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CLONING AND CHARACTERIZATION

OF THE TRPM-2 GENE IN THE RAT AND HUMAN

BY

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degree of

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in

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PREFACE

Le succès ne vient pas simplement du travail acharné, mais surtout de l'éclatement du jugement qui voile l'accès à la conscience universelle, de la naissance de l'âme humaine dans la lumière qui éclaire le chemin du présent, de la recherche de la vérité profonde qui enseigne le sens subtil des évènements et de la confiance inébranlable octroyée par la découverte de la mission de vie unique à chacun.

Jean Pineault

ABSTRACT

The removal of circulating androgens by castration has a marked effect on the physical size, protein, RNA and DNA contents of the rat ventral prostate. This androgen ablation also produces major changes in overall gene expression. Several castration induced mRNAs have previously been identified of which the major sequence is TRPM-2. The cDNA for the rat TRPM-2 has been previously cloned and its protein product characterized. This mRNA sequence is 1,640 bases in length. It codes for a protein of 70-80 kDa, that appears to play an important role in active cell death by blocking complement mediated cell lysis. TRPM-2 is present in a wide variety of species including rat and human. I have isolated genomic clones from two rat and human genomic libraries made from partial Mbo I digests in the EMBL3 vector. I have screened those libraries with the full length cDNA sequence corresponding to the rat TRPM-2, and have isolated overlapping clones which span the TRPM-2 locus in both species. The TRPM-2 gene has been characterized in both species by complete sequence analysis. The gene covers 13,750 bp in the rat and 16,570 bp in human, each having 9 exons. 5,700 bp of upstream sequences have also been sequenced for the rat gene and 1,300 bp for the human. The sequence similarity between the respective coding and 5' regions of the gene in both species is nearly 79%. There is also a striking exon/intron structural similarity between the two species. The rat TRPM-2 gene was also shown to be expressed in a wide variety of tissues at very different levels. Finally, the previously published sequence of SGP-2 cDNA is very similar to the TRPM-2 cDNA sequence except for their respective 5' ends. PCR analysis has clearly demonstrated that the SGP-2 leader is a cloning artifact.

DEDICATION

Cette thèse est dédiée à mon épouse, Céline, en appréciation de son support continu durant mes années d'études et à ma mère qui s'est efficacement servie de sa grande influence sur ses fils dans le but qu'ils se dépassent académiquement.

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I. INTRODUCTION

A. BACKGROUND: THE NEED FOR A SYSTEM TO STUDY CANCER

Cancer is the second leading cause of death in North America just after cardiovascular diseases. The prevalence of all forms of cancer will continue to rise, mainly due to improved health care in recent decades, increase in life expectancy and socio-environmental changes. Certainly, the best treatment for all forms of cancer would be prevention. However, at the state of present knowledge on the disease, prevention alone is far from being dependable. There are several ways presently used to approach cancer for treatment purposes. These therapies range from surgically removing the tumor, to diverse radiation therapies, to a set of chemotherapies, to a series hormonal/chemical remedies depending on the type of cancer and developmental stage of the disease. Nevertheless, all present methods of treatment are painstaking and inefficient. In most cases, even when the cancer is detected early, the metastases will recur after a disease-free interval that varies greatly in time with each type of cancer and patient. Refinements in the different methods of treatment are now imperative. Different systems to study the diverse forms of cancer that can be easily related to humans are needed to attempt achieve complete cure through a general understanding of metastasis.

B. THE RAT VENTRAL PROSTATE PROVIDES AN EXCELLENT SYSTEM FOR THE STUDY OF CANCER

1. The failure of current therapies

Several reasons point in the direction of prostate cancer as a designated system for the study of cancer in general and specifically. Among the different forms of this disease,

prostate cancer is the most common and the second leading cause of cancer related deaths in North American men (Carter and Coffey, 1990). Due to the slowly growing nature of this disease, standard treatments are ineffective. Hormonal therapy remains the first line of treatment. In spite of refinements in hormonal therapies, there has been a striking lack of improvement in the recurrence of a hormone insensitive form of prostate cancer after the usual disease-free interval of patients with prostatic carcinoma (Carter and Coffey, 1990; Iverson *et al.*, 1990; Iverson and Torp-Pedersen, 1991; Scott *et al.*, 1980; Schroeder, 1991; Slack *et al.* 1986).

Current studies aimed at developing and refining hormonal therapies originated from early reports that suggested that prostate cancer proliferation could be inhibited by castration (Huggins and Hodges, 1941; Huggins *et al.*, 1941). So far, a multitude of therapies have been developed to either prevent the synthesis, or inhibit the action of the active androgen, 5α-dihydrotestosterone. These include anti-androgens which disrupt the steroid receptor mechanism (Neumann, 1977; Tunn *et al.*, 1979; Tunn *et al.*, 1980), chemicals which interfere with steroid metabolism (Brooks *et al.*, 1986; Heyns *et al.*, 1985; Rittmaster *et al.*, 1989; Trachtenberg, 1984; Trachtenberg, 1987), and combination therapies involving a mixture of anti-androgens with either GnRH analogues, anti-prolactins (Dupont *et al.*, 1988; Labrie *et al.*, 1987; Lacoste *et al.*, 1989; Lefebvre *et al.*, 1982; Mathe *et al.*, 1986; Schroeder *et al.*, 1987; Waxman *et al.*, 1983) or aminoglutethimide (Drago *et al.*, 1984). To date, none of those therapies have produced any significant improvement on the usual variable disease-free interval. Still, the success

of those forms of treatment is based on the hormone responsiveness of the cancerous cells. Nevertheless, in every case, a subset of cells, either present in the early rounds of treatments and/or developing latter during the therapies, are completely insensitive to those hormonally based cures (Isaacs *et al.*, 1981; Isaacs and Kyprianou, 1987). Unfortunately, in these approaches, solely based on hormone responsiveness, may dwell the failure of the therapy. That is, the hormonal treatment may select for those *unresponsive cells* and provide the basis for recurrence. Thus, new therapies that eliminate not only hormone responsive, but also hormone unresponsive cells have to be developed. To achieve this goal we will require a more complete understanding of the inherent causes of the biochemical events that lead to metastasis.

2. The rat ventral prostate as a system

The rat ventral prostate undergoes drastic involution and loss of function upon removal of androgens by orchiectomy. The native size and original physiological functions of the gland is recovered when natural doses of testosterone are re-administered subcutaneously after castration (Bruchovsky, *et al.*, 1975). Furthermore, the prostate of the rat will react in a relatively similar fashion to its human counterpart when exposed to chemical, hormonal and combination therapies. The rat prostate is also already well characterized as a hormone dependent tissue (Bruchovsky, *et al.*, 1975) making it a very good candidate as a model to study prostate cancer.

3. Characteristics the rat ventral prostate

a. Physiology of the gland

The rat ventral prostate is a complex tissue with secretory functions. This exocrine gland under the control of androgens provides several constituents of the seminal fluid. These constituents include citrate, fructose, the secretory form of acid phosphatase, and prostate steroid binding protein (PSBP); the major seminal protein. This synthesis and secretion of the protein portion of the seminal fluid is critically dependent on androgens (Heyns *et al.*, 1977; Lea *et al.*, 1979; Parker and Scrace, 1979; Rennie *et al.*, 1978; Tenniswood *et al.*, 1978; Tenniswood *et al.*, 1981). The androgen receptor modulates the androgenic stimulation of transcription (Page and Parker, 1982; Peeters *et al.*, 1980; Perry *et al.*, 1985) and post-transcriptional stabilization of hnRNA (Zhang and Parker, 1985). Upon removal of androgens by castration the synthesis of androgen dependent proteins ceases. This is the time when the prostate undergoes significant involution (Bruchovsky *et al.*, 1975; Lee *et al.*, 1985). This atrophy of the prostate after castration results in the loss of approximately 90% of the epithelial cells with concomitant loss of DNA, RNA and protein content, within 8 days (DeKlerk and Coffey, 1978; English *et al.*, 1985; English *et al.*, 1987).

b. Functional morphology

Microdissection of the prostate demonstrates that the gland as an arborized array of ducts that lack true acini (Sugimura *et al.*, 1986). The gland contains a number of different cell types showing a clear proximo-distal heterogeneity along the ducts. Each

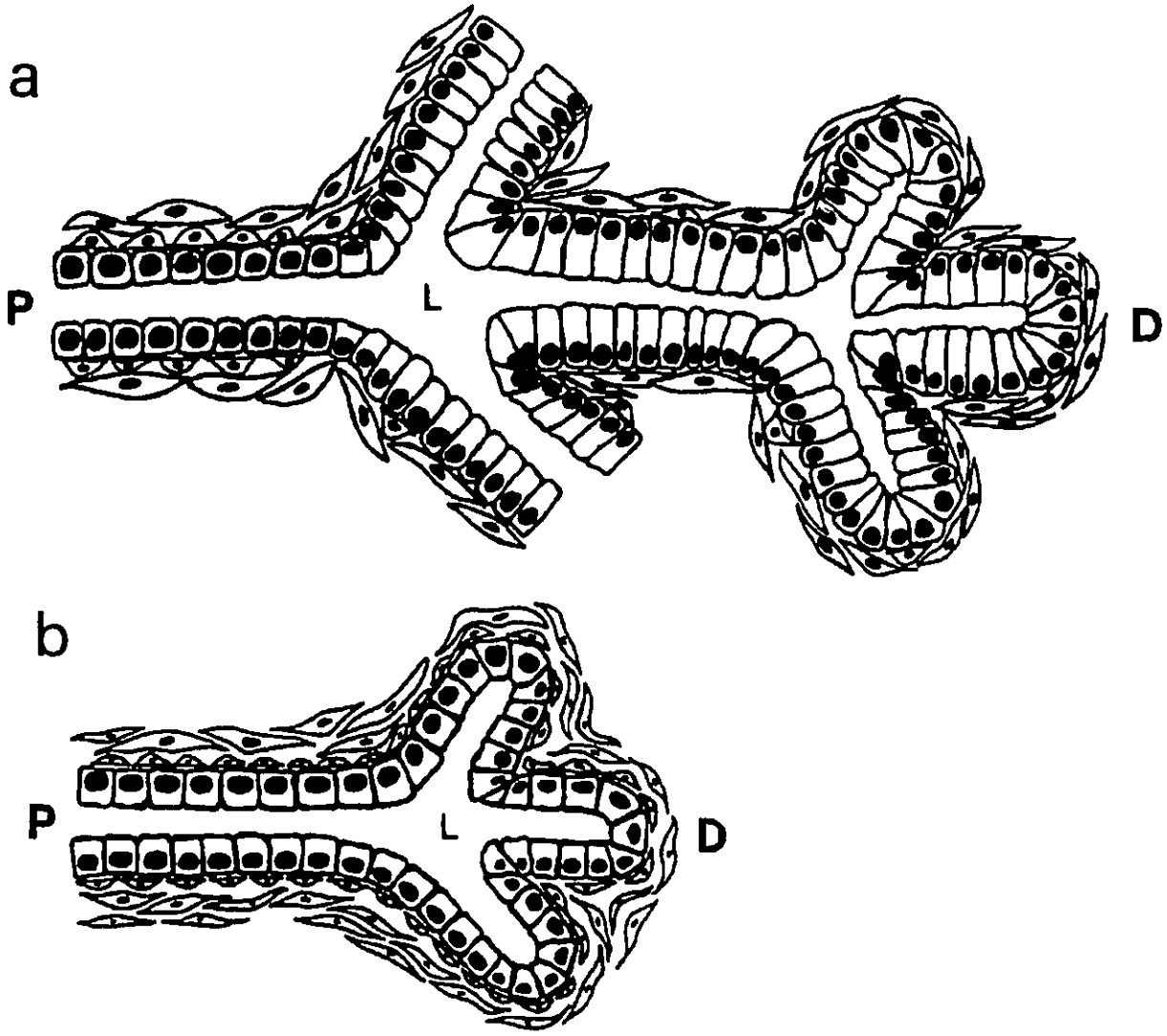
individual duct in the normal prostate is composed of several branch points that lead to the distal regions of the ducts or tips. As fig. 1 shows, within each duct there are at least three functional regions that can be identified by the proliferative zone (at the tip of the duct), the secretory zone (in the distal region of the duct) and the non-secretory zone (in the proximal region of the duct) (Sugimura *et al.*, 1986; Tenniswood, 1986; Tenniswood *et al.*, 1990). The base of the proximal zone originates at the urethra. These regions are also described as the distal, intermediate and proximal zones to clearly distinguish between the distal tips and the distal, or intermediate regions (Lee *et al.*, 1990).

c. Cellular morphology in normal and castrated rats

Analysis of cellular morphology and cytoskeletal signature in random sections of the rat ventral prostate demonstrates that the gland contains a mixed population of cells. The lumen of the ducts is lined with two different luminal epithelial cell types: cuboidal non-secretory epithelial cells in the proximal region of the ducts, and tall columnar secretory epithelial cells in the distal region of the ducts that account for the majority of the epithelial cells in the gland. Also, basal epithelial cells are found predominantly interspersed in the proximal region between the luminal cells and the fibroblasts (stromal cells), who in turn are found both in the proximal and distal regions of the ducts (Brandes, 1966; Ichihara *et al.*, 1985; Rouleau *et al.*, 1990; Verhagen *et al.*, 1988). Interestingly, gene expression from luminal epithelial cells seems to be dictated by their anatomical location and shape. The basal cells which are sparsely distributed throughout the distal region of the gland, in contrast with their continual layer that surrounds the

Figure 1. Ductal arrangement of the rat ventral prostate showing regional variation in epithelial cell morphology

a Prostatic ducts from normal rats, **b** Prostatic ducts from a castrated rat. The proximal (P) (near the urethra) and distal (D) regions are indicated. The ducts (L) are lined with epithelial cells which are lined with basal epithelial cells in the proximal region and stromal cells in the distal region. The basal epithelial cells are in contact with the stroma in the proximal region. Notice the shape of the lining epithelial cells; tall columnar in the distal region and cuboidal in the proximal region.


a
P
L
D
b
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L
D

luminal epithelial cells in the proximal region of the ducts, could have a regulatory function of the epithelial cells lining the lumen. It is important to note that other cell types may also be regionally distributed and could play essential roles in modulating the epithelial/stromal interactions in the proximal regions of the ducts (Verhagen *et al.*, 1988).

After androgen removal by castration the regression of the gland starts in the distal region of each duct, and the secretory epithelial cells undergo ACD (Active Cell Death) while the stromal cells are relatively unaffected (Rouleau *et al.*, 1990; Sensibar *et al.*, 1991). The cuboidal cells of the proximal region of the ducts are probably androgen independent as they are slightly affected by castration (Cunha *et al.*, 1985; Cunha *et al.*, 1987). After castration, the ducts greatly decrease in volume, length and number of branch points Sugimura *et al.*, 1986; Sugimura *et al.*, 1986a; Sugimura *et al.*, 1986b) giving the impression of a *melt back* (Rouleau *et al.*, 1990).

d. Biochemical aspects and gene expression

All the secretory functions of the prostate are carried out by the tall columnar epithelial cells of the distal region. A major component of the secretion, PSBP (Prostate Steroid Binding Protein), is only expressed and secreted by the tall columnar epithelial cells in the normal prostate, making this protein a good marker for normal prostatic functions. Several lines of evidence establish that prostate involution occurs specifically via induction of ACD in these same epithelial cells that express PSBP. This process of cell death is interesting as it requires both protein synthesis and active gene expression

as prostatic regression can partially be blocked by the inhibition of protein synthesis (Bruchovsky *et al.*, 1975; Lee *et al.*, 1985; Kerr and Searle, 1973; Sandford *et al.*, 1984). At the moment, the most widely used marker of ACD during prostate involution is TRPM-2. This gene has not only been shown to be involved in ACD, but is also expressed during regression in the same cells that synthesize PSBP in the normal state.

C. CELL DEATH MAY BE PART OF THE SOLUTION

One of the several approaches that could be used to challenge the hormone un-responsive cells problem of prostate cancer would be to invoke the active cell death (ACD) pathway in these cells. The idea of inducing self-death to cells or group of cells specifically is very appealing from a therapeutic point of view. However, more research on ACD is needed to achieve this goal.

1. Necrosis versus ACD

The visible characteristics of ACD were first described in 1907 by Collin (Collin, 1907). However, the physiological relevance of this observed phenomenon did not become apparent until the methods of study were more refined in the 1970s. This is when the term *apoptosis* was introduced to describe the way those cells died (Kerr *et al.*, 1972). As necrosis was already acknowledged, researchers then confirmed a second way cells could die. Presently, beside specific phenomena such as keratinization and reticulocyte formation, there are two recognized type of cell death. The first type is *necrosis*, which

occurs as a result of tissue damage and involves a sustained response from the immune system. This response is generated by the release of the cell contents into its surrounding environment. The second type of cell death is *apoptosis*. This process is differentiated from necrosis as it is an active one. Apoptosis requires the expression of a set of genes that will initiate a series of events responsible for the activation of some constitutively expressed proteins and the synthesis of new proteins responsible for the self-destruction of the affected cells. This process does not involve any leakage of cell content into their surroundings but rather the formation of apoptotic bodies, hence apoptosis, which look like microscopic *bags* containing the remains of the dying cells. Since there is no leakage of intracellular contents, this form of cell death does not invoke complement based immune response. Apoptosis is now designated as active cell death (ACD); a term that describes better the active nature of this phenomenon.

2. Morphology of ACD

Several distinct steps are histologically visible during the process of ACD as studied in the prostate (Bursch *et al.*, 1990; Bursch *et al.*, 1990a). The pre-condensation phase which is not histologically visible after the initial stimuli, lasts from only a few minutes to several hours. The first histologically visible stage of ACD in the prostate is the loss of cell-cell contacts as the desmosomal links between the epithelial cells are lost. This stage is referred to as cytoplasmic condensation. During this period the committed cells are released from their basement membrane, and the condensation of the intermediate filaments around the nucleus initiates the disruption of the cytoskeleton

(Wyllie, 1987). Therefore, this is the time when cell-cell contact and communication are upset and the dying cell is completely isolated from its neighbours. Chromatin condensation follows almost immediately. This step requires the activation of a Ca^{++}/Mg^{++} dependent endonuclease which is responsible for degrading the DNA to yield the hyperchromatic, pyknotic nucleus typical of apoptotic bodies (Kyprianou *et al.*, 1988; Wyllie *et al.*, 1981; Wyllie *et al.*, 1986). Each individual cell is then fragmented into several smaller bodies or *bags* of cytoplasmic membrane that contain its remains including the lysosomes, mitochondria, nuclear fragments, and the rest of the cell natural components. The essential feature of this fragmentation is that it occurs without any detectable leakage of the cell content into its surrounding environment. This *safe packaging* of the dead cell components insures that no inflammatory response will result from this process. Interestingly, the processus of ACD in the prostate is asynchronous; that is, not all cells that are destined to die at the same time. This asynchrony may be significant in preventing a major immune response which would result into an inflammatory reaction. The asynchronous death of the cells seems to give enough time for their surviving neighbours in the tissue and the invading macrophages to handle the bulk of apoptotic bodies resulting from ACD.

Recently several morphological variants of the late stages of ACD have been described and efforts have been made to further classify the possible different pathways of ACD (Clarke, 1990). The final stage of ACD differs in the way the apoptotic bodies

are degraded. *Type I ACD* is said to be *heterophagic* as the lysosomes of the recipient neighbouring cells are responsible for the digestion of the apoptotic bodies. In that case the lysosomes of the dead cells are not involved in the degradation process. *Type II ACD* was observed in some developing systems. In that case the lysosomes of the dead cells, contained within the apoptotic bodies, appear to be actively involved in the degradation of the remains. This type II ACD is said to be *autophagic* (Clarke, 1990). *Type III ACD*, or secondary necrosis, is seen during the regression of hormone dependent tissues, such as the prostate after castration, when the surviving cells are unable to cope with the large number of apoptotic bodies, and macrophages invade the regressing tissue to help out the neighbouring cells in their scavaging task. However, regardless of the type of ACD the processes are vary similar: the cells die actively (implying gene expression and protein synthesis), most of the biochemical steps preceding phagocytosis seem identical, and there is no leakage of the dying cells into their surroundings.

3. Hormonal induction of ACD in the rat ventral prostate

It is clear that the presence or absence of androgens in the rat prostate has drastic effects on its size and function. The gland is extremely sensitive to hormonal regulation and will respond to different factors which influence the presence and/or activity of androgens and the steroid metabolism of the androgen receptor. The removal of androgens by orchiectomy is the first method to induce ACD in the prostate. Anti-androgens such as flutamide and cyproterone acetate also induce ACD (Léger *et al.*, 1988) as well as in prostate derived tumor cell lines, like the rat Dunning 3327 H subline (Ramaekers *et al.*,

1989) and the human PC-82 and PC-EW lines (Kyprianou *et al.*, 1990). Lutenizing-hormone-releasing-hormone (LH-RH) and flutamide also induce ACD in the R3328H Dunning prostate cell line which is hormone dependent (Redding and Schally, 1985).

From the observed drastic regression of the prostate after androgen deprivation or blockade two general mechanisms for the induction of ACD are possible. The first possibility is that ACD could be induced by some unknown factor(s) which would be triggered by the sudden absence of androgens. Alternatively, ACD could be antagonized by androgens; in other words, the mere presence of androgens would be sufficient to block ACD induction. This latter mechanism of induction of ACD, androgen antagonization. has been demonstrated to be responsible for the onset of the phenomenon (Isaacs, 1984).

4. Biochemistry and gene expression of ACD in the rat ventral prostate

Changes in the overall architecture of the prostate during regression is associated with the activation of a constitutively expressed endonuclease and changes in gene expression. Northern hybridization analysis demonstrates that the steady states levels of the mRNAs coding for PSBP decrease rapidly during regression. As many as 30 uncharacterized *castration induced proteins* have been identified on two-dimensional gels of protein extracts or *in vitro* translation products isolated from castrated rats (Léger *et al.*, 1987; Lee and Sensibar, 1987; Sensibar *et al.*, 1990). These proteins all appear to be synthesized during the pre-condensation phase of ACD. At the same time, the steady state

levels of several mRNAs and proteins dramatically increase during regression. These latter mRNAs and proteins are probably repressed by the androgens in the normal prostate. These include among others, transglutaminase, poly(ADP-ribose)polymerase, HSP-27, the plasminogen activators, and TRPM-2.

a. Endonucleases

In the prostate, most biochemical events specific to ACD occur before any morphological changes are visible. The first step to produce any visible change is the activation of an endogenous Ca^{++}/Mg^{++} dependent nuclear endonuclease (Arends *et al.*, 1990; Cohen and Duke, 1984; English *et al.*, 1989; Kyprianou and Isaacs, 1988; Kyprianou *et al.*, 1988; McConkey *et al.*, 1988; Wyllie *et al.*, 1981; Wyllie *et al.*, 1984a; Wyllie *et al.*, 1986), resulting in cleavage of high molecular weight DNA and chromatin condensation. The cleavage of the DNA can be seen as a nucleosome ladder on agarose gel electrophoresis. This endonuclease activation also marks the commitment step of ACD (Bursch *et al.*, 1990; Bursch *et al.*, 1990a). Since the endonuclease(s) does not require gene transcription or protein synthesis for activation, the most likely method of activation is de-repression of a constitutively expressed protein. The nucleosome ladder is commonly, but not universally, associated with the activation of the endonuclease and nuclear condensation (Cohen and Duke, 1984; Laster *et al.*, 1988; Martz and Howell, 1989; Wyllie *et al.*, 1981; Wyllie *et al.*, 1986).

b. Transglutaminase

Tissue transglutaminase is involved in cross-linking of cytoskeletal components during the condensation phase of ACD (Fesus and Thomazy, 1988). This protein is regulated at the level of gene expression during ACD. This enzyme is expressed in several systems undergoing ACD such as the prostate, several tumor cell lines, and rat liver after treatment with lead nitrate which induces hyperplasia (Fesus *et al.*, 1987; Fesus *et al.*, 1989; Piacentini *et al.*, 1991). In this latter case, the enzyme has been immunohistochemically localized to apoptotic bodies containing increased ε-(γ-glutamyl)lysine and N^1,N^8-bis(γ-glutamyl)spermidine bonds (Fesus *et al.*, 1991). The biological reason underlying tissue transglutaminase activation is probably to provide additional mechanical strength conferred by crosslinking the cytoskeletal proteins, to maintain the integrity of apoptotic bodies (Fesus *et al.*, 1991).

c. Poly(ADP-ribose)polymerase

This enzyme is expressed in the nucleus and is tightly bound to chromatin and catalyzes the poly(ADP-ribosyl)ation of structural chromosomal proteins such as histones, non-histones (Burzio *et al.*, 1979; Ferro *et al.*, 1983; Ogata *et al.*, 1981; Okazaki *et al.*, 1980; Poirier *et al.*, 1982; Wong *et al.*, 1977) and nuclear enzymes, including topoisomerase (Ferro *et al.*, 1983; Jongstra-Bilen *et al.*, 1983) and DNA ligase (Ohashi *et al.*, 1983). Poly(ADP-ribose)polymerase is highly stimulated by DNA strand breakage (Zahradka and Ebisuzaki, 1982), an event that occurs in apoptotic cells during the formation of a nucleosomal ladder after activation of an endogenous Ca^{++}/Mg^{++}

endonuclease (Kyprianou *et al.*, 1988; Wyllie *et al.*, 1984a). Poly(ADP-ribose)polymerase can modify several of the enzymes involved in DNA repair (Ohashi *et al.*, 1983), and it is possible that its induction is a futile attempt by the dying cell to repair the damage caused by endonuclease activation. Conversely, the inhibition of a Ca^{++}/Mg^{++} endonuclease by ADP-ribosylation has been reported but it is not known if the endonuclease affected is the same as the enzyme responsible for DNA degradation during ACD (Welch, 1985). The experiments demonstrating inhibition of a Ca^{++}/Mg^{++} endonuclease by ADP-ribosylation were conducted using extracts from normal tissues, and suggested that a preliminary step in the activation of endonuclease activity during cell death could involve removal of poly(ADP-ribose).

In the regressing prostate, poly(ADP-ribose)polymerase is induced approximately 8-10 fold above normal levels following androgen deprivation. This induction peaks 2-3 days after castration. The mRNA for poly(ADP-ribose)polymerase is not induced in RDE cells treated with calcium ionophore, suggesting that the induction of poly(ADP-ribose)polymerase observed *in vivo* in the regressing prostate reflects an active role for the enzyme in ACD (Guenette, personal communication).

d. Heath shock protein 27

Hsp 27 belongs to a family of proteins induced in response to environmental stress. The transcriptional activation of the Hsp 27 gene seems to be regulated by a mechanism dependent on Ca^{++} suggesting a possible link between the expression of Hsp

27 and ACD. Hsp 27 mRNA is induced approximately 3-5 fold during ACD in the prostate, peaking between days 2 and 3 after castration (Guenette, personal communication). Therefore, gene expression of Hsp 27 parallels that of poly(ADP-ribose)polymerase. This is the time when there is maximal production of apoptotic bodies in the prostate (English *et al.*, 1989).

The induction of ACD as a result of hormone removal parallels the induction of a series of proteins usually involved in classical stress responses. Those proteins are *fos*, *myc*, the Hsp 70 family of chaperonin-like proteins (Buttyan *et al.*, 1988; Rennie *et al.*, 1988; Rennie *et al.*, 1989), and Hsp 27. Hsp 27 seems to play a more crucial role in dictating the fate of the cell, since the phosphorylation of the protein has profound consequences on cellular homeostasis.

e. Intra- and extra-cellular proteases

The destruction of the ECM underlying the cells committed to ACD is one of the key events during the regression of the prostate. This process requires the synthesis and secretion of several different proteases. The plasmin system, a complex cascade of enzymes, seems to play an important role in ECM degradation. The end product of the cascade, plasmin, is a serine protease that degrades many of the proteins found in the ECM (Vassalli *et al.*, 1991), and may also be critical for the activation of the latent form of TGFß (Rifkin *et al.*, 1990). Moreover, plasmin activates metalloproteases, such as collagenase IV, which are themselves involved in ECM degradation (He *et al.*, 1989).

Plasmin is activated by two enzymes: tissue type and urokinase type plasminogen activators (Dano *et al.*, 1985). The activity of these two types of plasminogen activators is modulated by a group of arginine-specific serine-protease inhibitors called plasminogen activator inhibitors (Seiffert *et al.*, 1990).

The homeostasis of ECM turnover is controlled by a delicate balance between, among other factors, the activities of the plasminogen activators and their inhibitors. During the pre-condensation phase of ACD in the prostate, the activity of both tissue type and urokinase type plasminogen activators is increased (Freeman *et al.*, 1990; Rennie *et al.*, 1984), leading to an imbalance in the relative levels of the plasminogen activators and their inhibitors. This results in the activation of plasmin and the metalloproteases. The degradation of the ECM then ensues. Glucocorticoids, which inhibit the induction of ACD in the prostate, have been shown to decrease the levels urokinase type plasminogen activator mRNA, and to increase the expression of the plasminogen activator inhibitor I gene (Vassalli *et al.*, 1991). Several other proteases, including cathepsin D (Sensibar *et al.*, 1991; Tanabe *et al.*, 1982), stromelysin (Basset *et al.*, 1990), and collagenase (Muntzing and Murphy, 1977; Muntzing *et al.*, 1979; Muntzing, 1980), have been implicated in the destruction of the basement membrane and have been shown to increase in activity during ACD in the prostate. This increase parallels with the peak of tissue involution, suggesting that these proteins are required for ACD.

f. Testosterone repressed prostate message 2 (TRPM-2)

At present, the most universally used genetic marker of ACD is the TRPM-2 mRNA. This sequence was originally identified and cloned from the regressing rat ventral prostate after castration (Montpetit *et al.*, 1986). The TRPM-2 gene is expressed *de novo* in the prostate after castration and the steady state levels of its mRNA during regression correlate well with the observed rate of tissue regression (Léger *et al.*, 1987). TRPM-2 mRNA is specifically induced in the prostate after castration or anti-androgen therapy, and at the same time course as tissue transglutaminase mRNA reaches a peak about 4 days after castration (Léger *et al.*, 1987; Léger *et al*,, 1988).

The TRPM-2 gene has been identified and cloned from the rat and human (this thesis), and the protein product characterized from several tissues and species. In addition to the regressing rat ventral prostate (Léger *et al.*, 1987), the cDNA and the protein has been isolated from rat Sertoli cells as sulfated glycoprotein 2 (SGP-2) (Collard and Griswold, 1987; Griswold *et al.*, 1986); the protein from the ram rete testes fluid as clusterin (Blaschuk *et al.*, 1983); from human serum, as apolipoprotein J (de Silva *et al.*, 1990); serum protein 40,40 (SP-40,40) (Kirszbaum *et al.*, 1989) and complement lysis inhibitor (CLI) (Jene and Tschopp, 1989); from Madin-Darby canine kidney cells as glycoprotein 80 (gp80) (Hartman *et al.*, 1991); from bovine adrenal medullary chromaffin granules as glycoprotein III (Palmer and Christie, 1990); and from Japanese quail neuroretinal cells as T64 (Michel *et al.*, 1989). The purified protein product is a secreted, heterodimeric, disulfide linked glycoprotein of 70-80 kDa, with subunits ranging from 29-

43 kDa by SDS PAGE (Blaschuk *et al.*, 1983; de Silva *et al.*, 1990; Griswold *et al.*, 1986; Jenne and Tschopp, 1989; Kirszbaum *et al.*, 1989; Palmer and Christie, 1990). The protein is translated from a single mRNA as a pre-pro-protein. The N terminal 21-22 amino acids predicted from the cDNAs are typical of a secretory signal sequence and are presumably removed during the secretory process. The pro-protein matures by limited proteolysis to the carboxyl side of a specific internal arginine. There are four absolutely conserved cysteines and several potential sites of N-linked glycosylation in each subunits. In SGP-2, a triantennary structure has been proposed for the oligosaccharide moieties which are further modified by sulfation (Griswold *et al.*, 1986). Tyrosine sulfation and serine phosphorylation has been documented for gp80 (Han *et al.*, 1991). The carbohydrate is important in vectorial transport of gp80 to the apical membrane in canine kidney cells (Parczyk and Koch-Brandt, 1991) but the significance of other post translational modifications is not clear. TRPM-2 is clearly a complex protein that may be modified in a tissue specific manner, and that may have more than one function. Since the only genomic clones of TRPM-2 were obtained using the rat TRPM-2 cDNA in the rat and human, the gene and protein product will be referred here to as TRPM-2.

The TRPM-2 gene is induced in most systems undergoing ACD, suggesting that the protein plays a significant role in the process. The gene is induced after castration in the rat ventral prostate and can also be induced by treatment with anti-androgens (Léger *et al.*, 1988) or calcium channel agonists (Kyprianou *et al.*, 1988), while both the rate of regression and the steady state levels of TRPM-2 mRNA can be decreased by the

administration of glucocorticoids (Rennie *et al.*, 1989) or calcium channel blockers (Connor *et al.*, 1986). The gene is also expressed in a variety of tissues during their natural regression; the weaning of the mammary gland (Corbeil *et al.*, in preparation), the granule neurons in the dentate gyrus of prepubescent rats (McNeill *et al.*, 1991), and cell lines following ablation of their trophic hormones (Kyprianou *et al.*, 1990; Kyprianou *et al.*, 1991; Parczyk and Koch-Brandt, 1991). Castration in the rat also leads to the accumulation of TRPM-2 mRNA in the hippocampus, specifically immunolocalized over astrocytes (Day *et al.*, 1990). The sensitivity of the hippocampus to hormone ablation is independent of, but synergistic with, entorhinal cortex lesions. Other neurodegenerative pathologies that are steroid hormone independent are associated with TRPM-2 up regulation including Scrapie infection in hamsters and in the hippocampus of Alzheimer patients (May *et al.*, 1990).

TRPM-2 is also induced in several other systems that undergo ACD in which the inductive signal is not hormone ablation. Androgen independent prostatic adenocarcinoma AT-3 cells undergo ACD in response to 5-fluorodeoxyuridine or trifluorothymidine treatments which is associated with an elevation of TRPM-2 mRNA levels (Kyprianou and Isaacs, 1989). Recombinant human tumor necrosis factor acts independently, and synergizes with, topoisomerase II inhibitors in induction of ACD of murine L929 tumor cells and elevation of TRPM-2 mRNA levels (Kyprianou *et al.*, 1991a). TRPM-2 mRNA is elevated in the rat kidney in association with kidney cell death following urethral obstruction in an experimental model of hydronephrosis (Sawczuk *et al.*, 1989). In this

case its expression is localized initially to the adventitial layers of the hilar arteries and intrarenal arterioles which are eventually depleted, and later shifts to the epithelial cells of the collecting ducts and distal tubules (Connor *et al.*, 1991). In addition, TRPM-2 mRNA is transiently up regulated in rat thymocytes undergoing ACD induced by administration of dexamethasone (Bettuzzi *et al.*, 1991).

Several observations, however, indicate that TRPM-2 is not always associated with ACD. In the developing rat, the spatial and temporal expression of TRPM-2 mRNA in the central nervous system does not correlate with the occurrence of developmental neuronal cell death, or with the developmental cell death of the nonneural palatial shelf epithelium (Rennie *et al.*, 1990). Also, TRPM-2 is expressed constitutively in several tissues under circumstances for which there is no documented occurrence of ACD, including the testis and epididymis of the rat (Grima *et al.*, 1990; Hermo *et al.*, 1991; Sylvester *et al.*, 1991), ram (Cheng *et al.*, 1988), and human (O'Bryan *et al.*, 1990), as a major glycoprotein in bovine adrenal chromaffin granules (Palmer *et al.*, 1990), and as a secretory product of Madin-Darby canine kidney cells (Hartmann *et al.*, 1991). It is also present constitutively in the serum of both rat and human (Grima *et al.*, 1990; Murphy *et al.*, 1988). TRPM-2 expression is also present at low levels in all rat tissues examined in this thesis.

TRPM-2 has been implicated in other numerous processes, including a role in chromaffin granule secretion (Palmer and Christie, 1990), in spermatogenesis (Collard and

Griswold, 1987; Griswold *et al.*, 1986; Kierszenbaum *et al.*, 1988; Kierszenbaum *et al.*, 1989), in immune regulation (Choi *et al.*, 1990; Eddy and Fritz, 1991; Jenne and Tschopp, 1989; Murphy *et al.*, 1988; Murphy *et al.*, 1989a; Murphy *et al.*, 1991; Wilson *et al.*, 1991), in regulation of lipid transport (de Silva *et al.*, 1990; James *et al.*, 1991.), and in morphological transformation (Michel *et al.*, 1989). In all but one of these systems there is no definitive biochemical evidence to support a specific function for TRPM-2. The exception is the role of SP-40,40/CLI in the modulation of the immune system. In this context TRPM-2 can inhibit complement mediated cytolysis of sheep erythrocytes *in vitro*, by binding to nascent soluble C5b-7 to form a cytolytically inactive complex, in a manner similar to S protein/vitronectin (Jenne and Tschopp, 1989; Murphy *et al.*, 1989). SP-40,40/CLI has been affinity purified from human blood using a non specific IgG column suggesting that it may bind to F_c effector controlling region of antibodies (Wilson *et al.*, 1989). Physiologically, TRPM-2 may function to ablate complement activity or some other aspect of the immune response in certain circumstances where such a response, for example an inflammatory one elicited by complement activation, could upset normal physiological functions. In ACD, the formation of apoptotic bodies requires substantial membrane remodelling, which must occur without leakage of the intracellular components into the extracellular space to avoid a large immune response that could lead to inflammation. Thus the induction of TRPM-2 during the pre-condensation phase of ACD may protect both the apoptotic cell and its vital neighbours from complement attack. The recent report that the transfection of anti-sense oligomers into the PC-3 and LNCaP prostate cell lines induces ACD, supports this hypothesized role of TRPM-2 in ACD

(Sensibar *et al.*, 1991a). The association of SGP-2 with the membrane of developing sperm (Kierszenbaum *et al.*, 1988; Kierszenbaum *et al.*, 1989; Sylvester *et al.*, 1991) in the male reproductive tract may also be essential for maintaining sperm integrity and the survival of the sperm in both the male and female reproductive tracts.

The lack of correlation between TRPM-2 expression and neuronal and some non-neuronal developmental cell deaths may reflect variations in developmental cell death compared to ACD in the adult animal. In particular, morphological dissimilarities have been noted in end stage processing of dying cells during developmental neuronal cell death compared to other examples of ACD (Clarke, 1990). Thus TRPM-2 is not required for all forms of ACD. Apolipoprotein J/SP-40,40/CLI forms a specific complex with apolipoprotein A-I in high density lipoproteins (de Silva *et al.*, 1990; James *et al.*, 1991; Jenne *et al.*, 1991). The function of TRPM-2 remains to be elucidated.

D. RELATIONSHIP OF ACD TO PROSTATE CANCER

Since ACD can be induced into tumor cells that are both hormonally responsive and unresponsive, the understanding of ACD aimed at improving prostate cancer therapies is crucial. The attractive feature of ACD from therapeutic point of view is that if the prostatic cells could be made to believe that they now have to die, cell death, hence the cure, would occur without any surgical intervention or major drug administrations. To date ACD remains the only known means of eliminating the recurrence of cancer after the variable disease-free interval. It is imperative to understand more about ACD so that

it will be possible to induce it specifically into metastatic cells that are either no longer or not responsive to hormonal therapies. This deeper understanding of ACD could even provide some important insights regarding the treatment of not only prostate cancer but of all forms of this deadly disease.

E. SPECIFIC AIMS

- To clone and characterize the full length rat and human TRPM-2 cDNAs.
- To clone and characterize the rat and human TRPM-2 genes.
- To determine the transcription start site of the TRPM-2 genes.
- To study the expression of the rat TRPM-2 gene in different tissues.
- To resolve the ambiguity regarding the leader sequence of the TRPM-2 and SGP-2 cDNAs
- To prepare the grounds for detailed analysis of the expression of the rat TRPM-2 gene via its 5' flanking region.

II. METHODS

A. NUCLEIC ACID EXTRACTIONS

1. DNA extractions

a. Genomic DNA extraction

DNA was extracted from nucleated white blood cells, using the method of Dr. Hear (University of Calgary, unpublished) of both rat and human. The blood was collected in 7 ml EDTA-containing lavender vacutainers, diluted five fold in 17 mM Tris-HCl (pH 7.65), 140 mM ammonium chloride at 37°C to insure specific lysis of the red blood cells. The nucleated intact cells were recovered by low speed centrifugation, washed twice in isotonic saline (0.85% NaCl) and lysed in 100 mM Tris-HCl (pH 8.0), 0.2% (w/v) SDS, 40 mM EDTA. The DNA was then extracted twice with freshly distilled phenol/chloroform (1:1) saturated with TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), once with chloroform, and precipitated with 2.5 volumes of ice cold 95% ethanol. The DNA was then treated with RNAse A (1 µg/100 µl final concentration) for 1 h at 37°C and re-extracted with phenol/chloroform and re-precipitated as above.

b. Phage DNA extractions

The recombinant phages/cells (MB 406 bacteria) were grown concomitantly in T broth (10 g/L tryptone, 5 g/L NaCl, 0.4% maltose, 10 mM $MgSO_4$ and the phage particles were purified on a 1.5 g/ml CsCl equilibrium gradient (Maniatis, 1982). The gradients were centrifuged in a Beckman SW 50.1 rotor for 22 h, 50,000 rpm, at 4°C. The phages were then disrupted in 20 mM EDTA, 0.5% SDS to release the DNA, which was subsequently diluted 5 fold with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and precipitated

with 2.5 volumes of ice cold 95% ethanol. The genomic inserts were subsequently restriction digested, Southern transferred and their identity verified by hybridization with p17H.

c. Plasmid extractions

The plasmid DNA was isolated using the standard alkaline procedure (Birnboim and Doly, 1979) and purified by a 1.7 g/ml equilibrium CsCl centrifugation gradient in a Beckman Vti65 rotor for 12 h, 55,000 rpm, at 20°C. The final concentration of ethidium bromide was 0.8 mg/ml. The ethidium bromide was removed by sequential extraction with n-butanol saturated with 5 M NaCl in water. The DNA was then diluted 5 times into 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and precipitated with 2.5 volumes of ice cold 95% ethanol. The DNA was redissolved into TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

d. Agarose gel electrophoresis of DNA

Every DNA sample preparation was checked by agarose gel electrophoresis on gels ranging from 0.5 to 4% depending on the size of the fragments. The genomic DNA was analyzed on 0.5% gels, the plasmid inserts on 1.5% gels and the smallest DNA fragments, down to 60 bp, on 4% NuSieve agarose gels. The voltage used was generally less than 50 V for 12 h or 100 V for 2 h. The gels were stained in 1 µg/ml ethidium bromide for 15 min, visualized and photographed using 306 nm U.V. transillumination with and orange filter and Ilford FP4 film. The molecular weights markers used were λ

DNA cut with Hind III or with Eco RI/Hind III or a mixture of both. The molar addition of linearized pTZ18R (size = 2.9 kb) to the mixture of λEco RI/Hind III+λHind III gave an all purpose molecular marker ranging between 0.5 to 20 kb.

2. RNA extractions

Total RNA was isolated from freshly excised rat tissues using the LiCl/urea method (Auffray and Rougeon, 1979) with modifications (Tenniswood & Simpson, 1982). Poly(A)$^+$RNA was isolated by oligo-dT cellulose column chromatography as described by Aviv and Leder (1972).

a. Agarose gel electrophoresis of RNA

Total and poly(A)$^+$RNA were electrophoresed through 1.5% agarose gels under denaturing condition with formamide using rat rRNA as size markers. Electrophoresis was carried out at room temperature at 100 V for 4 h. The gels were stained and photographed as above.

B. RAT AND HUMAN GENOMIC LIBRARIES

1. Mbo I restriction digests: 10-20 kb DNA preparation

Partial Mbo I genomic restriction digests were performed to yield DNA fragments mostly ranging between 10 and 20 kb as visualised on 0.5% agarose gels. 10 μg of genomic DNA were digested with 1 unit of Mbo I enzyme that was serially diluted to 0.1 U/μg of genomic DNA. The restriction digest was carried out at 37°C for 1 h, and

stopped by chilling on ice and by addition of EDTA to 10 mM. 1 µg of each serial dilutions of digested DNA was electrophoresed on a 0.5% agarose gel and visualized. Those digests who produced the predominant staining in the 10-20 kb range were pooled, run through a 0.5% low melting agarose gel (IBI). The lanes for the size markers were cut out, stained and realigned with the remaining of the gel to localize the genomic 10-20 kb fragments. This region was then cut out and the DNA purified from low melting agarose. The agarose plugs were chopped into small pieces, heated at 65°C in 5 volumes of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA for 1 h to allow the agarose to melt. The agarose solution was then extracted with an equal volume of phenol saturated with TE buffer, phenol:chloroform (1:1) saturated with TE buffer and then chloroform extracted. The genomic DNA was precipitated with 2.5 volumes of ice-cold ethanol at -70°C for 15 min and spun at 12,000 g for 30 min at 4°C. The DNA pellet was redissolved in 10 mM Tris-HCl (pH 8.0). This DNA was checked into a 0.5% agarose gel to determine its integrity.

2. EMBL3 genomic library construction

The Mbo I DNA has the advantage of having the same sticky ends of Bam HI. This makes possible the cloning of Mbo I genomic insets into the Bam HI site of EMBL3 vector (Promega, Toronto). A 3:1 EMBL3:genomic inserts ratio on a w/w basis was used for making all the libraries. This DNA mixture was ligated with 1 unit T4 ligase in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 5% PEG and 1 mM DTT at 21°C for 6 h. Then the properly ligated DNA was packaged into λ phage. The ligation mix was

added to one packaging extract (provided by Promega, Toronto) and incubated at 21°C for 2 h. MB 406 cells were then infected with the freshly packaged phages at 37°C for 20 min before plating on large LB plates (10 g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl, 10 mM $MgCl_2$, 10 g/L Bactoagar). To allow the screening of unamplified libraries, six libraries of each species were simultaneously prepared, giving a total of 3.6 X 10^6 independent recombinants for the rat and 3 X 10^6 for the human.

3. Screening of genomic libraries

500,000 plaques from each library were plated at high density and transferred to a hybridization membrane by plaque lift for screening. The lifts were probed with radiolabeled p17H (>10^8 cpm/μg). Twelve (12) rat and nine (9) human genomic clones ranging from 13 to 19 kb were identified on the basis of their hybridization signals to p17H. These clones were individually plaque purified and the size of their genomic inserts were established by Sal I restriction digestion.

C. MEMBRANES

1. Hybridizations and washes

All hybridizations were performed on Hybond N membranes in 6 X SSC, 0.1% SDS, 1 X Denhardt's solution (0.02% albumin, 0.02% ficoll, 0.2% polyvinyl pyrolidone), 100 μg/ml heterologous ssDNA, with 10^6 cpm/ml of radiolabeled probe (specific activity = 10^8 cpm/μg) at 65°C, overnight. The membranes were washed at 65°C, twice in 2 X SSC, 15 min, once in 2 X SSC, 0.1 SDS for 30 min, and once in 0.1 X SSC for 10 min.

2. Plaque lifting

Appropriate circular Hybond-N membranes were used for plaque lifts. The membranes were carefully deposited onto the plates, left blotting onto the agar for 1 min, peeled off gently and allowed to air dry. Each membrane was then separated by a 3 mm Whatman paper, wrapped into aluminium foil. The phages were disrupted, the DNA denatured and fixed by autoclaving at 131°C, under steam, 10 min. The packs were then dried 30 min under vacuum at 80°C. This plaque lifting method avoids the usual cumbersome treatment of the membranes in denaturing and neutralizing solutions and gives better resolution on the X-ray film.

3. Southern transfer

After restriction digests, the DNA was run into a 1% or 1.5% agarose gel, stained with ethidium bromide and photographed. If fragments larger than 10 kb were present, the DNA was partially depurinated by soaking the gel into 0.25 N HCl for 15 min before the denaturation step. The DNA was then denatured into 0.5 N NaOH, 1.5 M NaCl for 30 min and subsequently transferred to Hybond-N membranes by capillary transfer with 0.25 N NaOH, 1.5 M NaCl overnight. After the transfer the membrane was removed, soaked into 2 X SSC to remove any adhering agarose, and baked under vacuum at 80°C for 2 h.

4. Stripping of the membranes

To remove the radiolabeled probed, selected Southerns were stripped in 0.4 N NaOH for 45 min at 45°C, and neutralized in 100 mM Tris-HCl (pH 7.5), 0.1 X SSC, 0.1% SDS for 45 min at 45°C. The membranes were then air dried and exposed overnight to detect any residual radioactivity.

D. RESTRICTION MAPPING OF THE GENOMIC INSERTS

All EMBL3 inserts were restriction digested with Sal I, which was found to liberate all inserts intact. Single digests were then performed with the following enzymes: Eco RI, Bam HI, Hind III, Kpn I. These enzymes were selected because of their clearly distinguishable restriction patterns with EMBL3 alone, and thus made the restriction mapping of such large DNA fragments easier. Double digests were performed with Sal I and all four above enzymes. Systematic triple digests were also performed. A primary restriction map was then established by deduction. To complete and confirm this first restriction map, most genomic fragments were purified from a 1% agarose gel using GeneClean (Promega, Toronto) and hybridized, one against another, on several dot blots. Selected fragments were also hybridized against a series of Southerns blots. These Southerns were passed through sequential rounds of hybridizations and strippings. Dot blots and Southerns were performed until clear restriction maps were established for rat and human. The restriction map for the rat extends 30 kb. This genomic region is contained within two overlapping phage clones: λR8.1.1.1 and λR6.2.1.1. The restriction

mapped human locus is 18.5 kb and is also included in two overlapping genomic clones: λH5.1.1.1 and λH9.1.1.1.

E. SEQUENCING OF THE RAT AND HUMAN cDNAs AND GENOMIC FRAGMENTS

Both rat and human cDNAs and several selected genomic fragments were sequenced. Both strands of the cDNAs were sequenced. In the case of the genomic fragments when both strands were not sequenced usually more than three overlapping sequencing runs were used in the merging of the final sequence.

1. Exonuclease III deletions

Two strategies were used for all sequencing procedures: unidirectional Exo III deletion and primer extension. All exonuclease III digestions were performed with the Erase-a-base™ (Promega, Toronto). 10 μg of the selected intact plasmid were restriction digested with two enzymes; one leaving a blunt end or 5' overhang adjacent to the fragment from which deletions were to proceed and one leaving a 3' overhang from which exonuclease III cannot delete. In the case where no convenient 3' overhang could be produced an alternative strategy using α-phosphorothioate deoxynucleotides to fill the 3' recessed ends was used. In that case the filled in ends were produced before the 5' overhang. All four α-phosphorothioate-NTPs were added to the restriction digested DNA at a final concentration of 40 μM each. The fill in reaction was performed in presence of 20 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$, 1 mM DTT and 50 U/ml Klenow DNA polymerase. The mixture was incubated at 37°C for 10 min. To inactivate the klenow

fragment, the sample was heated at 70°C for 10 min. The DNA was then extracted with an equal volume of phenol:chloroform and precipitated with 2.5 volumes of ice-cold ethanol before the second digest. The double digested DNA was dissolved in 66 mM Tris-HCl (pH 8.0), 1.1 mM $MgCl_2$. The samples were prewarmed at 39°C which allowed an average deletion of 600 bp/min after the 25 second lag for the reaction to begin. To start the reaction, 300 U of Exo III were added, and 10 to 30 aliquots, depending of the size of insert, were taken out at 30 second intervals. The aliquots were transferred to 40 mM potassium acetate (pH 4.6), 330 mM NaCl, 1.4 mM $ZnSO_4$, 6.8% glycerol, 60 U S1 nuclease and left at room temperature for 30 min to allow complete digestion of the strand that Exo III left behind. The S1 nuclease was inactivated in the presence of 300 mM Tris-HCl (pH 8.0), 50 mM EDTA and heated at 70°C for 10 min. A 40 ng DNA sample was then checked on a 1% agarose gel to select those time points to be used in the transformation. The ends were then blunt ended by the addition of 10 mM of each dNTP and 1 U Klenow DNA polymerase at 30°C for 3 min. The plasmids were phenol:chloroform extracted and precipitated as usual. They were recircularized in the presence of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM ATP, 1 mM DTT, 5% PEG and 1 U of T4 DNA ligase at room temperature for 1 h. Each batch of circularized plasmids were transformed into DH5αF' cells to allow for single stranded sequencing. Plasmid streak preparations were used to size select the appropriate clones to be used for sequencing protocols. One clone was streak prepared for every 20 bp to be sequenced, and one deletion clone was sequenced for every 100 bp to be sequenced to ensure proper overlap and hence accuracy of the sequence data.

2. Primer extensions

Where needed, specific 17-mer sequencing primers were synthesized on an IBI oligonucleotide synthesizer to ensure sequencing of the few regions missed by exonuclease III deletions. In each case a set of two primers distanced by less than 50 bp was used to ensure the accuracy of the results.

3. Sequencing

a. Preparation of the ssDNA template from plasmid DNA

To prepare the ssDNA template for sequencing, M13KO7 helper phage was used to infect a 2 ml culture of DH5αF'. The cell were grown to an O.D. of 0.7 and the phages were added at an M.O.I. of 10. A 20 ml culture was then inoculated with the infected cells in the presence of 6 μg/ml kanamycin and incubated at 37°C, 18 h. The phages were precipitated with the presence of 5% PEG, 900 mM potassium acetate (pH 7.0) on ice, 30 min. The phages were recovered at 12,000 g, 4°C, 15 min. They were then phenol, phenol:chloroform, and chloroform extracted to remove the phage proteins and obtain the DNA to be sequenced.

b. The sequencing system

The annealing reactions contained 1 pmol of the appropriate primer from the MCS or from a specific location on the template (synthesized 17-mer), 3 μg ssDNA, 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$ and 50 mM NaCl. They were incubated at 65°C, 2 min, then at room temperature for 30 min. For the labeling reactions the annealed

templates were incubated with 10 mM DTT, 0.5 μM of each dGTP, dCTP and dTTP, 2.5 μCi of [α-^{35}S]dATP (>800 Ci/mmol) and 1 μl Sequenase®, a modified version of the T7 DNA polymerase (United State Biochemicals, Cleveland). These mixtures were incubated at 15°C, 2 min. The termination reactions were then executed in the presence of each set of ddNTP mix, 8 μM of each dNTP and 0.8 μM of either ddGTP, ddATP, ddCTP, ddTTP at 42°C for 5 min. The sequencing reactions were stopped 50% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.05% xylene cyanol FF. These were then ready to load into the sequencing gels.

The reaction mixtures were run into standard 6% (for short runs) or 5% (for long runs) 0.2 mm polyacrylamide sequencing gels (IBI). The acrylamide/bis-acrylamide ratio used was 19/1. For the short runs on 6% gels, the samples were electrophoresed at 2,500 V for 2.5 h, which resolved from 40 to 250 bases from each selected primer. For the long runs on 5% gel sequencing systems, the samples were electrophoresed at 2,500 V for 5.5 h, which resolved between 200 and 500 bases from the primer. Each gel was washed in 5% methanol/5% acetic acid (v/v), to remove urea, then dried onto the sequencing plate at 110°C before exposing directly to a Kodak X-ray film.

The sequence data was recorded manually and analyzed using the MicroGenie software (Beckman Instruments) on an IBM PC 386/25.

F. NUCLEASE S1 MAPPING OF GENOMIC DNA

Two oligomers of 60 nucleotides corresponding to exon I (TCCCGCGAAGGGGCCACGAGT-GGTGGGCGCAGTGGTCCTCCTCGCGTGACCTCGGTTCGG) and exon IV (AGAGGTACTTCCCACGAGATCTACTGTGGTCCCTAA-GACTTTACTTCGACTTCCGAAAGG) of the TRPM-2 gene, both overlapping the immediate 5' intron/exon genomic sequence, were used. They were designed such that the cleavage products after S1 digestion would be of different length: 40 nucleotides for the exon I probe and 54 nucleotides for the exon IV probe. The probes were end labeled with 5 U of polynucleotide kinase and 50μCi of [γ-^{32}P]ATP to a specific activity of not less than 10^8 cpm/μg. The radiolabeled oligomers were then hybridized to 100 μg total RNA in 100 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, and 80% deionized formamide at 30°C for 16 h. The mixture was then diluted into S1 buffer: 5 mM NaOAc (pH 4.5), 0.28 M NaCl, 4.5 mM $ZnSO_4$, 20 μg/ml denatured calf thymus DNA. 60 U nuclease S1 were added. The samples were incubated at 37°C for 2 h. The nucleic acids were precipitated with 2.5 volumes of ice-cold 95% ethanol. The precipitate was redissolved in 80% formamide containing 0.05% bromophenol blue, heated for at 80°C, 2 min, and loaded onto a standard 8% sequencing gel. The sequencing gel was treated as above and exposed overnight on Kodak X-ray film with one intensifying screen.

G. PRIMER EXTENSIONS OF THE TRPM-2 mRNA

Primer extension was performed using 5 U reverse transcriptase (Life Sciences, St. Petersburg, FL) using 100 μg of total RNA essentially as described (Sambrook *et al.*, 1989). The primers were end labeled with 5 U polynucleotide kinase and [γ-^{32}P]ATP to

a specific activity of not less than 10^8 cpm/μg and 10^6 cpm of labeled primer was used per reaction. The products of the primer extension reactions were RNase A treated and run on an 8% sequencing gel. Primers used to define the +1 site of TRPM-2 included (GAGAATCTTCATGTGTGGC) which hybridized to the non-coding strand of TRPM-2 from position 55-74. This region is common to both SGP-2 and TRPM-2. And the second primer (GGCTTGGCTCCAGTGCG) hybridized to the non-coding strand of TRPM-2 only from position 11-27. Those primers are complementary to TRPM-2 mRNA encoded by exon I and II respectively.

H. PCR ANALYSIS: A COMPARISON BETWEEN SGP-2 AND TRPM-2

Single stranded cDNA was synthesized from 10 μg poly(A)$^+$RNA as described above. PCR was performed using 1 μg of each TRPM-2 or SGP-2 specific primer in a buffer containing dATP (1 mM), [α^{32}P]-dATP (50 μCi), dCTP (1 mM), [α^{32}P]-dCTP (50 μCi), dGTP (1 mM), TTP (1 mM), in PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin), and 5 U Taq Polymerase (Bethesda Research Laboratories, Burlington, ON). Cycling times were as follows: 1 cycle of 94°C for 10 min; 25 cycles of 50°C for 2.5 min / 70°C for 4 min / 94°C for 1.5 min and 1 cycle of 50°C for 2.5 min / 70°C for 13 min. Unincorporated nucleotides were removed by passing the reaction through a Sephadex G-50 spin column. The primers were designed to examine potential alternative exon I usage and were either common to both the reported SGP-2 and TRPM-2 sequences (forward: GGACAATGGCATGGTCCTGGGAGAG; REVERSE: GGCTTACACTCTTCCCAGAGGGCCA); one primer specific to the TRPM-2 reported sequence

(forward: GGAGGAGCGCACTGGAGCCAAGCCG) and one common primer (reverse: GGCTTACACTCTTCCCA-GAGGGCCA) and two primers specific to the reported sequence of SGP-2 (forward: GGCTGGACTTCCTCGCCATGACAGT; and reverse: TGGAGACAACGGTGGTGTTTACTTC). The PCR products were transferred to Hybond N membranes, and hybridized to a probe specific for the common exon II SGP-2 and TRPM-2 (GAGAATCTTCATGGTGTGGC) or a probe specific for the predicted exon I of SGP-2 (GGCGAGAAGAACCACCCCAAATGTAGGTGGTATCTCGTTACCGTGACTC).

III. RESULTS

A. CLONING AND SEQUENCING OF THE RAT TRPM-2 cDNA AND GENE

1. Sequence of the rat TRPM-2 cDNA

A potential full length TRPM-2 cDNA was isolated from a λgt-10 library by Dr. Chiayeng Wang on the basis of the cross-hybridization to pG21-04, the partial cDNA clone described by Léger et al. (1987). This cDNA, designated p17H, appeared to be slightly longer than 1.6 kb on a 1% agarose gel. I subsequently sequenced this cDNA in full and a single open reading frame (ORF), coding for 447 amino acids was identified (fig 2). Further primer extension and nuclease S1 analysis (see below) demonstrated that p17H was indeed the full length rat TRPM-2 cDNA missing only 10 bp on its 5' end. From this 1,630 bp cDNA it can be deduced that the 5' untranslated portion of the mRNA is 72 nucleotides long, the 3' untranslated region is 228 nucleotides and the putative polyadenylation signal is 19 nucleotides from the poly(A) tail.

2. Screening strategy of the rat TRPM-2 gene

500,000 EMBL3 plaques from the rat libraries were screened on the basis of their hybridization signal with pG21-04, the partial length TRPM-2 cDNA. Twelve positive regions on the plates were identified and further plaque purified. Absolute purity of each positive was confirmed after four rounds of plaque purification. All subsequent work was performed with the full length cDNA, p17H, being then available. Two overlapping phage inserts from those positives were latter found to contain the complete rat gene (fig. 3 and below).

Figure 2. Sequence of the rat TRPM-2 cDNA and its ORF.

The figure shows the complete sequence of 17H, the rat TRPM-2 cDNA, and its ORF. Nucleotides 63, 64, 65 (ATG) were selected as the first codon (a methionine) since it yields the longest ORF. The putative polyadenylation site is underlined. The first and last of the 447 amino acids are in bold case. The number of nucleotides and amino acids are indicated on each of their respective lines.

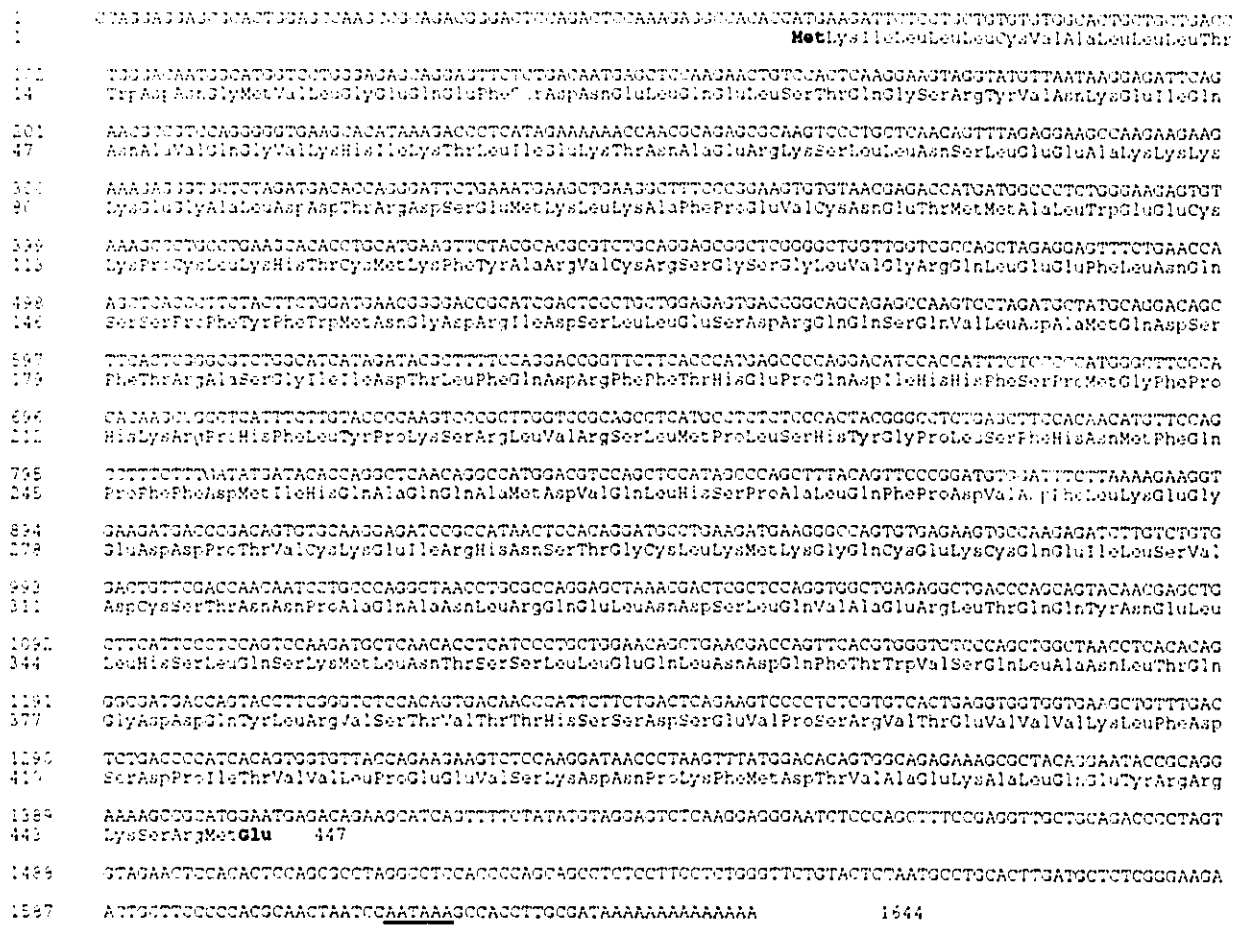
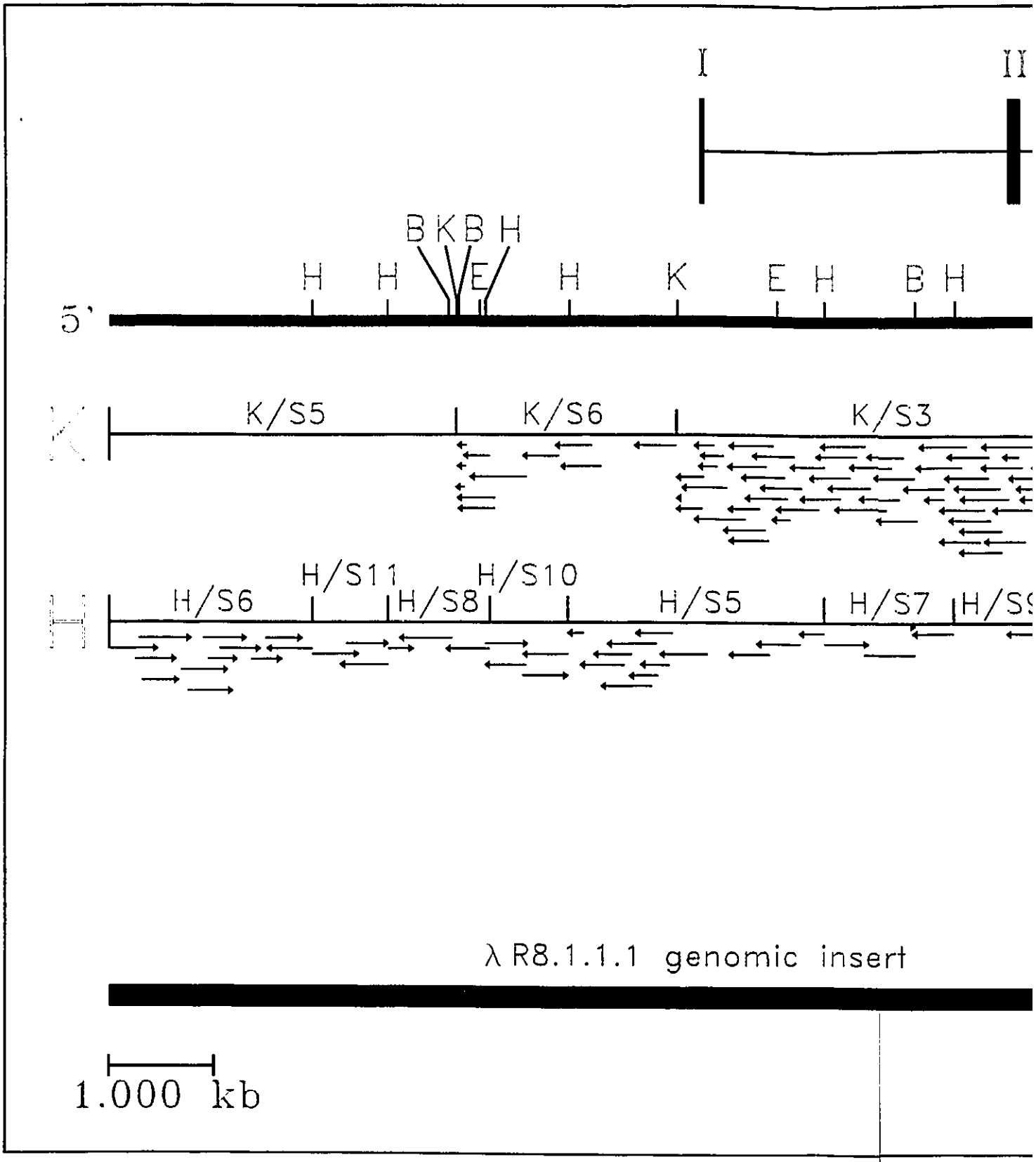
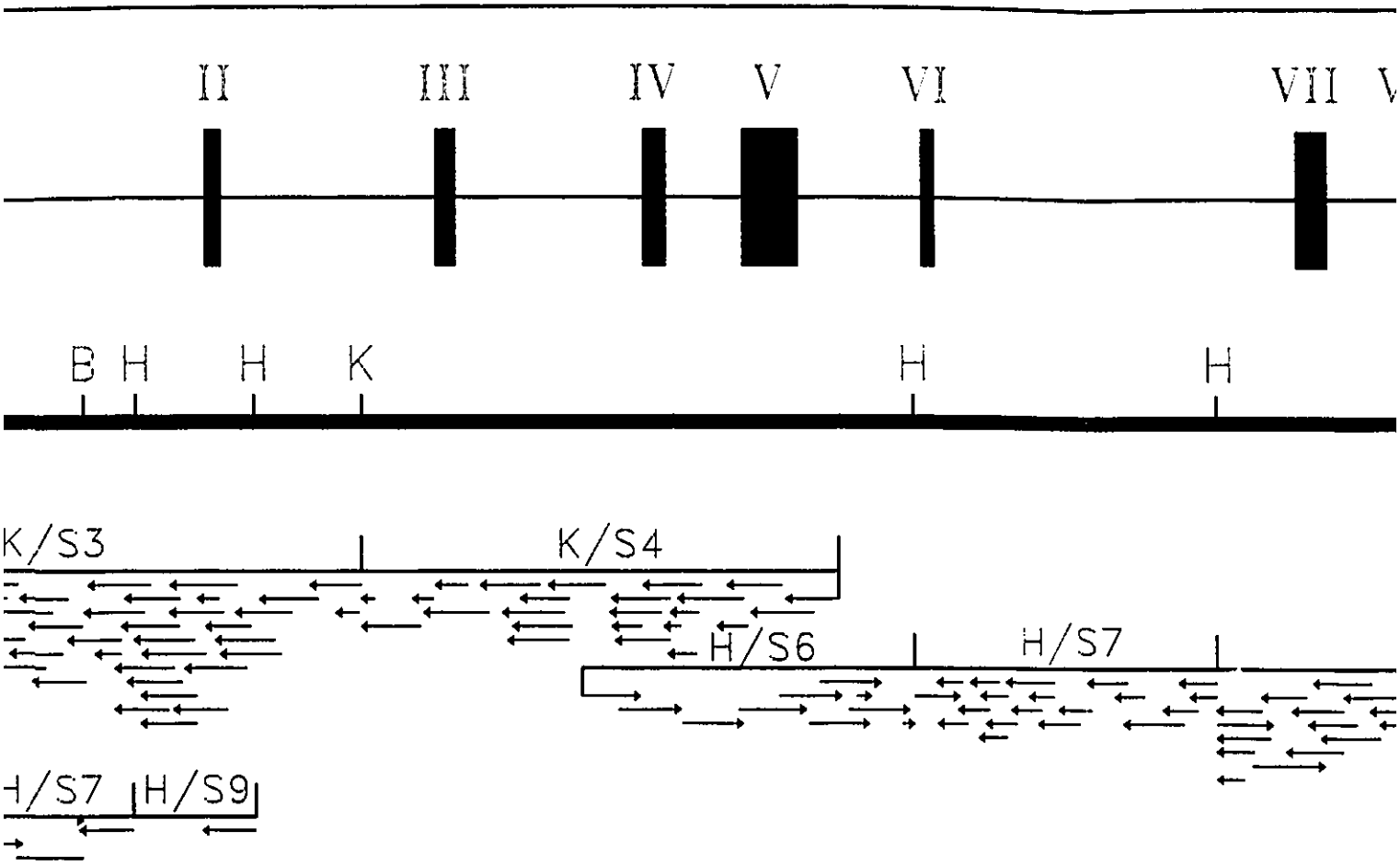


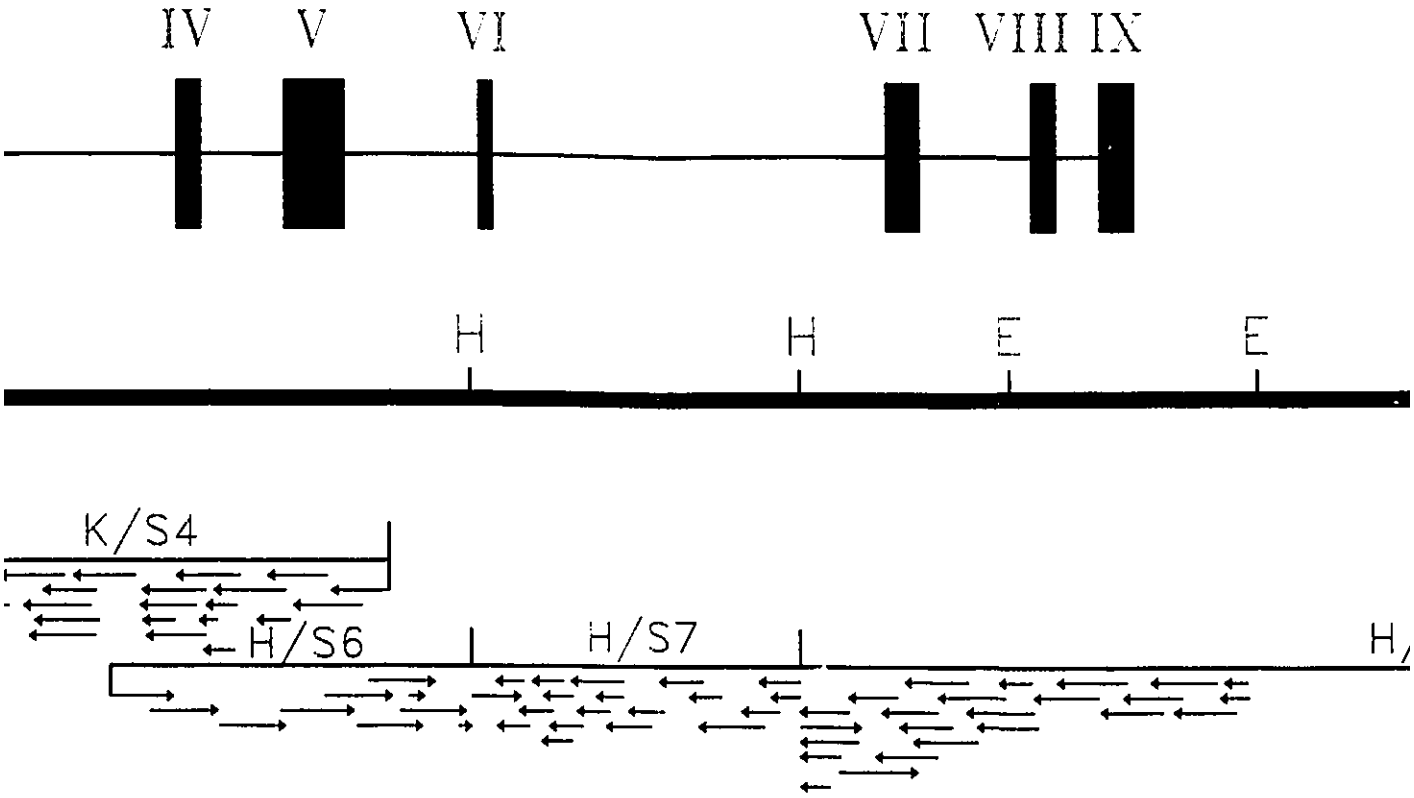
Figure 3. Genomic organization of the rat TRPM-2 gene, with subclones and sequencing strategy.

The exons are depicted as filled boxes. The restriction enzymes are E, Eco RI; B, Bam HI; H, Hind III; K, Kpn I. The extent of each subclone used is displayed under the restriction map. The sequencing strategy is displayed under its corresponding subclone. The pointing direction of the arrows indicate the direction of sequencing from the primer as well as the sequencing run length. The extent of the phage inserts from which the subclones were derived is depicted as two long black rectangles at the bottom of the figure. Notre that when only one strand was sequenced, the region was sequenced several times from different unidirectional exonuclease III deletion clones or genomic subclones. The scale is 1 kb = 2 cm.

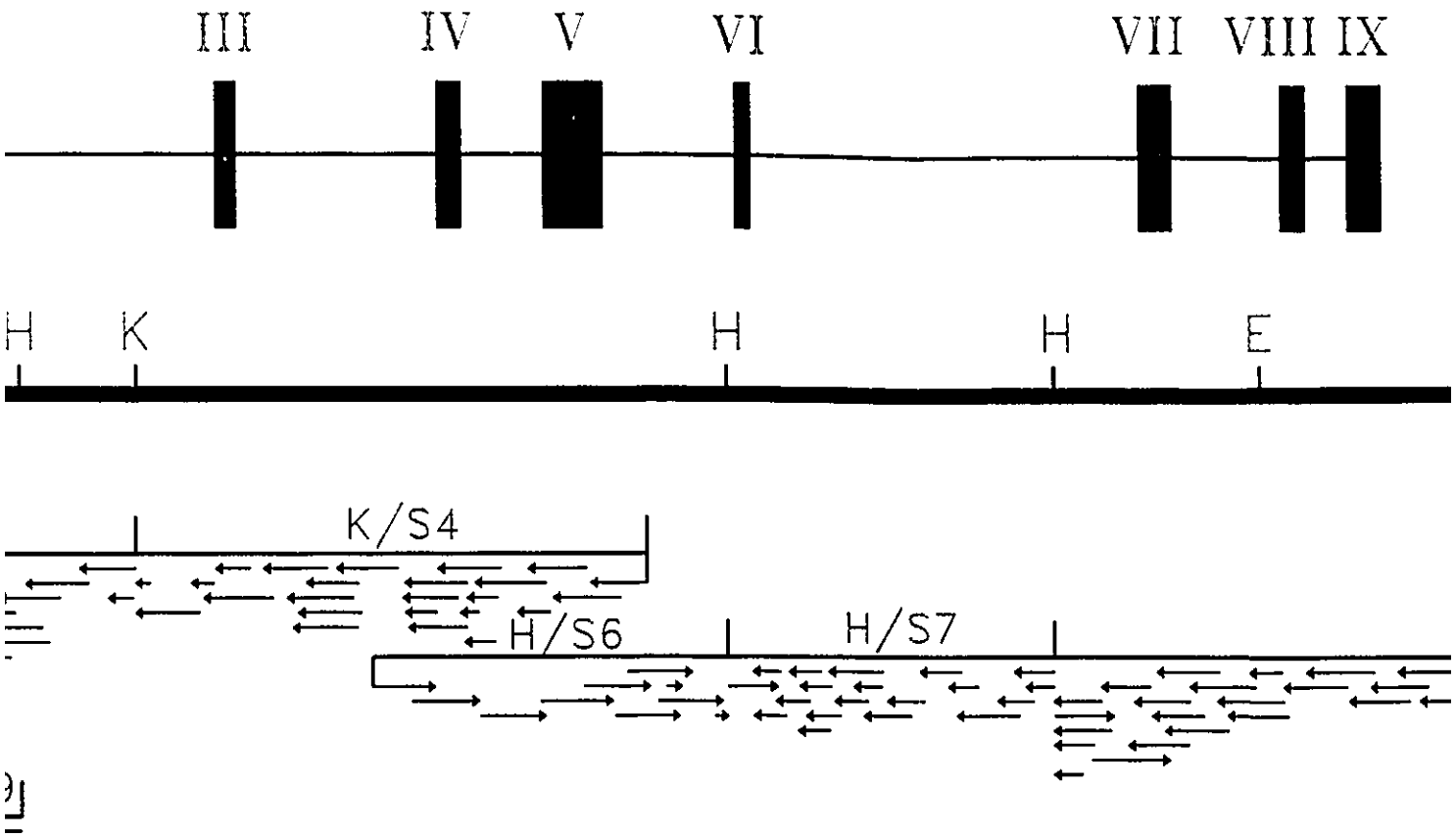

I
II
B K B H
H H E H K E H B H
5'
K
K/S5
K/S6
K/S3
H
H/S6
H/S11
H/S8
H/S10
H/S5
H/S7
λ R8.1.1.1 genomic insert
1.000 kb

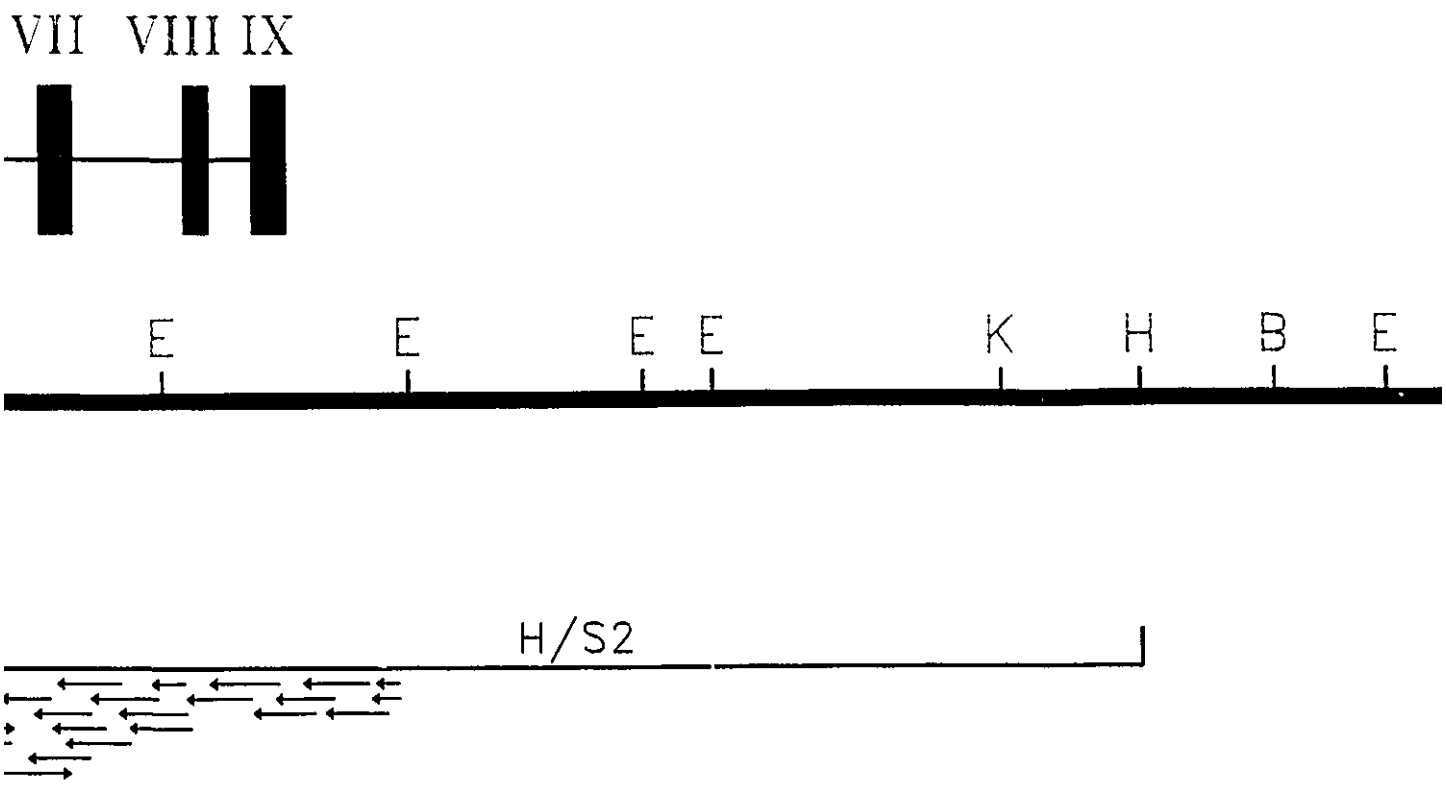


sert

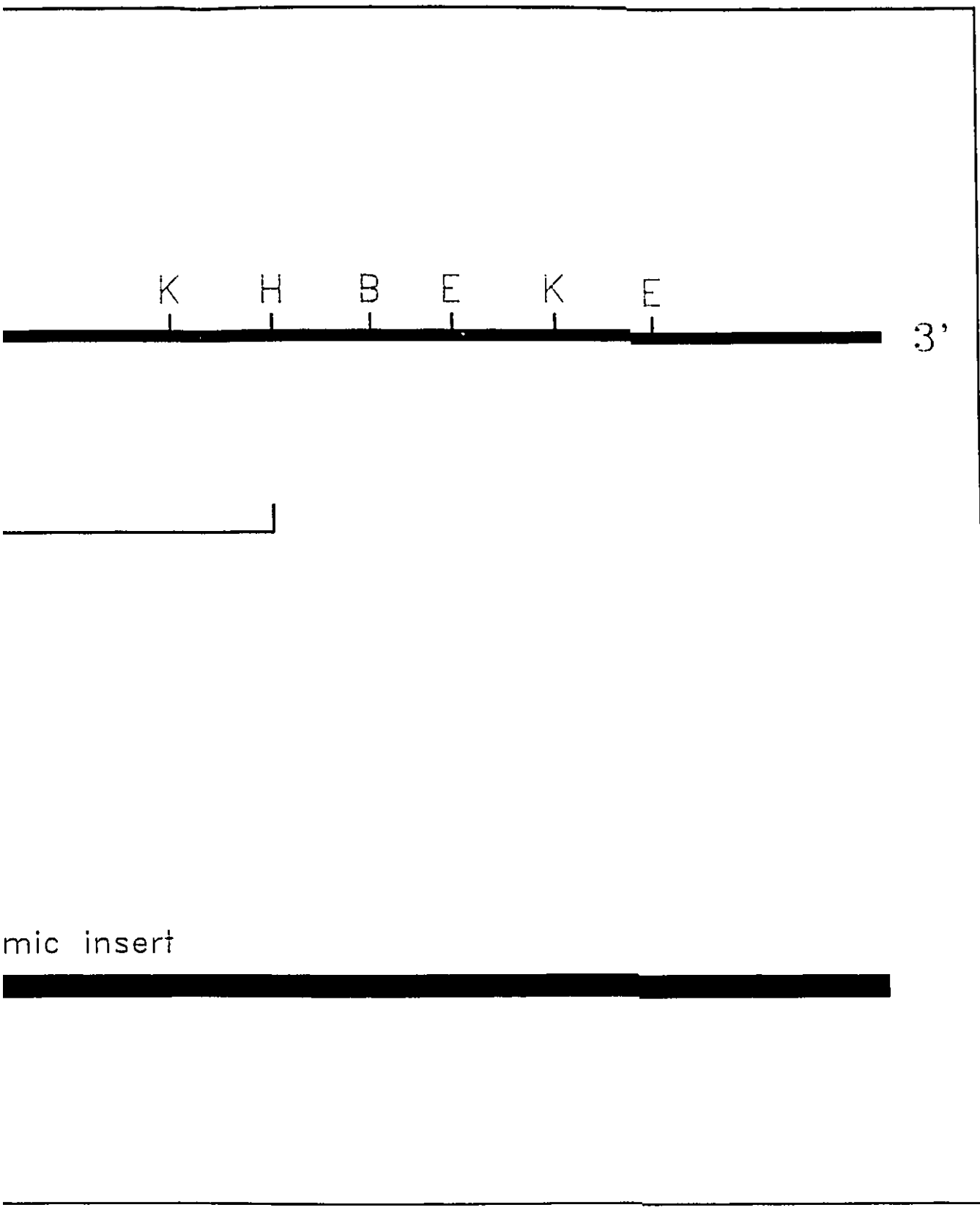


λ R6.2.


III
IV
V
VI
VII
VIII
IX
H
K
H
H
E
K/S4
H/S6
H/S7



λR6.2.1.1 genomic insert


K
H
B
E
K
E
3'
mic insert

3. Subcloning strategy of the rat TRPM-2 gene

Initially all phage inserts were restriction mapped with four enzymes: Eco RI, Bam HI, Hind III, Kpn I to determine the extent of overlap of the genomic clones. On this basis two overlapping phage inserts were selected for subcloning: λR8.1.1.1 (13.5 kb) and λR6.2.1.1 (17.7 kb). Almost every restriction fragments were subcloned in either pTZ18R or pTZ19R as these vector systems were found to be the most stable with the genomic inserts. Most restriction fragments subcloned hybridized to p17H, but with different strengths in the signal, indicating that the gene was made up of several exons. To enhance the restriction map and to facilitate sequencing various sub-subclones were produced in the same vector system.

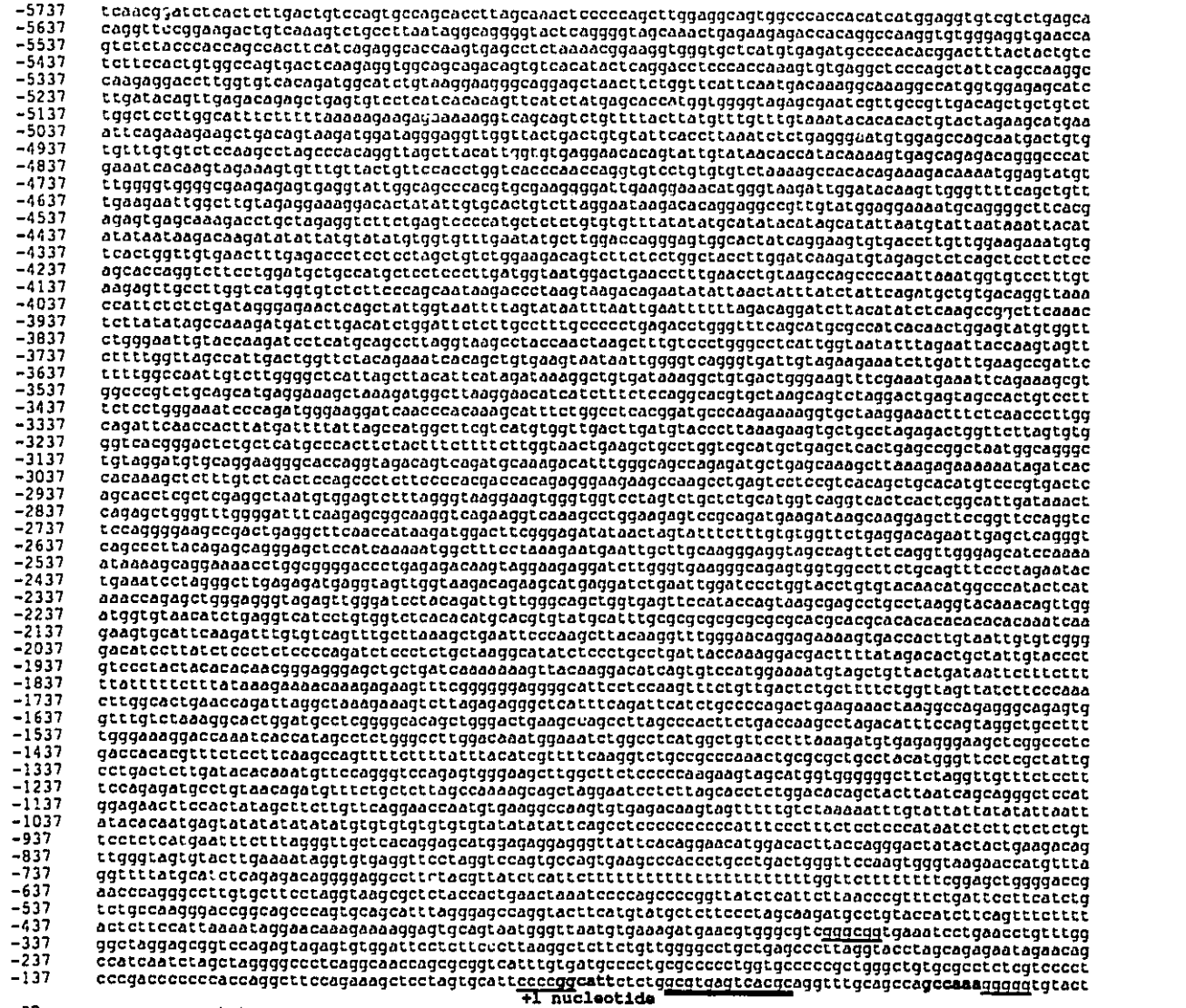
4. Sequencing results

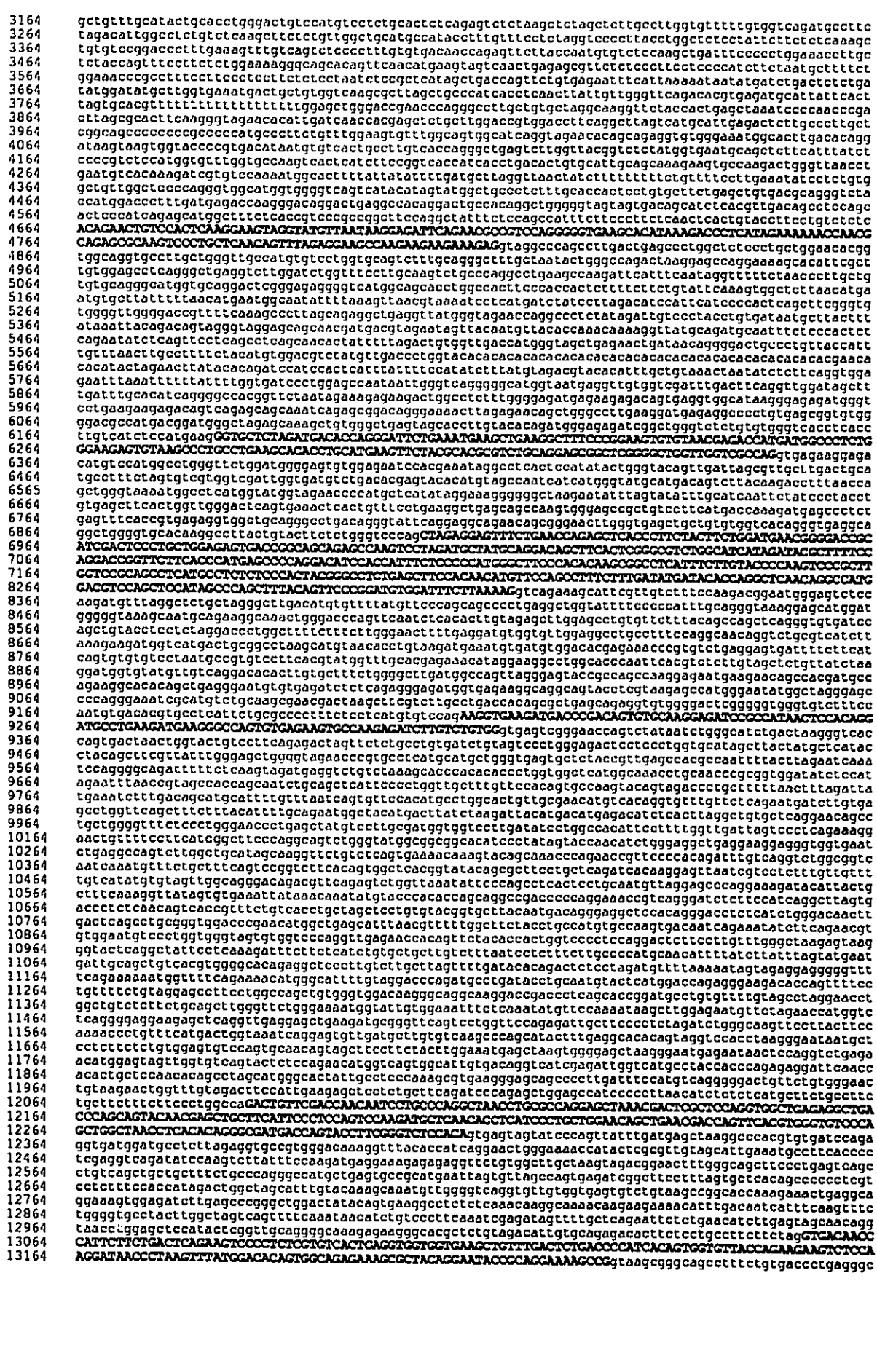
Systematic sequencing of all restriction fragments that hybridized to p17H (fig. 4) was performed using unidirectional exonuclease III deletions. To cover the regions missed by this latter method a series synthesized primers (17-mers) were used. These two techniques allowed the complete sequencing of the rat TRPM-2 gene including all exons and introns.

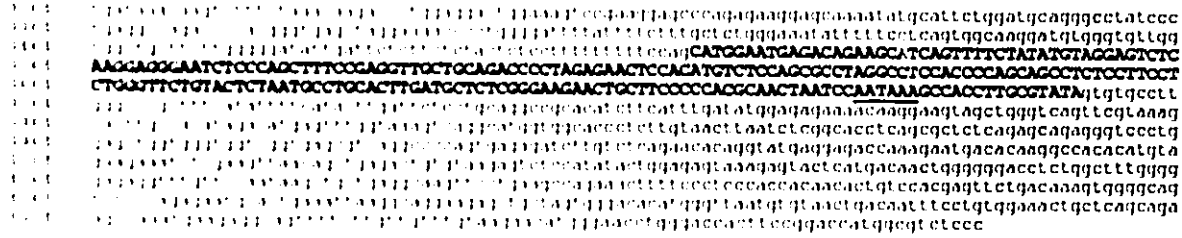
Computer sequence comparison of the full length rat cDNA and the rat genomic sequence confirmed the presence of 9 exons, spanning 13,750 bp from the +1 nucleotide to the polyadenylation signal. See table 1 for the exon/intron statistics.

Figure 4. Complete sequence of the rat TRPM-2 gene.

The intervening, upstream, and downstream sequences are in lower case letters. The sequence predicted in the mature mRNA is in uppercase and bold. The potential putative TATA and CAT boxes are in lower case and bold. Other interesting sequences such as GC rich, and polyadenylation signal are underlined. The palindrome identified in the 5' region is double underlined.







5. Positioning of the +1 nucleotide

Two complementary techniques were used to locate with the most accuracy possible which nucleotide(s) is the first to be transcribed (the +1 nucleotide) in the rat TRPM-2 gene: Primer extension and nuclease S1 mapping of exon I.

a. Primer extension analysis of the rat TRPM-2 exon 1

Due to the discrepancy between the SGP-2 and TRPM-2 cDNAs in their respective 5' regions, the primer extension analysis was repeated with two probes: One common to SGP-2 and TRPM-2 (GAGAATCTTCATGGTGTGGC starting at position 75 on the TRPM-2 cDNA, fig. 2 and 5) and one closer to the 5' end specific to TRPM-2 (CGGCTTGGCTCCAGTGCGCT starting at position 28). Also, since SGP-2 was initially cloned from a rat testis cDNA library and TRPM-2 from the rat prostate four days after castration, there was a possibility that a different transcription site was used in those two tissues. This is the reason why the primer extensions were done using RNA from both tissues.

Fig. 5 shows three bands at 85, 86, and 87 nucleotides from the position 75 primer that is common to both SGP-2 and TRPM-2 clearly indicating that the TRPM-2 cDNA (p17H) is missing only 10 nucleotides compared to the putative mRNA. The same result was obtained in the testis except for a weaker signal. This was to be expected since TRPM-2 is expressed at lower levels in the testis compared to the prostate. The use of the primer located closer to the 5' end and specific to TRPM-2 yielded the same result (data not shown). This same fig. 5 also shows the possibility that transcription of the

TRPM-2 gene may start at one of several nucleotides, however to simplify further discussion, based on consensus transcription start sites an adenosine has been selected as the probable +1 nucleotide for the rat gene.

b. Nuclease S1 analysis of the rat TRPM-2 exon I

The nuclease S1 confirmation of the +1 nucleotide came from the study of the expression levels of TRPM-2 in different tissues (see below). This series of experiments not only confirmed the +1 transcription start but also indicated that the same start site is used in all tissues examined (see below).

6. Detailed analysis of the functional elements in the rat TRPM-2 gene

a. Identification of known regulatory sequences

The first exon of the rat gene is preceded by a TATAAA box at position -30. Two potential CAT boxes were identified at -59 (GCCAAA) and -96 (GGCATT). These boxes are very similar to the consensus GGCAAT. Within the first 1 kb upstream of the transcription start site there are two GC rich regions at positions -100 and -339. These sequences are not perfect SP1 consensus sites (fig. 6). The remainder of the 5' upstream region is interesting primarily for the lack of known conventional hormone regulatory elements. Thus there are no consensus sequences for the ARE (androgen responsive element), ERE (estrogen responsive element), GRE (glucocorticoid responsive element), or CRE (cAMP responsive element). The 3' untranscribed region of the rat gene contains a unique polyadenylation signal.

Figure 5. Primer extension of the rat TRPM-2 mRNA.

The upper strand is the coding strand. The experiment was performed on 100μg day four prostate and testis total RNA. The gel was exposed 12 hours at room temperature, as described in the methods. In the extension reactions, the non-coding strand was synthesized from the primer whose sequence is underlined with the arrow. The sequence predicted from the cDNA and genomic sequences is shown in uppercase to the transcription start site. Non-transcribed sequences are shown in lower case. The arrows point to possible +1 nucleotides as estimate from primer extension analysis and the solid circles from the nuclease S1 analysis and a second set of primer extensions (data not shown). The number of nucleotides from the start of the primer that correspond to the bands are indicated: 85, 86, 87.

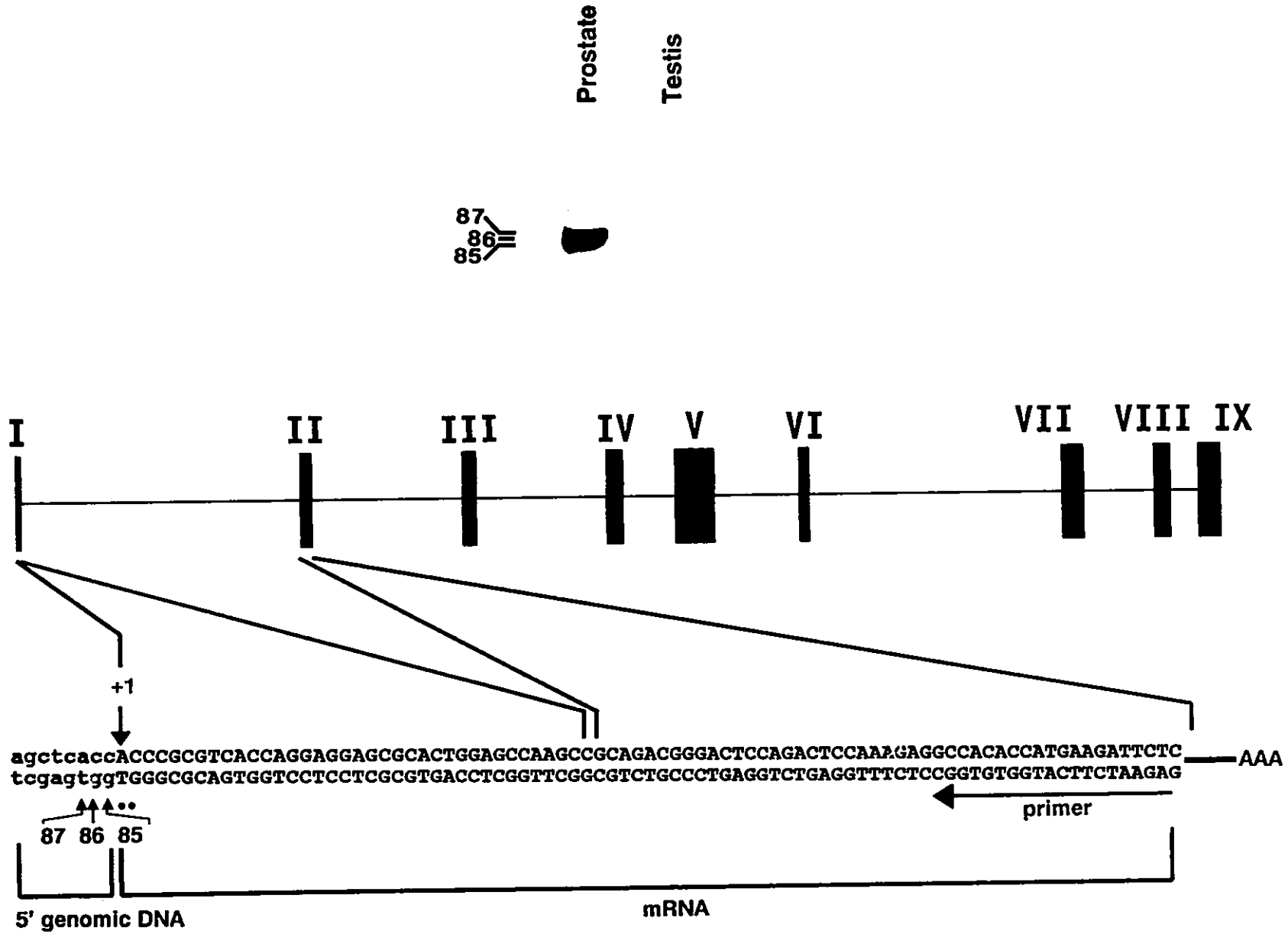
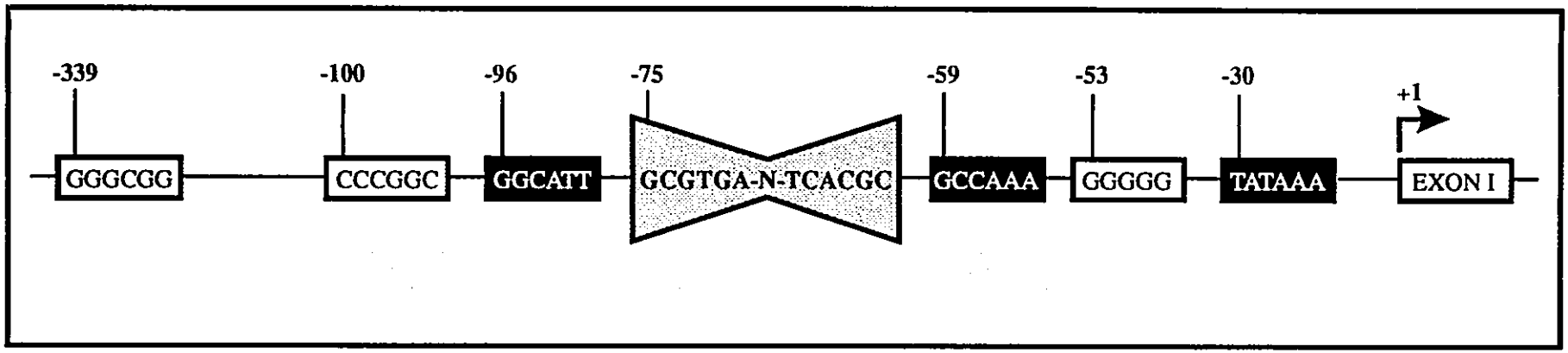

Prostate
Testis
87
86
85
I
II
III
IV
V
VI
VII
VIII
IX
+1
agctcaccACCCGCGTCACCAGGAGGAGCGCACTGGAGCCAAGCCGCAGACGGGACTCCAGACTCCAAAGAGGCCACACCATGAAGATTCTC
tcgagtggTGGGCGCAGTGGTCCTCCTCGCGTGACCTCGGTTCGGCGTCTGCCCTGAGGTCTGAGGTTTCTCCGGTGTGGTACTTCTAAGAG
AAA
primer
87 86 85
5' genomic DNA
mRNA

Figure 6. 5' upstream elements of the rat TRPM-2 gene.

The start of transcription and exon I are represented graphically. The TATA and CAT boxes are shown as black boxes. The GC rich clusters are shown inside white boxes. The palindrome is depicted as a stipled double triangle. The long stippled bar represents the region of sequence similarity (79%) with its human counterpart.


-339
-100
-96
-75
-59
-53
-30
+1
GGGCGG
CCCGGC
GGCATT
GCGTGA-N-TCACGC
GCCAAA
GGGGG
TATAAA
EXON I

b. Identification of unknown regulatory sequences

Since most of the regulatory elements of highly induced genes that have been identified to date are palindromic, the 5' upstream region was searched for short inverted repeats with a close axis of symmetry. One such sequence was identified in the 5' upstream region for the TRPM-2 gene: GCGTGA-(N_1)-TCACGC at position -75 (fig. 6).

c. Comparison of rat TRPM-2 genomic sequences with other rodent sequences in GENBANK

Comparison of the TRPM-2 sequence to GENBANK has revealed that the cDNA and/or the protein have been identified from several tissues and species. The cDNA and the protein has been isolated from rat Sertoli cells as sulfated glycoprotein 2 (SGP-2) (Collard and Griswold, 1987; Griswold *et al.*, 1986); the protein from the ram rete testes fluid as clusterin (Blaschuk *et al.*, 1983); from human serum, as apolipoprotein J (de Silva *et al.*, 1990); serum protein 40,40 (SP-40,40) (Kirszbaum *et al.*, 1989) and complement lysis inhibitor (CLI) (Jenne and Tschopp, 1989); from Madin-Darby canine kidney cells as glycoprotein 80 (gp80) (Hartmann *et al.*, 1991)); from bovine adrenal medullary chromaffin granules as glycoprotein III (Palmer and Christie, 1990); and from Japanese quail neuroretinal cells as T64 (Michel *et al.*, 1989). The comparison of the sequence of the TRPM-2 cDNA to the sequences has revealed that TRPM-2 possessed the same ORF in every case. Only minor single nucleotides discrepancies between TRPM-2 and other published sequences were identified except for SGP-2. SGP-2 has a 269 bp leader sequence that is not found in any other clones including TRPM-2.

d. Analysis of exons/introns borders

The sequence borders of the exons/introns were deduced from published consensus sequences (Mount *et al.*, 1982) and from S1 nuclease analysis in the case of exon I and IV. Table 1 shows all exon/intron borders compared to the consensus sequences.

B. CLONING AND SEQUENCING OF THE HUMAN TRPM-2 cDNA AND GENE

1. Sequence of the human TRPM-2 cDNA

The full length human TRPM-2 cDNA was isolated from a human testis cDNA library purchases from ClonTech, California. This cDNA, designated pHT7 appeared to be a near full length cDNA of 1,648 bp based on a comparison with its rat counterpart. The full sequence of pHT7 is shown in fig. 7. The ORF is 449 aa starting with two Met. HT7 does not have a poly(A) tail, however the published sequence of SP-40,40 (Kirszbaum *et al.*, 1989) does have one. On this basis the 3' untranslated region has 250 nucleotides. the putative polyadenylation signal is 22 nucleotides from the poly(A) tail.

2. Screening strategy of the human TRPM-2 gene

At the time genomic screening of the human libraries was performed, the human cDNA, pHT7, was not available. Therefore 500,000 EMBL3 plaques from the human libraries were screened on the basis of the hybridization signal with p21-04, the partial length rat TRPM-2 cDNA. The extent of conservation was sufficient to identify nine positive regions on the plates. The four rounds of plaque purifications were done in parallel with the rat. Subsequent work was then performed with p17H, the full length rat

Table 1. Splice junctions of the rat TRPM-2 gene compared to the consensus sequences.

The consensus sequence from Mount, 1982 is compared to all splice junction of the rat TRPM-2 gene.

Predicted exon/intron splice sites

Consensus	$\begin{smallmatrix}C\\A\end{smallmatrix}$**AG/**gt$\begin{smallmatrix}a\\c\end{smallmatrix}$agt
I /1	**CCG/**gtgaga
II /2	**AGG/**gtgagt
III /3	**GAG/**gtcagg
IV /4	**CAG/**gtgaaa
V /5	**GAG/**gtgaga
VI /6	**TGG/**gtgagc
VII /7	**ACG/**gtgagc
VIII/8	**CCG/**gtaagc

Predicted intron/exon splice sites

Consensus	$(\begin{smallmatrix}c\\t\end{smallmatrix})_n$ n$\begin{smallmatrix}t\\c\end{smallmatrix}$ag/**G**
1 /II	ctctgcag/**A**
2 /III	tcttgcag/**A**
3 /IV	tcctgtag/**G**
4 /V	tgtcccag/**C**
5 /VI	gtccccag/**A**
6 /VII	ctgggcag/**A**
7 /VIII	ccatttag/**G**
8 /IX	tatttcag/**G**

cDNA and then pHT7, the full length human cDNA (see below). Also similar to the rat, two overlapping phage inserts from those positives were latter found to contain the complete human gene (fig. 8 and 9 and below).

3. Subcloning strategy of the human TRPM-2 gene

The subcloning strategy for the human gene was similar to the rat. The restriction enzyme Sal I also released all inserts intact. Restriction mapping with the same four enzymes (Eco RI, Bam HI, Hind III, Kpn I) was therefore straight forward. Also two overlapping phage inserts contained the complete gene and were selected for subcloning: λH5.1.1.1 (15.1 kb) and λH9.1.1.1 (13.3 kb). The same vector systems were used: either pTZ18R and pTZ19R (Pharmacia). These vectors seemed to yield stable inserts for the human clones as well. Cross-hybridization to p17H also revealed a multi-exonic gene that appeared similar in size as compared to its rat counterpart.

4. Sequencing results

The sequencing strategy for the human subclones was very similar to the rat, the major difference being the fact that there were much more intronic repetitive sequences in the human subclones. This made complete sequencing more difficult. Where some of the exonulease III deletion clones missed, primer extensions could not be performed since the 17-mers hybridized to several regions simultaneously. Parts of three intronic regions were not sequenced. A 1,560 bp stretch is missing in intron 3, a 150 bp gap in intron 4 and 3,100 bp in intron 6. However, all exons and their borders were sequenced (fig 9).

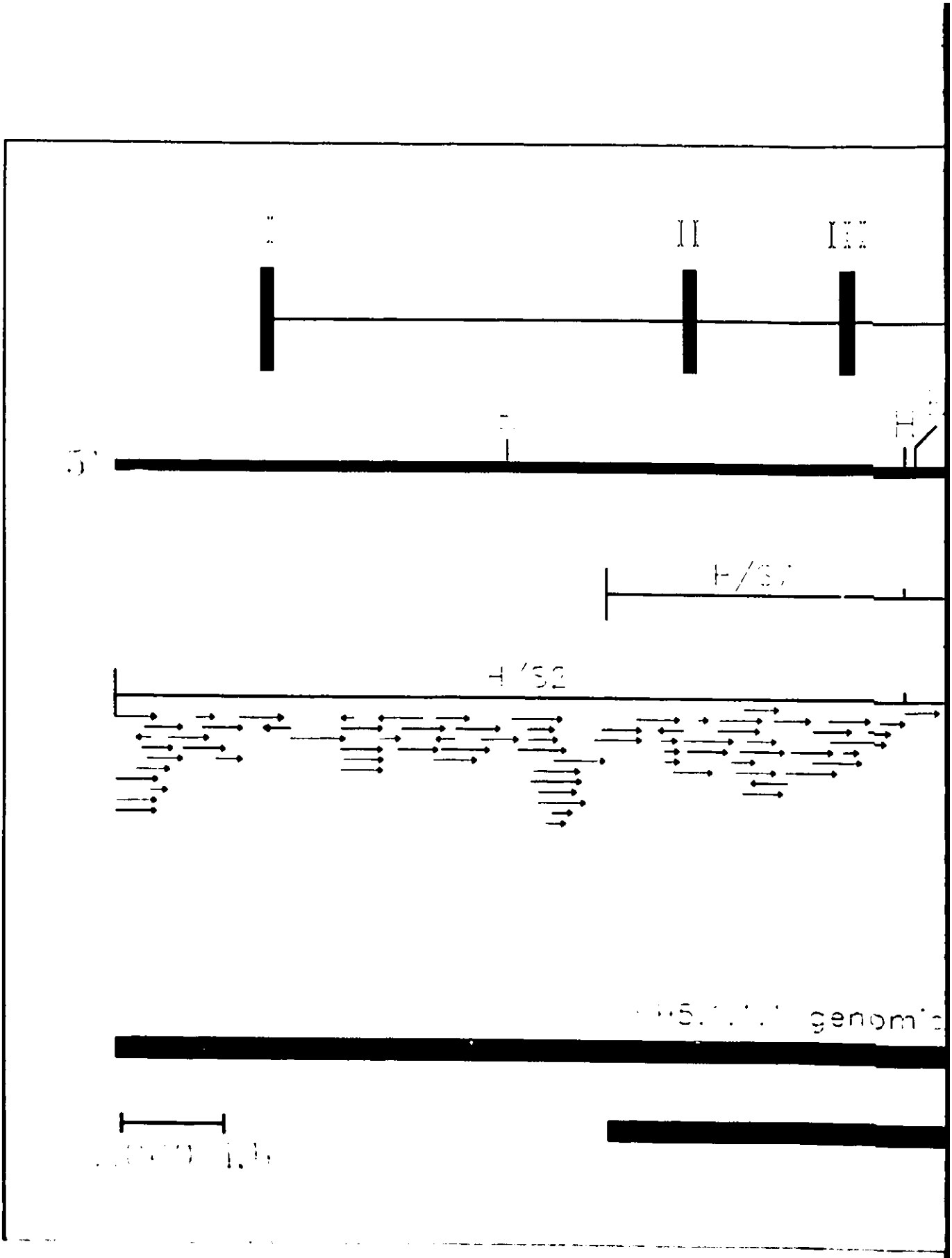
Figure 7. Sequence of the human TRPM-2 cDNA and its ORF.

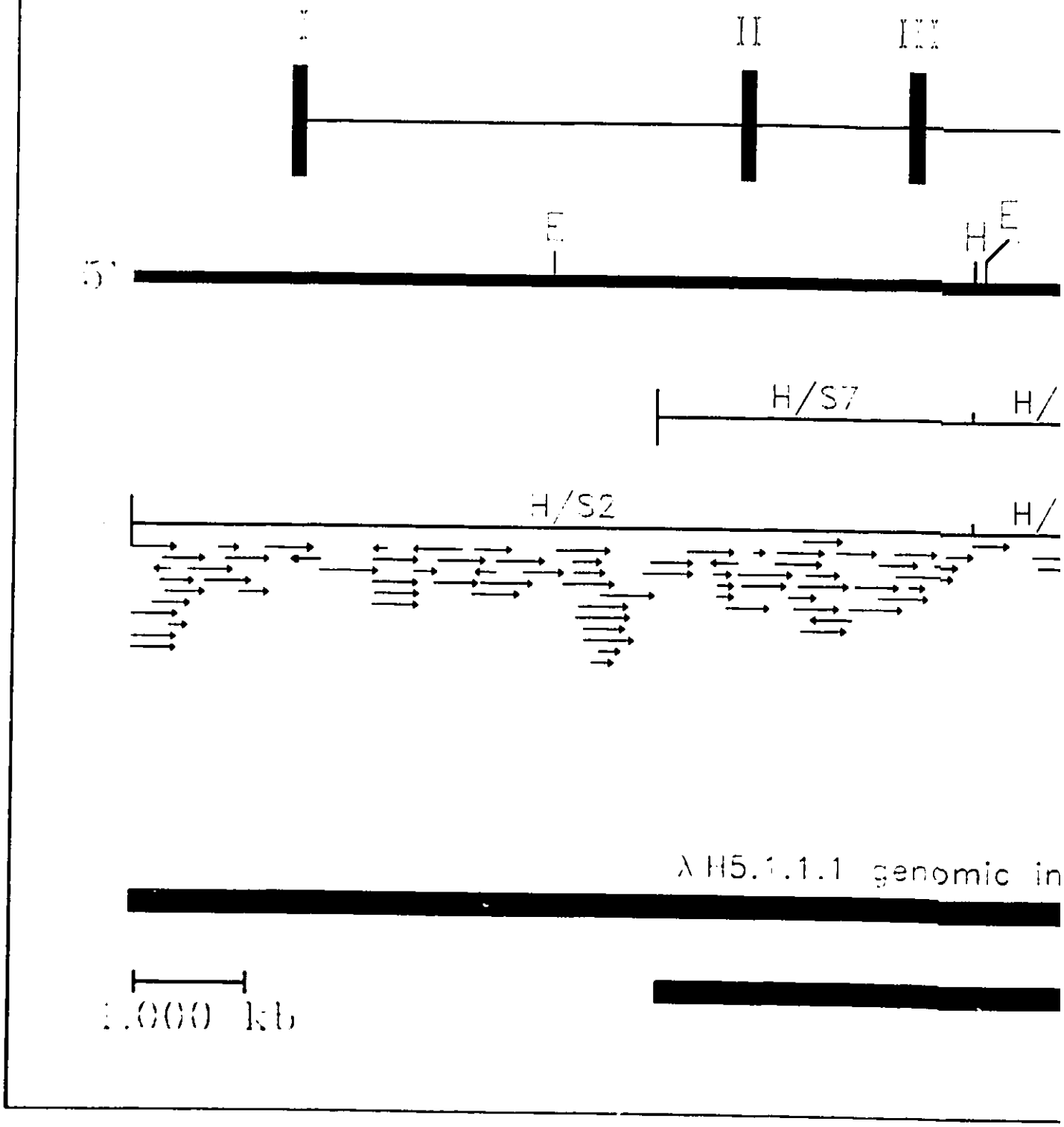
The figure shows the complete sequence of HT7, the human TRPM-2 cDNA, and its ORF. Nucleotides 53, 54, 55 (ATG) were selected as the first codon (a methionine) since it yields the longest ORF. The putative polyadenylation site is underlined. The first and last of the 447 amino acids are in bold case. The number of nucleotides and amino acids are indicated on each of their respective lines.

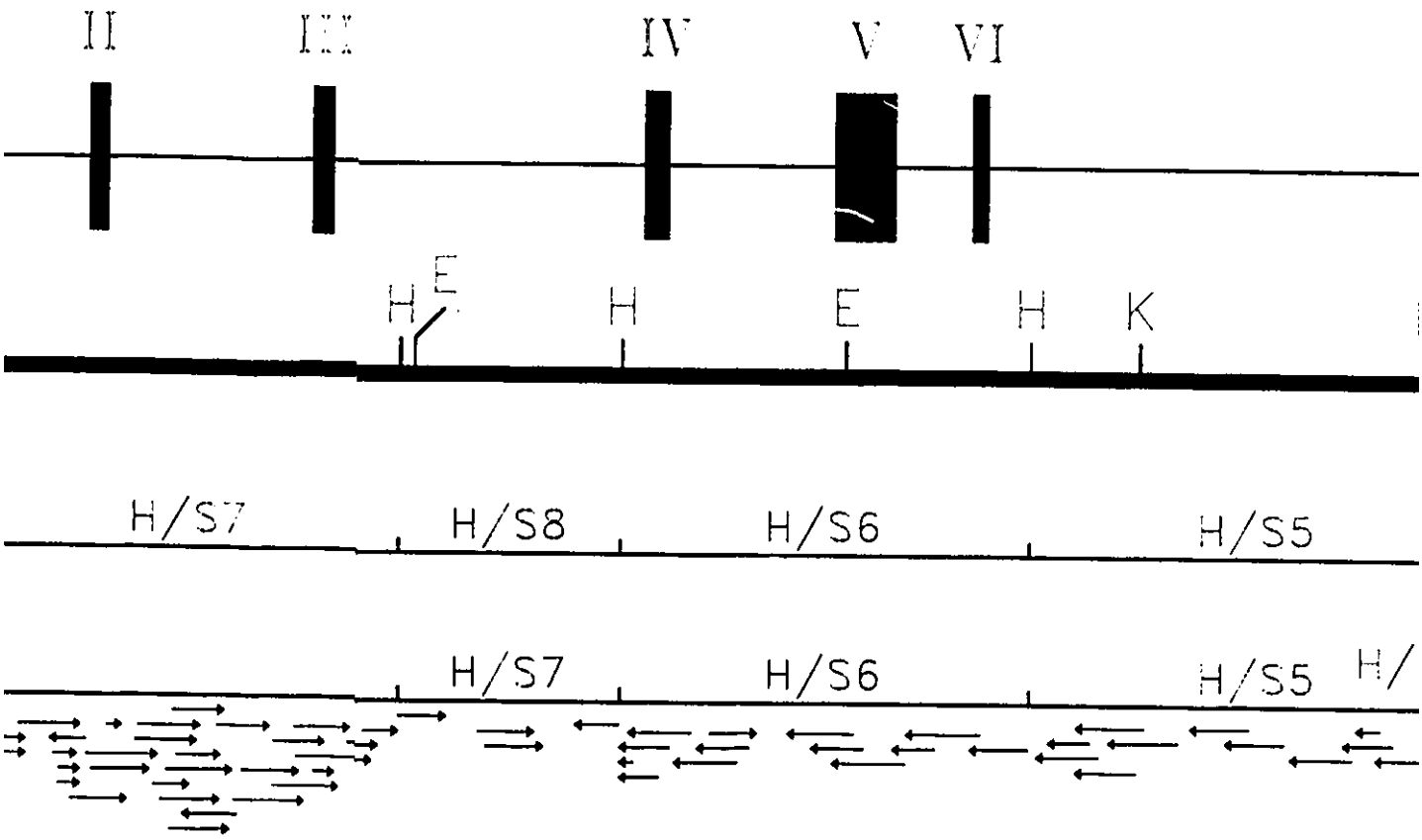
```
1       CGCCGACAGGGTGCCGCTGACCGAGGCGTGCAAAGACTCCAGAATTGGAGGCATGATGAAGACTCTGCTGCTGTTTGTGGGGCTGCTGCTGACCTGGGAG
1                                                                 MetMetLysThrLeuLeuLeuPheValGlyLeuLeuLeuThrTrpGlu

101     AGTGGGCAGGTCCTGGGGGACCAGACGGTCTCAGACAATGAGCTCCAGGAAATGTCCAATCAGGGAAGTAAGTACGTCAATAAGGAAATTCAAAATGCT
17      SerGlyGlnValLeuGlyAspGlnThrValSerAspAsnGluLeuGlnGluMetSerAsnGlnGlySerLysTyrValAsnLysGluIleGlnAsnAla

200     GTCAACGGGGTGAAACAGATAAAGACTCTCATAGAAAAAACAAACGAAGAGCGCAAGACACTGCTCAGCAACCTAGAAGAAGCCAAGAAGAAGAAAGAG
50      ValAsnGlyValLysGlnIleLysThrLeuIleGluLysThrAsnGluGluArgLysThrLeuLeuSerAsnLeuGluGluAlaLysLysLysLysGlu

299     GATGCCCTAAATGAGACCAGGGAATCAGAGACAAAGCTGAAGGAGCTCCCAGGAGTGTGCAATGAGACCATGATGGCCCTCTGGGAAGAGTGTAAGCCC
83      AspAlaLeuAsnGluThrArgGluSerGluThrLysLeuLysGluLeuProGlyValCysAsnGluThrMetMetAlaLeuTrpGluGluCysLysPro

398     TGCCTGAAACAGACCTGCATGAAGTTCTACGCACGCGTCTGCAGAAGTGGCTCAGGCCTGGTTGGCCGCCAGCTTGAGGAGTTCCTGAACCAGAGCTCG
116     CysLeuLysGlnThrCysMetLysPheTyrAlaArgValCysArgSerGlySerGlyLeuValGlyArgGlnLeuGluGluPheLeuAsnGlnSerSer

497     CCCTTCTACTTCTGGATGAATGGTGACCGCATCGACTCCCTGCTGGAGAACGACCGGCAGCAGACGCACATGCTGGATGTCATGCAGGACCACTTCAGC
149     ProPheTyrPheTrpMetAsnGlyAspArgIleAspSerLeuLeuGluAsnAspArgGlnGlnThrHisMetLeuAspValMetGlnAspHisPheSer

596     CGCGCGTCCAGCATCATAGACGAGCTCTTCCAGGACAGGTTCTTCACCCGGGAGCCCCAGGATACCTACCACTACCTGCCCTTCAGCCTGCCCCACCGG
182     ArgAlaSerSerIleIleAspGluLeuPheGlnAspArgPhePheThrArgGluProGlnAspThrTyrHisTyrLeuProPheSerLeuProHisArg

695     AGGCCTCACTTCTTCTTTCCCAAGTCCCGCATCGTCCGCAGCTTGATGCCCTTCTCTCCGTACGAGCCCCTGAACTTCCACGCCATGTTCCAGCCCTTC
215     ArgProHisPhePhePheProLysSerArgIleValArgSerLeuMetProPheSerProTyrGluProLeuAsnPheHisAlaMetPheGlnProPhe

794     CTTGAGATGATACACGAGGCTCAGCAGGCCATGGACATCCACTTCCACAGCCCGGCCTTCCAGCACCCGCCAACAGAATTCATACGAGAAGGCGACCAT
248     LeuGluMetIleHisGluAlaGlnGlnAlaMetAspIleHisPheHisSerProAlaPheGlnHisProProThrGluPheIleArgGluGlyAspAsp

893     GACCGGACTGTGTGCCGGGAGATCCCCCACAACTCCACGGGCTGCCTTCGGATGAAGGACCAGTGTGACAAGTGCCGGGAGATCTTGTCTGTGGACTGT
281     AspArgThrValCysArgGluIleArgHisAsnSerThrGlyCysLeuArgMetLysAspGlnCysAspLysCysArgGluIleLeuSerValAspCys

992     TCCACCAACAACCCCTCCCAGGCTAAGCTGCGGCGGGAGCTCGACGAATCCCTGCAGGTCGCTGAGAGGTTGACCAGGAAATACAACGAGCTGCTAAAG
314     SerThrAsnAsnProSerGlnAlaLysLeuArgArgGluLeuAspGluSerLeuGlnValAlaGluArgLeuThrArgLysTyrAsnGluLeuLeuLys

1091    TCCTACCAGTGGAAGATGCTCAACACCTCCTCCTTGCTGGAGCAGCTGAACGAGCAGTTTAACTGGGTGTCCCGGCTGGCAAACCTCACGCAAGGCGAA
347     SerTyrGlnTrpLysMetLeuAsnThrSerSerLeuLeuGluGlnLeuAsnGluGlnPheAsnTrpValSerArgLeuAlaAsnLeuThrGlnGlyGlu

1190    GACCAGTACTATCTGCGGGTCACCACGGTGGCTTCCCACACTTCTGACTCGGACGTTCCTTCCGGTGTCACTGAGGTGGTCGTGAAGCTCTTTGACTCT
380     AspGlnTyrTyrLeuArgValThrThrValAlaSerHisThrSerAspSerAspValProSerGlyValThrGluValValValLysLeuPheAspSer

1289    GATCCCATCACTGTGACGGTCCCTGTAGAAGTCTCCAGGAAGAACCCTAAATTTATGGAGACCGTGGCGGAGAAAGCGCTGCAGGAATACCGCAAAAAG
413     AspProIleThrValThrValProValGluValSerArgLysAsnProLysPheMetGluThrValAlaGluLysAlaLeuGlnGluTyrArgLysLys

1388    CACCGGGAGGAGTGAGATGTGGATGTTGCTTTTGCACCTTACGGGGGCATCTTGAGTCCAGCTCCCCCCAAGATGAGCTGCAGCCCCCCAGAGAGAGCT
446     HisArgGluGlu   449

1487    CTGCACGTCACCAAGTAACCAGGCCCCAGCCTCCAGGCCCCCAACTCCCCCCAGCCTCTCCCCGCTCTGGATCCTGCACTCTAACACTCGACTCTGCTG

1586    CTCATGGGAAGAACAGAATTGCTCCTGCATGCAACTAATTCAATAAAACTGTCTTGTGAGCTG   1648
```

Figure 8 Genomic organization of the human TRPM-2 gene, with subclones and sequencing strategy.

The exons are depicted as filled boxes. The restriction enzymes are E, Eco RI; B, Bam HI; H, Hind III; K, Kpn I. The extent of each subclone used is displayed under the restriction map. The sequencing strategy is displayed under its corresponding subclone. The pointing direction of the arrows indicate the direction of sequencing from the primer as well as the sequencing run length. The extent of the phage inserts from which the subclones were derived is depicted as two long black rectangles at the bottom of the figure. Note that when only one strand was sequenced, the region was sequenced several times from different unidirectional exonuclease III deletion clones of genomic subclones. The scale is 1 kb = [illegible]

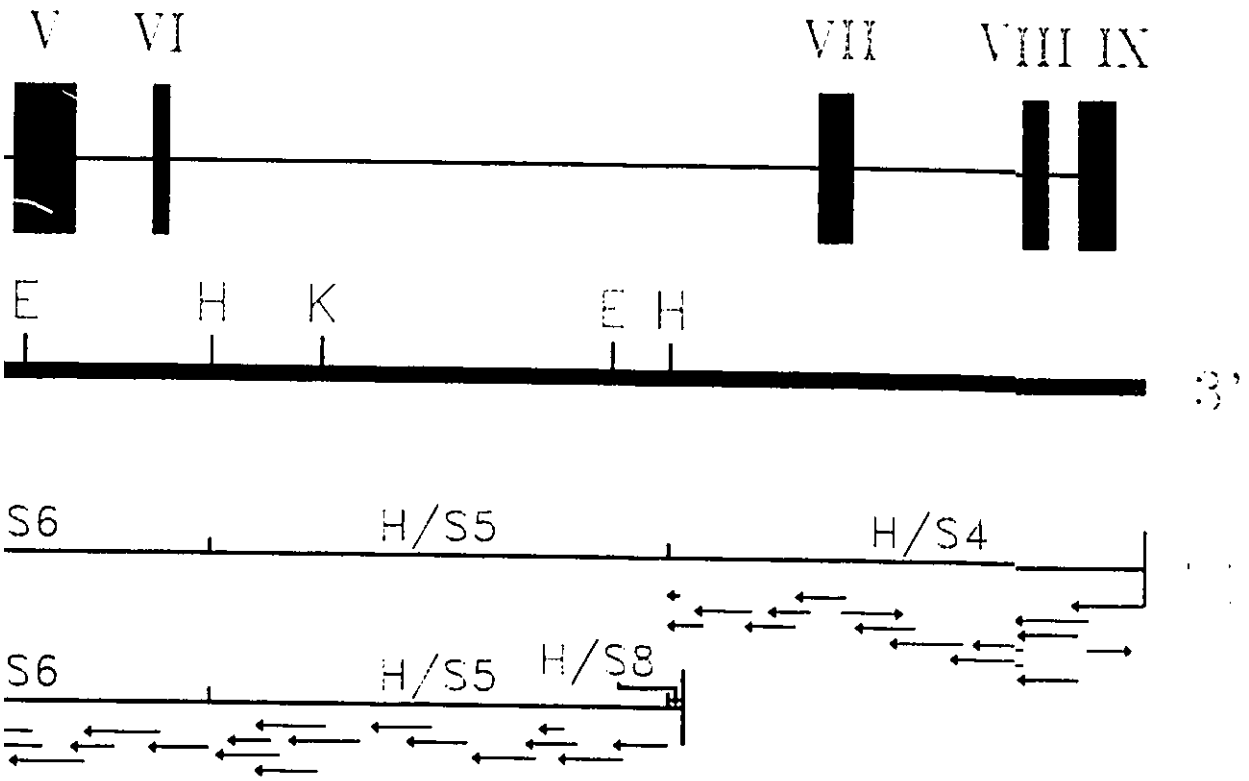

I
II
III
5'
H/32
genomic


I
II
III
E
H
E
5'
H/S7
H/
H/S2
H/
λH5.1.1.1 genomic in
1,000 kb



λ H5.1.1.1 genomic insert

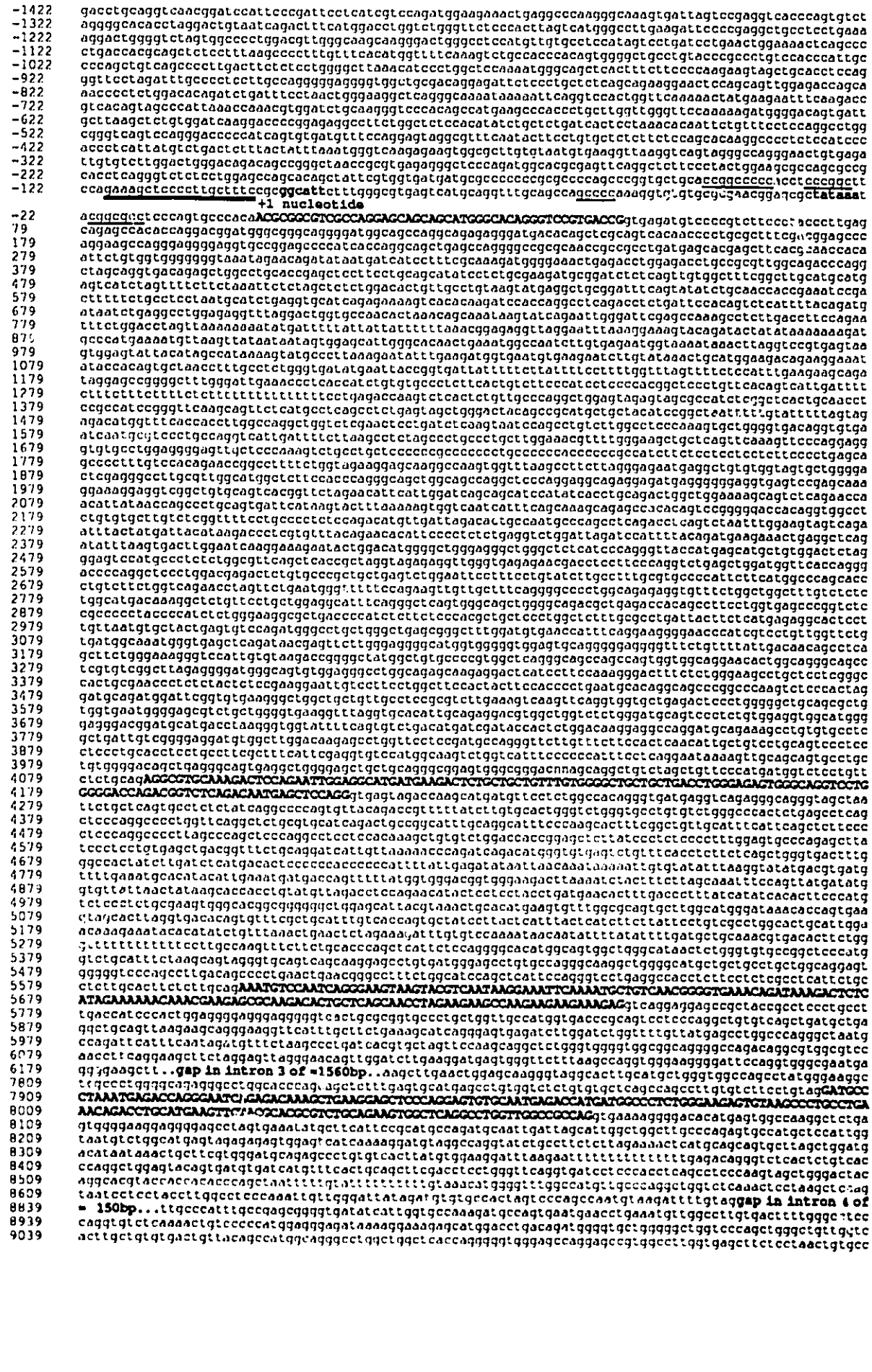
λ H.9.1.1.1 genomic insert

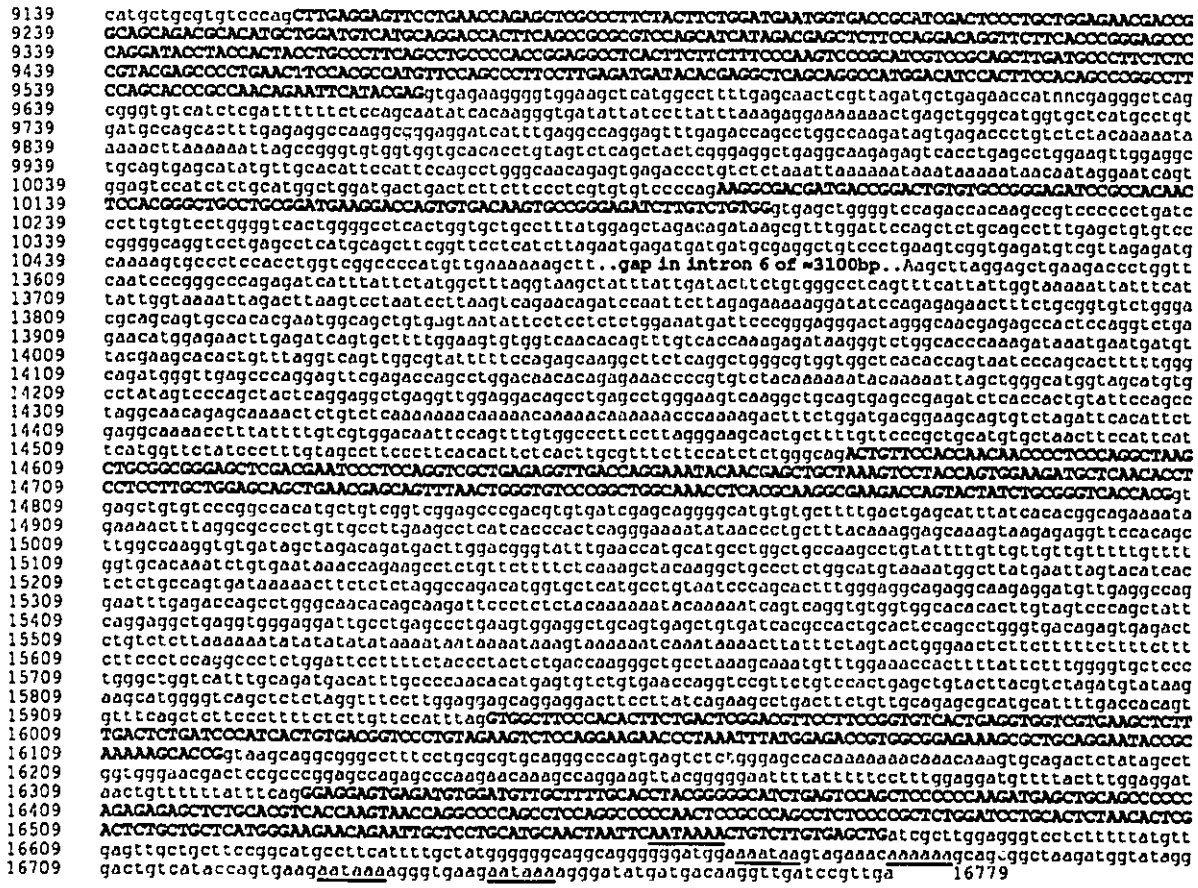


i genomic insert

Figure 9. Sequence of the human TRPM-2 gene.

The complete sequence of the human gene could not be generated due to multiple repeats in introns 3, 4, and 6. The intervening, upstream, and downstream sequences are in lower case letters. The sequence predicted in the mature mRNA is in uppercase and bold. The potential putative TATA and CAT boxes are in lower case and bold. Other interesting sequences such as GC rich, and polyadenylation signal are underlined. The palindrome identified in the 5' region is double underlined.





Computer sequence comparison of the full length human cDNA and the human genomic sequence initially confirmed the presence of 8 exons, spanning 12,370 bp. However, no consensus TATA or CAT boxes could be found. Since this gene was very well conserved compared to the rat (79% in the exonic sequences) a ninth exon upstream from the initial first exon was suspected to be missing. This exon I was found 4.2 kb from the initially designed exon I, which now becomes exon II (see below). Therefore the human TRPM-2 gene spans from its +1 nucleotide to the polyadenylation site (22 bp from the first nucleotide of the first polyadenylation signal) 16,570 bp. See table 2 for the exon/intron statistics.

5. Positioning and confirmation of human exon I and +1 nucleotide

The exon I of the human gene could not be confirmed based on sequence comparison to pHT7 (the human cDNA). This cDNA does not have enough sequence information at its 5' region to either hybridize or validate a computer search, with the genomic sequence. Therefore systematic sequence comparison using the MicroGenie software (Beckman) was performed using the 5' upstream sequence of the rat gene including exon I to all the available human sequence upstream to the initially designated exon I, now known to be exon II. The minimum logistical stringency used in the computer comparisons was 75% of 20 nucleotides. The inter-species conservation of the upstream DNA sequence between the rat and human is nearly 79%. This great conservation facilitated the search of exon I and the human upstream sequences. Also, since there is a significant sequence match (18/22) between the first 22 nucleotides of

pHT7 to the sequence identified as exon I by cross species computer comparison in the human genomic clones, and since this sequence also demonstrates the necessary consensus splice junction at its 3', it is very likely that the actual assignment of human exon I is correct (fig. 10).

6. Detailed analysis of the functional elements in the human TRPM-2 gene

a. Identification of known regulatory sequences

The 5' region contain a consensus TATAAA box at position -29 and a near consensus CAT box (GGCATT instead of GGCAAT) at position -96. The upstream region also contains multiple GC rich boxes as in its rat counterpart at positions -21, -59,-129, -142, -174, none of which is a consensus SP1 site. The remainder of the human 5' upstream region also lacks known conventional hormone regulatory elements.

Of interest, the 3' downstream region contains four polyadenylation signals, of which only the first one is transcribed (fig. 11).

b. Identification of unknown regulatory sequences

As for the rat, a short inverted repeat with a close axis of symmetry was found in the 5' upstream region. However, this human palindromic sequence bears no similarity with the rat sequence. The palindrome is located at -118: GAAAGC-$(N)_7$-GCTTTC. The significance of this palindrome was not determined .

Figure 10. Positioning of the human exon I and +1 nucleotide

The exon I of the human gene was positioned based on the sequence similarity between the rat and human genomic sequences and the potential TATA and CAT boxes. The 5' upstream sequence is shown in lower case letters and the transcribed sequence is shown in uppercase. The putative TATA and CAT boxes are underlined.

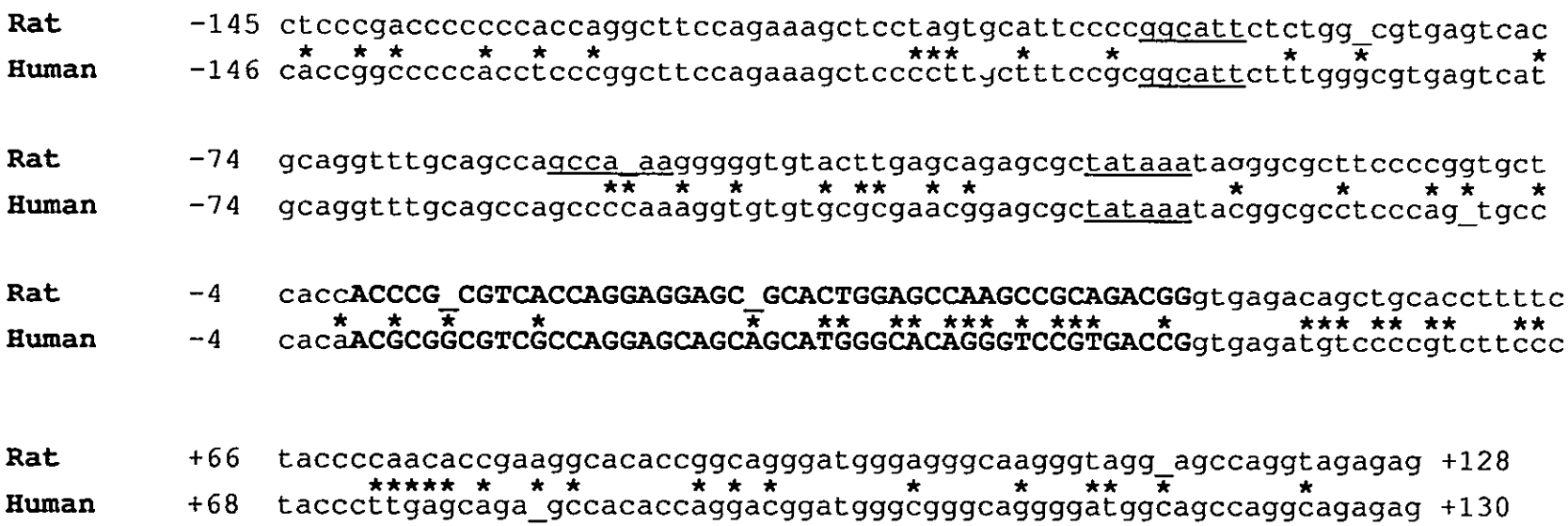
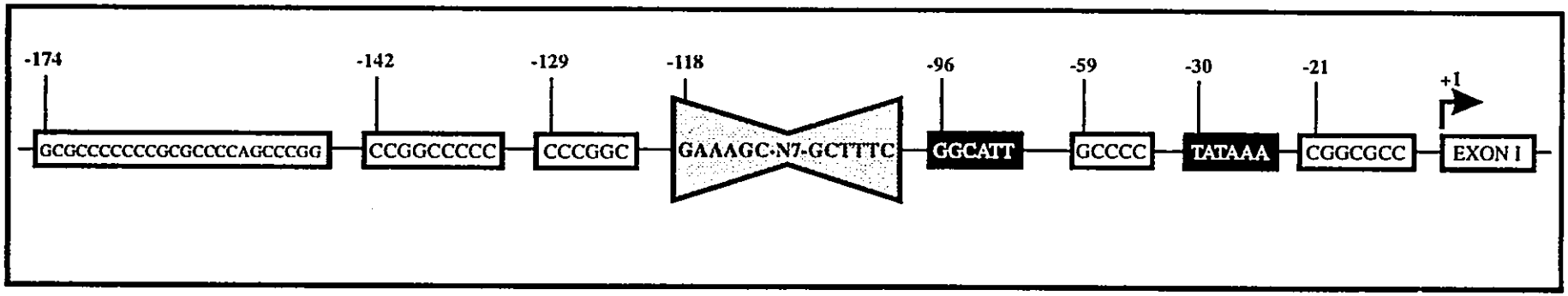

Rat -145 ctcccgacccccccaccaggcttccagaaagctcctagtgcattcccc ggcatt ctctgg cgtgagtcac
Human -146 caccggcccccacctcccggcttccagaaagctcccctt ctttccgc ggcatt ctttgggcgtgagtcat
Rat -74 gcaggtttgcagccagcca aaggggtgtacttgagcagagcgc tataaa taggcgcttccccggtgct
Human -74 gcaggtttgcagccagccccaaaggtgtgtgcgcgaacggagcgc tataaa tacggcgcctcccag tgcc
Rat -4 cacc ACCCG CGTCACCAGGAGGAGC GCACTGGAGCCAAGCCGCAGACGG gtgagacagctgcacctttc
Human -4 caca ACGCGGCGTCGCCAGGAGCAGCAGCATGGGCACAGGGTCCGTGACCG gtgagatgtccccgtcttccc
Rat +66 taccccaacaccgaaggcacaccggcagggatgggagggcaagggtagg agccaggtagagag +128
Human +68 taccccttgagcaga gccacaccaggacggatgggcgggcaggggatggcagccaggcagagag +130

Figure 11. 5' upstream elements of the human TRPM-2 gene.

The start of transcription and exon I are represented graphically. The TATA and CAT boxes are shown as black boxes. The GC rich regions are inside white boxes. The palindrome is depicted as a stipled double triangle. The long stippled bar represents the extent of sequence similarity (79%) with its rat counterpart.


-174
-142
-129
-118
-96
-59
-30
-21
+1
GCGCCCCCCCGCGCCCCAGCCCGG
CCGGCCCCC
CCCGGC
GAAAGC-N7-GCTTTC
GGCATT
GCCCC
TATAAA
CGGCGCC
EXON I

c. Comparison of human TRPM-2 genomic sequences with other human sequences in GENBANK

The sequence similarity between the rat and human TRPM-2 genes lies mostly in the transcribed regions and in the 5' upstream segment. This homology is 79%. Comparison of the human sequences against GENBANK revealed the same several sequences as in the rat. Of interest, SP-40,40 and CLI cDNAs were cloned from human RNA. No other group has actually cloned the genomic portion of human TRPM-2.

d. Analysis of exons/introns borders

The sequence borders of the exons/introns were deduced from published consensus sequences (Mount *et al.*, 1982). As no testis RNA could be obtained, no S1 nuclease analysis was performed to confirm the exon/intron borders and the +1 nucleotide. Table 2 shows all exon/intron borders compared to the published consensus sequences.

C. THE RAT TRPM-2 GENE IS EXPRESSED IN A WIDE VARIETY OF TISSUES AT VERY DIFFERENT LEVELS

Nuclease S1 protection assays using a single stranded 60-mer complementary to the 5' region of exon I to position -19 (8 nucleotides downstream of the putative TATA box) were performed on a variety of tissues (fig. 12). A second series of nuclease S1 digestions were performed using a second probe specific for the intron 3/exon IV boundary (fig. 13). In every tissue examined TRPM-2 transcription appears to be initiated from the same transcription start site, appears to have the same splicing pattern at the intron 3/exon IV border. Furthermore, the level of expression of exon I and exon IV is

Table 2. Splice junctions of the human TRPM-2 gene compared to the consensus sequences.

The consensus sequence from Mount, 1982 is compared to all splice junction of the human TRPM-2 gene.

Predicted exon/intron splice sites

Consensus	$^{C}_{A}$**AG**/gt$^{a}_{c}$agt
I /1	**CGG**/gtgaga
II /2	**CAA**/ggtaga
III /3	**GAG**/gtaggc
IV /4	**CAG**/gtgaga
V /5	**AAG**/gtcaga
VI /6	**GTG**/ggtgag
VII /7	**ACA**/gtgagt
VIII/8	**CCG**/gtaagc

Predicted intron/exon splice sites

Consensus	$(^{c}_{t})_{n}$ n$^{t}_{c}$ag/**G**
1 /II	ctttgcag/**G**
2 /III	ctctcaca/**G**
3 /IV	ccatgaag/**G**
4 /V	ggtcccag/**C**
5 /VI	gtgtccag/**A**
6 /VII	cctggcca/**G**
7 /VIII	tcttctag/**G**
8 /IX	ttttccag/**C**

Figure 12. Nuclease S1 mapping of the TRPM-2 rat gene in different tissues with its exon I.

The relative position in the gene and the sequence of the 60-mer probe used is shown. As molecular weight marker a sequencing ladder was generated using a primer from the first 17 nucleotides of the 60-mer probe. 100µg of total RNA extracted from each of the tissues before, and four days after castration, was incubated with the radiolabeled exon I probe. After nuclease S1 digestion the samples were run through an 8% polyacrylamide gel and subsequently exposed to an X-ray film for 5 h at room temperature.

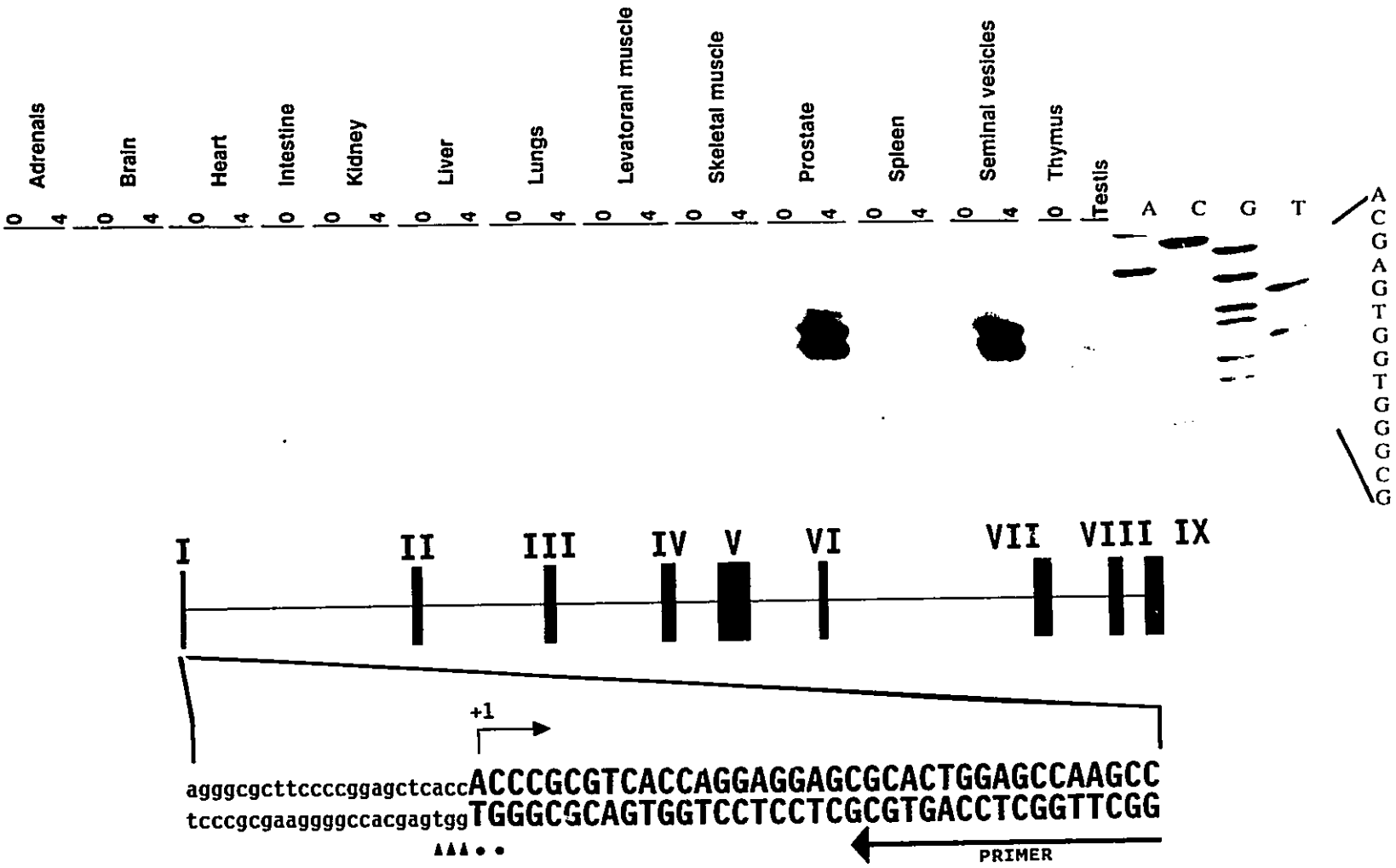
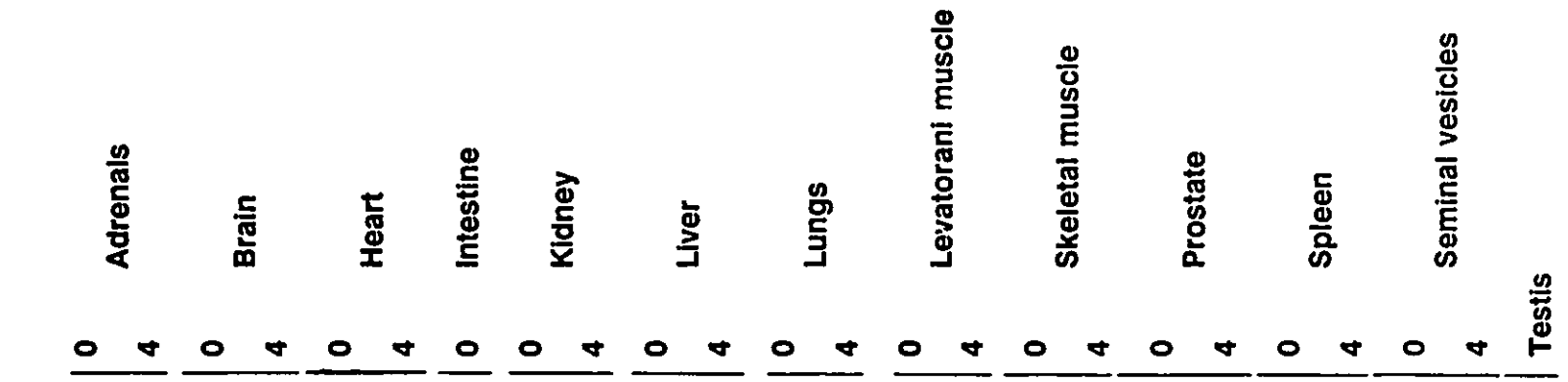
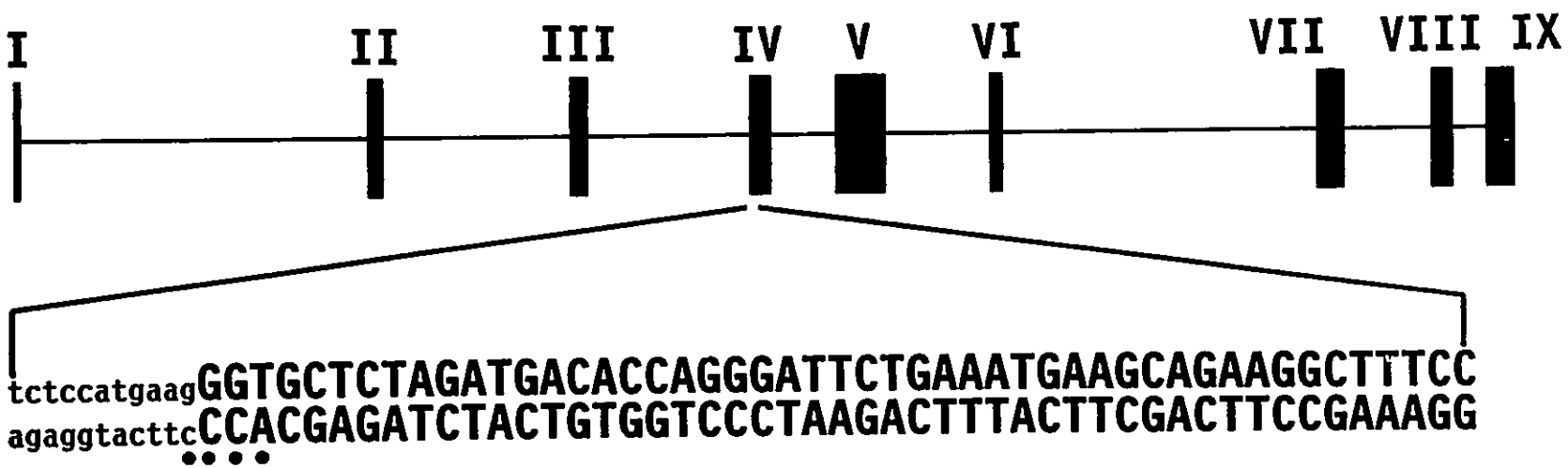

Adrenals
Brain
Heart
Intestine
Kidney
Liver
Lungs
Levatorani muscle
Skeletal muscle
Prostate
Spleen
Seminal vesicles
Thymus
Testis
0
4
A C G T
A C G A G T G G T G G C G
I II III IV V VI VII VIII IX
+1
agggcgcttccccggagctcacc
tcccgcgaaggggccacgagtgg
ACCCGCGTCACCAGGAGGAGCGCACTGGAGCCAAGCC
TGGGCGCAGTGGTCCTCCTCGCGTGACCTCGGTTCGG
PRIMER

Figure 13. Nuclease S1 mapping of the TRPM-2 rat gene in different tissues with exon IV

The relative position in the gene and the sequence of the 60-mer probe used is shown. As molecular weight marker a sequencing ladder was generated using a primer from the first 17 nucleotides of the 60-mer probe. 100µg of total RNA extracted from each of the tissues before, and four days after castration, was incubated with the radiolabeled exon I probe. After nuclease S1 digestion the samples were run through an 8% polyacrylamide gel and subsequently exposed to an X-ray film for 5 h at room temperature.


Adrenals
Brain
Heart
Intestine
Kidney
Liver
Lungs
Levatorani muscle
Skeletal muscle
Prostate
Spleen
Seminal vesicles
Testis
0 4


I
II
III
IV
V
VI
VII
VIII
IX
tctccatgaagGGTGCTCTAGATGACACCAGGGATTCTGAAATGAAGCAGAAGGCTTTCC
agaggtacttcCCACGAGATCTACTGTGGTCCCTAAGACTTTACTTCGACTTCCGAAAGG

proportionate in each tissue, adding further support to the fact that alternate exon I splicing does not occur. It is also clear from these results that the gene is markedly induced in the prostate after castration (as is expected), and to the same extent in the seminal vesicles. It is also expressed constitutively in the testis, although at a level that is less than 2% of that observed in the prostate. Very low levels of TRPM-2 expression (less than 0.2% of the level of the prostate) are detectable in several of the other tissues (heart, liver, brain). Since the level of expression in these tissues is not altered after castration it probably relates to the naturally occurring cell death that is necessary for tissue homeostasis.

D. THE LEADER SEQUENCES BETWEEN THE SGP-2 AND TRPM-2 cDNAs ARE DIFFERENT

The sequence homology between SGP-2 and TRPM-2 is essentially identical within the coding region. However there is a striking difference in the untranslated leader sequence of SGP-2 on 269 nucleotides that has no similarity to the corresponding sequence in TRPM-2 which is 34 nucleotides in length (fig. 14). It is not possible that the cause for the discrepancy between SGP-2 and TRPM-2 arose from the presence of two or more allelic genes in the genome since the TRPM-2 gene has already been located on the rat chromosome 8 (Wong *et al.*, 1992). Therefore only two explanations are possible: either TRPM-2 is alternatively or trans-spliced or a mechanism of differential promoter usage in different tissues, or one of the two report leader sequences is an artifact.

1. Possible alternative or trans splicing or differential promoter usage

To address the possibility of alternative splicing, sequence comparisons using the published SGP-2 sequence (Collard and Griswold, 1987) with the available rat TRPM-2 genomic sequence (5,739 bp upstream exon I, and complete intron 1) were computerized and showed no sequence similarity, even at lowest logistical stringency. Therefore, if alternative splicing occurs, the sequence unique to SGP-2 must lie more than 5.7 kb upstream of TRPM-2 exon I. Because the two sequences diverge within only one base pair of exon I/II border of the TRPM-2 cDNA (fig. 14), it is not possible to rule out an alternative or trans splicing or differential promoter usage mechanism solely based on available sequence data. This possibility was therefore examined by primer extension analysis, nuclease S1 mapping and specific PCR amplification experiments.

The primer extension analysis (fig. 5) clearly shows that no minor RNA species are detectable in the prostate after castration or in the testis. In addition, nuclease S1 mapping using an overlap of the immediate 5' region and exon I (described above) confirmed the primer extension results (fig. 12). In fact neither of these two methods conclusively demonstrate that SGP-2 is not a trans-spliced product of TRPM-2 due to the detection level of these methods. It is still possible that very low levels of alternatively spliced or trans-spliced or differential promoter usage products are normally present *in vivo*.

Figure 14. Sequence comparison between the rat TRPM-2 cDNA (p17H) and the published SGP-2 cDNA.

Panel **A**. The figure shows the sequence data difference between SGP-2 and TRPM-2 cDNAs compared to the sequence of the rat gene exon I. The region of mismatch is in uppercase and bold and the mismatching nucleotides are indicated with stars. It is interesting to note that the SGP-2 leader sequence discrepancy starts one nucleotide short of the exon I/II splice junction suggesting possible alternative or trans-splicing for the SGP-2 transcript. However, the sequence discrepancy is a cloning artifact. Panel **B**. The list of other sequence mismatches are presented with the position and the mismatching nucleotide. Where the nucleotide is a "Δ", it is missing in the published sequence of SGP-2 (Collar and Griswold, 1987). SGP-2 cDNA does not have a poly(A)tail. This indicated by "end Δ" in the SGP-2 column. The differences in sequence data between SGP-2 and p17H have all been confirmed at the genomic level in favour to p17H.

A

```
SGP-2  226 CACCGTTGTCTCCATGGAAATGCTTAACTACGGCTTGCTAGTAAGGACTCCAGACTCCAA
           **    ****** * ** * **  ******* ********  ***
p17H     1            CCAGGAGGAGCGCACTGGAGCCAAGCCGCAGACGGGACTCCAGACTCCAA
ExonI    1 ACCCGCGTCACCAGGAGGAGCGCACTGGAGCCAAGCCGCAGACGGgt-intron1-
```

B

SGP-2		p17H		
528	A	293	G	
856	C	621	G	
1729	Δ	1494	C	
1813	Δ	1579	C	
1837	Δ	1604	A	
1852	Δ	1620	C	
end	Δ	1626	GCGATAAAAAAAAAAAAA	1644

2. The SGP-2 cDNA 5' end is an artifact

To definitively rule out that alternative of trans-splicing of the TRPM-2 transcript occurs in different tissues (prostate and testis) to produce the mature SGP-2 and TRPM-2 mRNAs respectively, PCR analysis of the 5' region of the TRPM-2 transcript was performed (fig. 15).

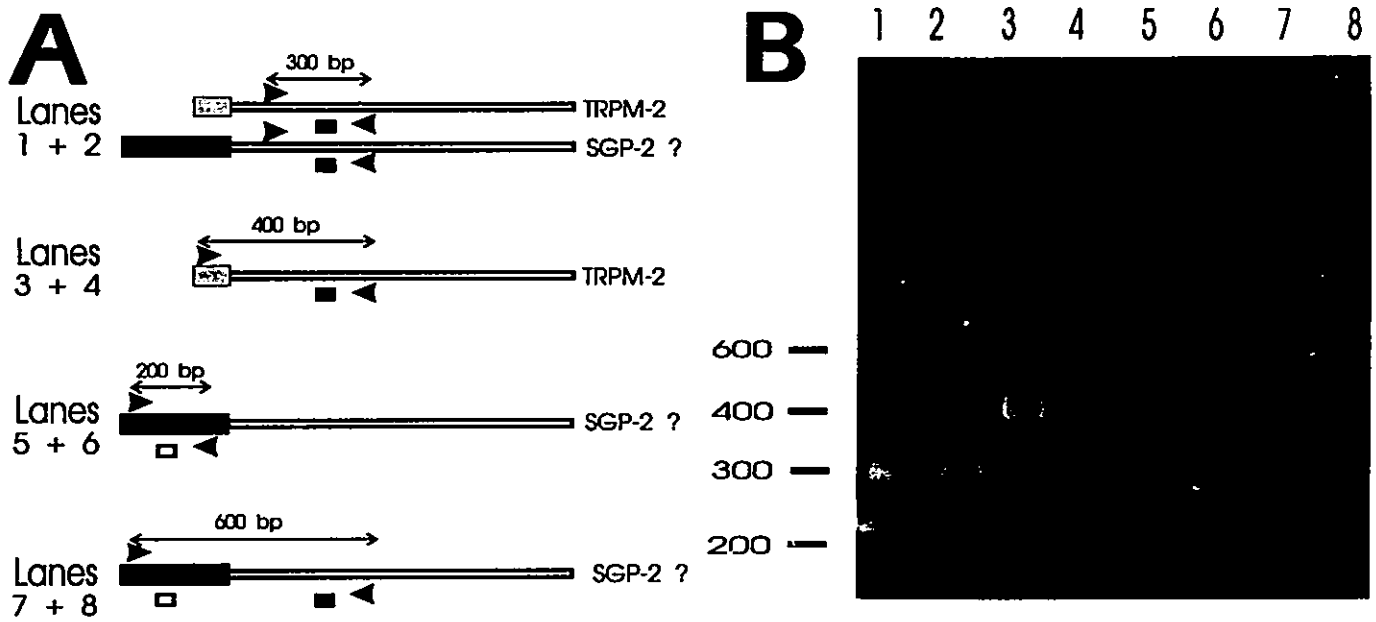
Four primers were synthesized and used in pairs to amplify:

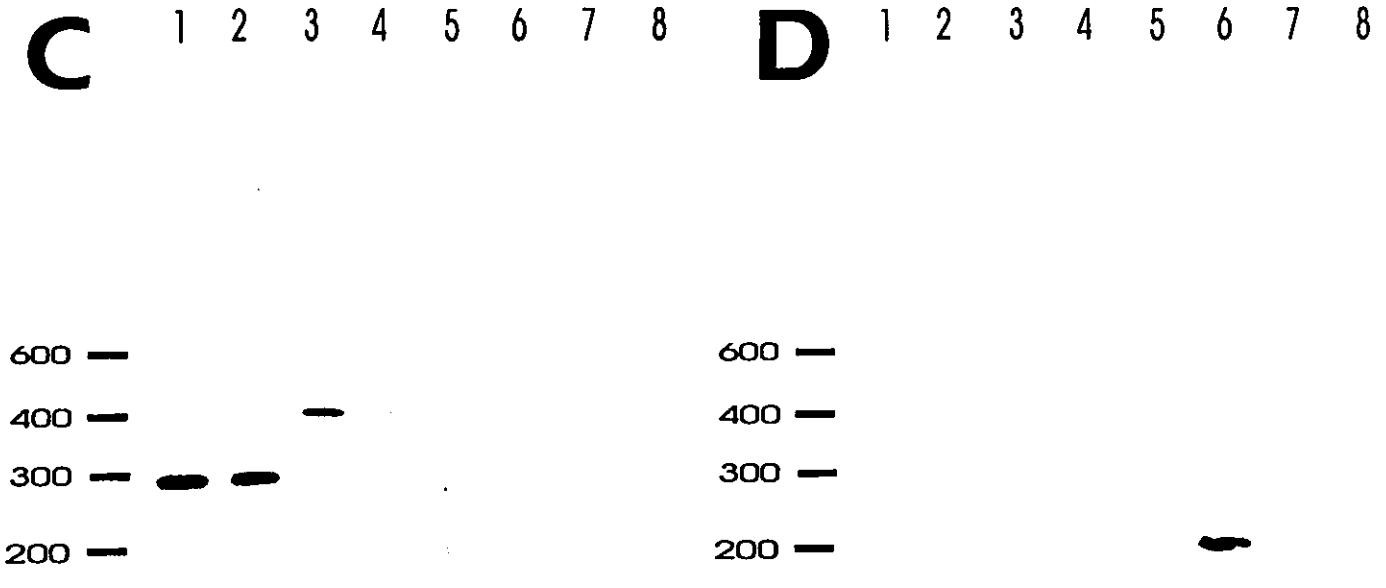
1. The common region of SGP-2 and TRPM-2;
2. The region overlapping the specific and common sequence of TRPM-2;
3. The specific region of SGP-2;
4. The region overlapping the specific and common sequence of SGP-2.

The much higher sensitivity of the PCR technique served well to detect the presence of the specific end of SGP-2 (fig. 15) which could not be detected by Northern or nuclease S1 analysis (Gleeson, personal communication). This experiment also clearly demonstrates that the published sequence of SGP-2 is not physically associated with the common region. This PCR experiment along with primer extension and nuclease S1 analysis rule out the possibility of alternative of trans-splicing explaining the discrepancy between SGP-2 and TRPM-2 and shows that the 5' leader sequence of SGP-2 is a cloning artifact.

Figure 15. PCR analysis comparison between SGP-2 and TRPM-2

Panel **A** depicts the TRPM-2 cDNA and the possible SGP-2 cDNA aligned. The stippled box in the TRPM-2 cDNA indicates the region that is unique to TRPM-2. The solid box on the SGP-2 cDNA depicts the region that is unique to the SGP-2 published sequence. The arrow heads indicate the relative positions of the different primers used the PCR amplification experiments. The filled and empty boxes under the schematic cDNAs represent the two different probes that were used to confirm the nature of the amplified fragments and indicate the approximate relative positions of those probes, The lengths of the predicted amplified fragments are shown above the pairs of cDNAs. In subsequent panels: Lanes 1, 3, 4, 7 are from prostate RNA and lanes 2, 4, 6, 8 are from testis RNA. Panel **B** is the 4% agarose gel obtained after PCR amplification. Panel **C** is the resulting Southern X-ray after hybridization with the probe depicted by the filled boxes in panel A. Panel **D** is the resulting Southern X-ray after hybridization with the probe corresponding to the empty boxes in panel A.


A
300 bp
Lanes
1 + 2
TRPM-2
SGP-2 ?
400 bp
Lanes
3 + 4
TRPM-2
200 bp
Lanes
5 + 6
SGP-2 ?
600 bp
Lanes
7 + 8
SGP-2 ?
B
1 2 3 4 5 6 7 8
600
400
300
200


C
1 2 3 4 5 6 7 8
600
400
300
200
D
1 2 3 4 5 6 7 8
600
400
300
200

IV. DISCUSSION

A. GENOMIC LIBRARIES

The cloning of the rat and human TRPM-2 genes required the preparation of two genomic libraries. These libraries were prepared from Mbo I digests in the EMBL3 vector. Mbo I was selected for two reasons. Firstly, it is a 4 bp cutter that digests at an average of 300 bp in both rat and human genomes as seen from complete digests on a 1% agarose gel (data not shown). Therefore, its activity on genomic DNA does not seem to be very specific and makes it a good candidate for the construction of unbiased libraries. Secondly, Mbo I is an isoschizomer of Bam HI which has one of the restriction sites in the EMBL3 MCS. Since amplified libraries have been shown to be biased in their sequence retention, I chose to screen three unamplified genomic libraries for each species. This provided enough independent clones to allow the screening. A total of more than 3 million independent clones were generated that way. In the process of building the libraries, I aimed at generating enough genomic clone to cover between 5 and 10 times the genome of each species. The actual libraries contained clones with an average size insert of 14 kb in length. Since the libraries contained more that 3 million independents, the genome was mathematically covered 6.8 times, which was sufficient to initiate the screening process and to be sure that unique sequences were adequately represented in the libraries.

The screening of both genomic libraries was initially performed with pG21-04, the first partial length cDNA clone of TRPM-2 (Léger *et al.*, 1987). Twelve rat genomic inserts were initially pulled out, two of those were found to include the complete gene by

overlap: λR.8.1.1.1 (13.5 kb) and λR6.2.1.1 (17.7 kb). The sequence conservation between the rat and the human is such (79%) that nine cloned were originally screened out with pG21-04; two of those were overlapping and contained the complete human TRPM-2 gene: λH5.1.1.1 (15.1) and λH9.1.1.1 (13.3 kb).

B. THE RAT TRPM-2 GENE

pG21-04 was only 1,340 bp in length and to characterize the TRPM-2 gene properly, the full length cDNA was required. p17H then was cloned and sequenced. It is the full length TRPM-2 cDNA (1,630 bp) missing only 10 nucleotides on its 5' end compared to the putative mRNA. This cDNA clone was used for all subsequent work on the rat TRPM-2 gene.

The gene is 13,750 bp in length and is composed of 9 exons. 5,737 bp upstream of exon I have been sequenced (fig. 4). The spliced product yields an mRNA of 1,640 nucleotides with the same transcription start in all tissues examined. The TATA and CAT boxes are located at positions -30 and -96 respectively and their nucleotide composition fits consensus sequences. An additional near consensus CAT box is also found at -59. A single palindromic sequence has been found at -75 and its biological significance is being currently investigated. The gene also has a single polyadenylation signal.

C. THE HUMAN TRPM-2 GENE

The human complete cDNA clone, pHT7, was also cloned and used for all subsequent work on the human TRPM-2 gene. This cDNA did not however contain exon

I. The mRNA is approximately 1,660 bp in length. The human TRPM-2 gene is 16,570 bp in length and is also composed of 9 exons (fig. 8 and 9). 1,300 bp upstream of exon I have been sequenced. Because of the difficulty of obtaining human testis RNA, exon I and the +1 nucleotide were located by computer comparisons with its rat counterpart (fig. 10). These searches confirmed the presence of consensus TATA (-29) and CAT (-96) as well as a single palindrome at -118.

D. COMPARISON BETWEEN THE RAT AND HUMAN TRPM-2 GENES

1. Exon/intron structures

Visual inspection of the exon/intron structure of both rat and human genes indicates remarkable similarities. Not only the sequence conservation between the exonic sequence of the two genes and their 5' regions homologous at 79%, the distribution of the exons is almost identical between the two genes. While the human gene has a total of 2.8 kb of additional intervening sequences and while no introns show any sequence similarity between the two species, the interspacing between both sets of exons is very similar as well the length of each individual introns. By comparing fig. 3 and 8, one can observe the stand alone exon I, the cluster of 5 exons in the middle part of the gene and the cluster of three exons on the most 3' end of the genes. The explanation for those similar TRPM-2 exons lengths from one species to another and the relative spacing of the intervening sequence alike is unknown at the moment.

2. 5' upstream sequences

The extent of sequence homology in the 5' region of both genes is the same as within the exonic regions. This suggests a similar mechanism of control. Both TATA and CAT boxes in both genes are spaced identically being at -30 and -96 for the rat, -29 and -96 for the human. The sequence of both consensus is also identical. However the rat gene has an additional near consensus CAT box at -59. The presence of this additional sequence could merely be coincidental or has a presently unknown biological function. Both genes have palindromes whose sequence are however totally different. Also, while the human unique palindrome is upstream of the -96 CAT box, the rat palindrome is in between the TATA and CAT boxes. The presence of the palindromes suggest that the gene may be controlled through them, but the significance of the differential positioning and the lack of similarity between the sequence data are not known. However, DNA band shift assays have made clear that proteins bind specifically the rat palindrome, suggesting the presence of a new regulatory palindromic sequence (Taillefer, personal communication).

3. Polyadenylation signals

Finally, the rat gene has a unique polyadenylation signal while there are four for the human; only the first being transcribed.

E. THE RAT AND HUMAN cDNAs AND/OR PROTEINS IN OTHER SYSTEMS

The rat and human cDNA and/or protein products have been cloned and characterized in other systems. However, the only report for the cloning of the actual gene to date is this thesis. The cDNA has been cloned as sulfated glycoprotein-2 (SGP-2) from rat Sertoli cells (Collard and Griswold, 1987; Griswold *et al.*, 1986); as clusterin from the ram rete testes fluid (Blaschuk *et al.*, 1983); from human serum, as apolipoprotein J (de Silva *et al.*, 1990); human serum protein 40,40 (SP-40,40) (Kirszbaum *et al.*, 1989) and human complement lysis inhibitor (CLI) (Jene and Tschopp, 1989); from Madin-Darby canine kidney cells as glycoprotein 80 (gp80) (Hartmann *et al.*, 1991); from bovine adrenal medullary chromaffin granules as glycoprotein III (Palmer and Christie, 1990); and from Japanese quail neuroretinal cells as T64 (Michel *et al.*, 1989). The rat TRPM-2 cDNA sequence is nearly identical to its rat counterparts except for SGP-2 and the human cDNA is almost identical to its human counterparts without exception, the only difference being point mutations that probably arose in the cloning and/or sequencing procedures and represent species differences. However the 5' end of SGP-2 bears as 269 nucleotides leader not seen in any other clones and the rat TRPM-2 has a 34 nucleotides leader that is different from SGP-2 and that is absent in other clones.

1. Discrepancy between the reported cDNA sequence of SGP-2 and TRPM-2

I have taken four complementary steps to resolve the 5' end ambiguity between the reported full length cDNA clone designated SGP-2, and the full length sequence of the rat TRPM-2 reported here.

First, computer screening of the sequence data of the complete TRPM-2 gene and especially intron 1 and the available upstream sequence (5.7 kb) has failed to locate the 269 nucleotides leader of SGP-2 or any part of it, at any logistical stringencies. Since, the full contingent of control sequence in the 5' upstream sequences are present, if the 269 nucleotides sequence is biologically associated with the rest of the cDNA, it must come from alternative or trans-splicing and would involve differential promoter usage. The possibility of a multi allelic gene is ruled out because the rat TRPM-2 has been located as a single allelic gene to the rat chromosome 8 and human chromosome 15 (Wong *et al.*, 1992, submitted).

Second, to resolve the nature of this discrepancy I have performed nuclease S1 analysis. The S1 analysis were two fold. Since this method is at least 10 time more sensitive than Northern analysis, it was used to detect the presence in rat testis RNA of the 269 nucleotides sequence with a specific oligonucleotide (data not shown). This analysis failed to identify to the 269 nucleotides leader (Gleeson, personal communication). S1 analysis was also used to locate the +1 nucleotide, or the possible splice junction of exon I (fig. 12). This type of analysis accurately pin points which

nucleotide(s) at which transcription initiates or splicing occurs, but cannot differentiate between the two. Nuclease S1 experiments have shown that exon I is present in testis RNA pointing to either the start of transcription or a splicing site.

Third, to distinguish between the possibilities of exon I being the start of transcription or merely a spliced exon, primer extensions analysis were performed. This experiment involved the use of a specific antisense primer to the TRPM-2 mRNA to allow AMV reverse transcriptase to run off the 5' end of the mRNA. This technique allows one to determine the distance in nucleotides between the first nucleotides of the primer and the most 5' nucleotide of the mRNA. If alternative or trans-splicing, or differential promoter usage were the case in TRPM-2, more than one band or a band of a different size than the one predicted by the actual structure of exon I, should appear. Even after extensive exposure of the sequencing gel, the rat testis RNA showed only the expected band and 86 nucleotides from the primer (fig. 5). Therefore, the combination of nuclease S1 and primer extension analysis show that alternative or trans-splicing or differential promoter usage in the TRPM-2 gene is very unlikely. However, the detection limit of these techniques is not absolute; both of these methods could have missed very low levels of alternative or trans-splicing or differential promoter usage. A more sensitive detection system was required to definitively rule out the unlikely possibility of alternative or trans-splicing or differential promoter usage.

Fourth, to definitively show that the TRPM-2 gene is transcribed from a single promoter and the structure presented here includes all exons, specific PCR amplification analysis was used. PCR analysis using a set of primers specific to the putative SGP-2 mRNA demonstrated that this latter sequence exists, at very low levels, in both prostate and testis. However, specific PCR amplification using primers from the common sequence of SGP-2 and TRPM-2, and the unique SGP-2 leader sequence failed to demonstrate any association between the SGP-2 269 nucleotides leader and their common cDNA sequences (fig. 15). Therefore, the 269 nucleotides SGP-2 leader sequence is a cloning artifact.

Finally, these results also support the fact that the TRPM-2 gene is not multi allelic (Buttyan *et al.*, 1989; Grima *et al.*, 1990) and exists as presented in this thesis.

F. EXPRESSION OF THE RAT TRPM-2 GENE IN DIFFERENT TISSUES

Fig. 12 and 13 show the nuclease S1 mapping of exon I and IV respectively in different tissues of the rat. These experiments demonstrate primarily that TRPM-2 is expressed in all tissues examined at different levels. Tissues like the intestines do not show any band on the picture but those very weak bands were visible on the actual X-ray film. It is interesting to note that the seminal vesicles show similar induction after androgen removal compared to the prostate. This is not surprising since the seminal vesicles are an androgen dependent tissue and probably also undergo drastic involution after castration of the rat. The fact that TRPM-2 is expressed constitutively, at low levels,

in all tissues examined suggests that the protein product may be required for normal tissue homeostasis.

G. POSSIBLE FUNCTION OF THE TRPM-2 PROTEIN

Since the synthesis and secretion of TRPM-2 by the Sertoli cells does not appear to be associated with the process of apoptosis within the testis, it is important to determine the role of the TRPM-2 gene products in the testis, and the regressing prostate. The sequence similarity between the TRPM-2 sequence and the sequence of a component of the inhibited human complement cascade, that is referred to as CLI (complement cytolysis inhibitor) (Jenne & Tschopp, 1989) or SP-40,40 (Kirszbaum *et al.*, 1989), probably provides an important clue as to the function of TRPM-2. This protein is a component of the C5b-9 complex in its inactivated state, which binds to the final MAC complex, inhibiting the lysis of the cell membrane of attacked cells.

More clues to as the possible function of the TRPM-2 protein come from recent studies using the RDE (Montpetit *et al.*, 1988) rat prostate cell line (Lakins and Tenniswood submitted) have demonstrated that TRPM-2 is expressed at low, but clearly detectable, levels in proliferating cells. *In situ* hybridization experiments suggest that the TRPM-2 gene is expressed prior to the G_2/M interface, based on the time of expression after re-addition of serum. However, without more detailed analysis of the cells cycle it is not possible to categorically state that TRPM-2 is involved in the G_2/M transition. What is evident is that TRPM-2 appears to be expressed during replication just prior to

membrane remodelling that occurs during cell division. At the morphological and biochemical level, proliferation and apoptosis share many characteristics in common, in particular the entire cell membrane is remodelled, and divided into two (cell division) or more (apoptosis) discrete bodies. This occurs without any leakage of the cellular components into the extracellular space, and requires the integrity of the membrane be maintained at all times during the remodelling process.

It is clear that TRPM-2 is involved in both proliferation and in apoptosis. Compiling all of the available data suggests that the role of TRPM-2 in these processes (apoptosis, proliferation and sperm maturation) is to ensure the integrity of the cell membrane during the extensive remodelling that occurs in each case and to prevent the complement mediated lysis of the membrane. Immunofluorescence microscopy, using an anti-clusterin antibody, has demonstrated that the TRPM-2 gene product is associated with the membrane of cells undergoing apoptosis (Grima *et al.* 1990), giving more weight to this hypothesis.

However, some neuronal and non-neuronal developmental cell deaths failed to identify the expression of TRPM-2 in the process. These processes seem to reflect variations of ACD compared to the adult animal (Clarke, 1990). Since, in those developing systems, due to the lack of a complement system, no complement response is possible, TRPM-2 as a protector of cellular integrity from complement associated lysis would not need to be expressed. This correlates well with natural functions of a

developing animal, where a selection of genes to be expressed is tightly made both spatially and temporally.

V. CONCLUSION

The research supporting this thesis dealt with the cloning of the TRPM-2 gene, both in rat and human; the complete sequencing of both genes; the initial determination of characterization of functional elements within the rat gene; the study of expression of the rat TRPM-2 gene in different tissues; and the resolution of the discrepancy between the published sequence of SGP-2 cDNA (Collard and Griswold, 1987) and the TRPM-2 cDNA.

The rat TRPM-2 gene is 13,750 bp in length and is composed of 9 exons. 5,737 bp upstream of exon I have been sequenced. The spliced product of this gene produces an mRNA of 1,640 bp with the same transcription start in all tissues examined. Consensus TATA and CAT boxes have been found at positions -30 and -96 respectively. A single palindromic sequence has been found at -75 and is its biological significance is being currently investigated. The gene also has a single polyadenylation signal.

The human TRPM-2 gene is 16,570 bp in length and is composed of also 9 exons. 1,300 bp upstream of exon I have been sequenced. The mRNA is approximately 1,660 bp in length. Consensus TATA, at -29, and CAT, at -96, have been identified as well as a single palindrome at -118 that bears to sequence similarity with its rat counterpart. Also, in the rat palindrome is between the TATA and CAT boxes and the human the palindrome is upstream of the CAT consensus sequence.

The rat TRPM-2 mRNA was detected in all tissues examined (fig. 12 and 13) at very different levels. The gene seems to be constitutively expressed in all tissues being highly induced after hormonal ablation in the prostate and seminal vesicles.

PCR analysis confirmed the presence of the SGP-2 leader sequence in the rat testis RNA. However this same set of experiments also confirmed that the published 269 nucleotides leader of SGP-2 is not associated either by alternative or trans-splicing to the rest of the SGP-2 sequence that is similar to TRPM-2. Therefore the 269 bp leader of SGP-2 is a cloning artifact and serves no biological function when physically associated with TRPM-2

The next logical step to this research is to characterize possible regulatory elements lying either in the 5' region or any of the TRPM-2 intervening sequences. The sequence data provided in this thesis, and in particular, the palindromes, provide solid grounds to achieve this step.

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APPENDIX I: List of abbreviations

aa	amino acid(s)
ACD	active cell death
ATP	adenosine 5'-triphosphate
bp	base pair(s)
cDNA	complementary DNA
CLI	complement cytolysis inhibitor
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
GnRH	gonodotropin releasing hormone
hnRNA	heteronuclear RNA
kb	kilobase(s)
kDa	kilo daltons
MAC	membrane attack complex
MCS	multi-cloning site
MOI	multiplicity of infection
mRNA	messenger RNA
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
poly(A)$^+$RNA	polyadenylated RNA
RDE cells	rapidly dividing epithelial cells
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SGP-2	sulfated glycoprotein 2
SSC	saline sodium citrate
ssDNA	single stranded DNA
TGFß	transforming growth factor ß
Tris	2-amino-2-hydromethylpropane-1,3-diol
TRPM-2	testosterone repressed prostates message 2
w/v	weight/volume
pH	$\log_{10}[H^+]$

APPENDIX II: Materials

The following items were supplied by Amersham corp. (Oakville, ON); α-[^{32}P]-dCTP (3000 Ci/mmol), γ-[^{32}P]-dATP (3000 Ci/mmol), [^{35}S]-methionine (>800 Ci/mmol), Multiprime labeling kit, Hind III, Eco RI, Kpn I, Bam HI, Sal I, Mbo I, Hybond-N membranes, RNas I "A"

From Sigma Chemical (St-Louis, Mo). Oligo $(dT)_{12-18}$, acrylamide, bis-acrylamide, TEMED.

From Boehringer Mannheim Canada Ltd (Dorval, QC). Calf thymus DNA, Klenow fragment of DNA polymerase I, T4 ligase, polynucleotide kinase, calf intestinal phosphatase, PST I, Sma I.

From Life Sciences (St-Petersberg, FL). AMV reverse transcriptase.

From International Biotechnologies Inc (Toronto, ON). Electrophoresis grade agasose, Hind III and Eco RI/Hind III λ DNA molecular weigth marker.

From Pharmacia (Montréal, QC). pTZ18R, pTZ19R, M13KO7, oligo(dT)-cellulose, Sephades G-50, Sepharose 4B.

From Oxoid Canada (Nepean, ON). Agar, tryptone, bacto-yeast extract.

From BDH Canada (Toronto, ON). Ficoll, polyvinylpyrrolidone.

From Beckman Canada (Mississauga, ON). All centrifuges and rotors, all ultra-centrifuge tubes, Microgenie software, ready-Solv ET scintillation fluid, all scintillation counters.

From Picker International (Ottawa, ON). Cronex X-ray film.

Fisher Scientific Co. Ltd (Nepean, ON). All other chemicals and materials.

APPENDIX III: Curriculum vitae

CURRICULUM VITAE

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EDUCATION

1993	M.Sc. in Biochemistry, University of Ottawa, Ottawa, ON Thesis title: Cloning and characterization of the TRPM-2 gene in the rat and human.
1987	BSc. Hon. in Biochemistry University of Ottawa, Ottawa, ON
1984	College Diploma in health Science Séminaire St-Augustin, St-Augustin-des-Maures, QC.

STUDENTSHIP

09/89-06/91	F.C.A.R. (Les fonds pour la formation de chercheurs et aide à la recherche).

RELEVANT EXPERIENCE

10/92-PRESENT	Production Manager. CME Television Inc. Montréal, QC
07/91-10/92	Production Manager The Sales Professionnals Group. Montréal, QC

09/87-06/91	MSc. Student, (Supervisor: Dr. M. Tenniswood) Department of Biochemistry, Faculty of Health Sciences, University of Ottawa, Ottawa, ON. Involved mastering current techniques in Molecular Biology relating to nucleic acids manipulation.
05/87-08/87	Laboratory technician, in the lab of Dr. M. Tenniswood. Department of Biochemistry, Faculty of Health Sciences, University of Ottawa, Ottawa, ON Participated in a study regarding the possible presence of different malignant human papilloma viruses (HPVs) in the etiology of prostate cancer and benign prostatic hyperplasia.
05/86-08/86	Laboratory technician, in the lab of Dr. Jean Himms-Hagen. Department of Biochemistry, Faculty of Health Sciences, University of Ottawa, Ottawa, ON. Participated in a study of energy expenditure by the brown adipose tissue of hamsters.
01/87-04/87	Undergraduate Lab demonstrator. Second year general Biochemistry lab course. Department of Biochemistry, Faculty of Sciences, University of Ottawa, Ottawa, ON.
09/87-12/87	Undergraduate Lab demonstrator. Third year metabolism lab course. Department of Biochemistry, Faculty of Sciences, University of Ottawa, Ottawa, ON.
01/88-04/88	Undergraduate Lab demonstrator. Third year Physical Biochemistry lab course. Department of Biochemistry, Faculty of Sciences, University of Ottawa, Ottawa, ON.
09/88-12/88	Undergraduate Lab demonstrator. Fourth year Molecular Biology lab course. Departments of Biology & Biochemistry, Faculty of Sciences, University of Ottawa, Ottawa, ON.
09/89-12/89	Undergraduate Lab demonstrator. Fourth year Molecular Biology lab course. Departments of Biology & Biochemistry, Faculty of Sciences, University faculty Ottawa, Ottawa, ON.

01/90-04/90	Undergraduate Lab demonstrator. Third year Physical Biochemistry lab course. Department of Biochemistry, Faculty of Sciences, University of Ottawa, Ottawa, ON.

PUBLICATIONS AND ABSTRACTS

1. Paul Wong, **Jean Pineault**, Johnathon Lakins, Dan Taillefer, Jocelyne Léger, Chiayeng Wang, and Martin Tenniswood. (1992). Genomic organization and expression of the rat TRPM-2 gene, a gene implicated in apoptosis. Submitted to J. Biol. Chem.

2. Johnathon Lakins, **Jean Pineault**, Paul Wong, Daniel Taillefer, and Martin Tenniswood. (1992). Molecular structure, expression, and sequence comparison of the human TRPM-2 gene. Submitted to J. Biol. Chem.

3. **J.M. Pineault**, R. Pilon, and M. Tenniswood. Genomic and cDNA structure of the rat and human TRPM-2 gene: a comparative analysis. Presented at the 25th FEBS meeting in Budapest (Hungary), August 1990.

4. **J.M. Pineault**, and M. Tenniswood. Genomic organisation of the rat TRPM-2 gene. Presented at the CFBS meeting in Halifax, June 1990.

5. M. Tenniswood, M.F. Montpetit, J. Léger, P. Wong, **J.M. Pineault** and M. Rouleau. Cell death in the prostate. CRC press, 1988.

6. M. Levesque, **J.M. Pineault**, M. Tenniswood and D. Johnson. Genomic organization of a soybean gene involved in nodule senescence. Presented by M.L. at the XVIth International Congress of Genetics, August 1988, Toronto.

7. **J.M. Pineault**, D. Johnson et M. Tenniswood. Structure génomique du gène TRPM-2. Présenté dans le cadre du colloque de l'ACFAS, mai 1988, à Moncton.

8. **J.M. Pineault**, D. Johnson and M. Tenniswood. Genomic organization of the TRPM-2 gene. Presented at the CFBS meeting, June 1988, Québec.