en Genetica, met jullie hulp heb ik de fusie eiwitten kunnen opzuiveren voor het ontwikkelen van de antisera. En Rien, je aanbod om het *TGM4* gen uit jouw genomische bank (genetische achtergrond: Rik) te vissen kwam precies op het juiste moment en heeft uiteindelijk geresulteerd in twee artikelen! Jan Vreeburg van Endocrinologie en Voortplanting, bedankt voor het leggen van het contact met de Andrologie.
De promotiecommissie wil ik bedanken voor de snelle beoordeling van het manuscript. De SUWO wil ik expliciet bedanken als voornaamste sponsor van mijn proefschrift en van een groot aantal congres- en werkbezoeken. Overigens gaat mijn dank ook uit naar NWO, het Trustfonds, en Yamanouchi Nederland BV voor hun bijdragen hieraan.
Mama, ik wil u bedanken dat u, samen met papa, mij de mogelijkheid gegeven heeft het onderwijs te volgen dat nodig was om tot dit proefschrift te komen. Roely, bedankt voor alle support die je tot op het neerzetten van de laatste punt gegeven hebt.