THE UNIVERSITY OF MANITOBA

ISOLATION AND CHARACTERIZATION OF RHAMM GENOMIC CLONES

by

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Right at this moment, I recall a great song of the lab. " I'm too sexy for the lab..."

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LIST OF ABBREVIATIONS

AMF	Autocrine Motility Factor
BLOTTO	Bovine Lacto Transfer Technique Optimizer
CSF	Colony Stimulating Factor
cpm	Counts per minute
DC	Detergent Compatible
DMEM	Dulbecco's Modified Engles Medium
DMSO	Dimethyl Sulfoxide
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Growth medium - calf serum free
EDTA	Ethylene-diaminetetraacetic acid
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
FBS	Fetal Bovine Serum
Glu MTs	Detyorsinated Microtubules
IFN	Interferon
IGF	Insulin Growth Factor
IL	Interleukin
IPTG	Isopropylthio-ß-D-galactoside
kb	Kilobase
kDa	KiloDalton
MSF	Migration Stimulating Factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-Buffered Saline
PDGF	Platlet-derived Growth Factor
PEG	Polyethylene Glycol
PD-ECGF	Platelet derived extracellular growth factor
pfu	Plaque forming unit
SDS	Sodium dodecyl sulphate
SF	Scatter Factor
TBS	TRIS-Buffered Saline
TGF-ß	Transforming Growth Factor-ß
TNF	Tumor Necrosis Factor
X-GAL	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

REVIEW OF LITERATURE

1.1 INTRODUCTION

Cell locomotion is an innate capacity of most cells, that is exhibited by somatic cells as amoeboid-like movement and by sperm as flagellum based locomotion (Trinkhaus, 1976). It is vital to many normal physiological processes such as embryogenesis, morphogenesis, nerve regeneration, angiogenesis, homeostasis, immune surveillance and wound repair. Aberrant cell locomotion contributes to such pathological situations as arthritis (Nykanen et al., 1990), atherosclerosis (Scott-Burden and Bugler, 1988), the expression of many inherent developmental abnormalities including cleft lip and palate (Longaker et al., 1992), endocardial cushion defects (Krug et al., 1987) neuronal migration syndrome (Erickson, 1990); and tumor progression (Strauli and Weiss, 1977).

Cell locomotion can be detected as chemotaxis (directional migration in response to a gradient of a diffusible signal), haptotaxis (directional migration in response to a gradient of substrate adhesivitiy), chemokinesis (random movement) or, galvanotaxis (directional migration in response to electrical field); and is a result of several characteristics including signal transduction, cell-cell and cell-substratum adhesion as well as organization of cytoskeleton.

Cell locomotion appears to be regulated by chemotactic factors, growth factors, motility factors, and contact behaviour. Several factors have been shown to stimulate cell movement without promotion of proliferation including scatter factor (hepatocyte growth factor) (Stoker et al., 1985, 1987), autocrine motility factor (Liotta et al., 1986), tumor growth factor-ß1, (Yang and Moses, 1990; Nickoloff et al., 1988) and migration-stimulating factor (Schor et al., 1988; Grey et al., 1989); suggesting that the two phenomena can be mechanistically separate.

Although it isn't fully understood, cell migration is thought to involve recurring and

regulated attachment and detachment. Cell fragments (without the nucleus, Golgi apparatus, or rough ER) are still able to respond to a plethora of chemotactic stimuli (Stossel, 1989) and can locomote for a few hours; demonstrating that genetic activation, which may only be compulsory for the chronic maintenance of cell locomotion and invasion, is not necessary for acute migratory responses.

A repertoire of adhesive behaviour of the cell is essential for cell movement to occur and the level of the cell-substratum adhesivity is critical. Too strong cell-substratum adhesion will inhibit movement as will too weak cell-substratum adhesion (Erickson, 1990). Equally important, cell-cell adhesion must be weakened to allow movement away from cell groups. Such cell adhesive diversity is typically controlled by a balance of proteoglycans / glucosaminoglycans, collagens, and adhesive glycoproteins of the extracellular matrix (ecm) and cell surface; and by regulated activities of activators and inhibitors of proteases (i.e. plasmin), which are needed to break down the localized cell substrate attachments termed focal adhesions.

Most motile cells exhibit few focal contacts. Indeed, the occurrence of large focal contacts appears to be associated with maintenace of a static, differentiated spread-cell shape (Kolega et al., 1982; Duband et al., 1988). Focal contacts appear to form at the leading edge, diminishing in number as the cells move forward. Only a few focal adhesions are found in the rear region of the cell. Disruption of focal adhesions and stress fibers is thought to be an important primary step for cell translocation to begin.. This event seems to be mediated by intracellular signalling and the role of ecm receptors will be mainly considered here.

The intracellular machinery for cell movement resides in the cortical cytoplasm where a diversity of proteins including actin, myosin, microtubules and intermediate filament are contained (Pollard and Goldman, 1992; Erickson, 1992). Depletion in one of these proteins has selective defects in cell motilty (see below). A number of models have

been proposed to explain an interplay among the cytoskeleton proteins in locomotion. How ecm molecules give rise to the signalling event and subsequent alternation of cytoskeleton will be discussed.

1.2 REGULATORS OF CELL LOCOMOTION

a) Contact inhibition

In most cell types, an immediate paralysis occurs when the leading edges of two migrating cells encounter on another. This is the well known phenomenon of contact inhibition that affects both locomotion and growth. Cells may use contact inhibition as a mechanism to orient or guide themselves to their destinations i.e. during embryogenesis (contact-induced directional migration). The selective cell-cell adhesion may be involved to provide the information and strategy to restrain different cells to their own territories (Erickson, 1990). Cellular transformation often involves loss of contact inhibition and although research on this fundamental mechanism has been ongoing for a long time, little is known about it's molecular mechanisms.

It has been considered that both contact inhibition of cell movement and division involve intracellular signalling that leads to reorganization of cytoskeleton and loss of focal adhesions (Hall et al, 1994). Several observations indicate that the physiological states of a cell differs in the contact inhibited vs non-contact inhibited state. For instance, contact inhibited cells show a reduced level of serum-stimulated hydrolysis of phosphatidylinositol bisphosphate (PIP2) (Miloszewska et al., 1991). Cell-cell contacts also affect intracellular pH, which increases with cell density. Once cells reach a confluent (topoinhibited) monolayer, the intracellular pH decreases (Galkina et al., 1992). Moreover, it has been illustrated when cells completely surround each other, extensive arrays of detyrosinated microtubules (Glu MTs) are found coiled around the nucleus. When the contact was confined to a portion of the cell margin, Glu MTs were absent from the area behind the

contact site, yet were still oriented toward the noncontacting and ruffling margins (Nagasaki et al., 1992).

b) Motogens

Substances capable of stimulating cell motility have been designated as "motogens" (Stoker and Gheradi, 1991). To date, as many as fourteen motogenic factors have been reported. In addition, two inhibitory motility factors have also been described (see below). Autocrine motility factor, (AMF) widely produced by tumor cells, is an example of a motogen. It is a 55 kD single protein with intrachain disulphide bonds which are necessary for stimulation of motility (Liotta et al., 1986). AMF appears to act in autocrine mode and its effects are mediated by a specific membrane receptor of gp78 (Nabi et al., 1992 and 1990). Migration stimulating factor (MSF), a 70 kD protein, is another cytokine involved in the autocrine stimulation of tumor cell motility. Its mechanism of presentation and action in vivo may correlate to its capability of binding to heparin (a feature shared by a number of cytokines) (Stoker and Gheradi, 1991). Scatter factor, capable of inducing the disruption and scattering of epithelial colonies as a result of increasing the movement of individual epithelial cells, is a heterodimer composed of a 57 kDa and a 30 kDa subunits. It is highly homologous to and is likely the same as HGF/hepatopoietin A, a potent mitogen for rat hepatocytes in culture. In contrast to the above motility factors, scatter factor primarily acts in a paracrine fashion.

Many well known mitogens are also capable of affecting cell motility. PDGF was the first well studied growth factor found to induce locomotion of 3T3 and haematopoietic cells (Matsui et al., 1989; Hosang et al., 1989). Several studies indicate that the states of the cells and ligand are influential factors for the chemotactic response of sensitive cells to PDGF. That is, the chemotactic response of density-arrested 3T3 cells is much greater than that of exponentially growing cells (Grotendorst, 1984). Further, treatment with the

reducing agents affect motogenic responses elicited by PDGF. Interestingly, the number of PDGF receptors is higher in sparse cells than that of confluent cells. The number of receptors expressed on target cells may be an important regulatory mechanism of motogen induced motility.

The motilty of human keratinocytes, dermal fibroblasts and ras-transformed fibroblasts can be stimulated by TGF-ß. The motility effect of TGF-ß seems to be brought about by inducing synthesis of fibronectin as it can be inhibited by anti-fibronectin antibodies; and by stimulating the production of both hyaluronan and its receptor of RHAMM (see below). EGF and TGF bind to the same transmembrane receptor with a cytoplasmic tyrosine kinase domain and both can induce the migration of keratinocytes in culture. EGF also stimulates the motility of intestinal epithelial cells, but only those cells from sparse culture show a strong chemotactic response. aFGF stimulates the motility of endothelial cells, human and bovine fibroblasts and astroglial cells. Stimulation of bovine capillary endothelium motility has also been observed with bFGF. The concentration of the cytokine is a crucial factor in the cellular motility response. High concentrations of aFGF markedly induce the motility of a rat bladder carcinoma line and 3T3 fibroblasts while low concentration of aFGF contributes to DNA synthesis (Valles et al., 1990). The motility of the metastatic melanoma cell line A2058 can be induced by IGF-I and this motility response in A2058 cells can be enhanced by AMF.

PD-ECGF, a human platelet growth factor, stimulates only the directed cell migration of endothelial cells. IL-6 enhances the motility of two human breast carcinoma lines in a way that is similar to the effect of scatter factor on epithelia. Two haemopoietic growth factors, GM-CSF and G-CSF, increase endothelial migration in autocrine manner. Bombesin, a short peptide released by CNS in response to electrical stimulation, promotes motility of a number of small lung carcinoma lines in an autocrine fashion.

Both TNF-α and IFN-γ inhibit motility. TNF-α has been reported to inhibit the

migration of endothelial cells induced by tumor-derived cytokines without affecting their basal motility. IFN- appears to inhibit the motility of human keratinocytes by decreasing synthesis of fibronectin and thromospondin both of which stimulate motilty (see below). Collectively, these results indicate that motogens stimulate cell locomotion in part by regulating expression of ecm and their receptors.

1.3 THE EXTRACELLULAR MATRIX MOLECULES INVOLVED IN CELL LOCOMOTION

Several adhesive glycoproteins have been identified that support either cell chemotactic or chemokinetic motilty. Well studied molecules include fibronectin, laminin, collagen IV, and thrombospondin. The acid solubilized A-elastin and non-cross-linked tropoelastin monomer have also been demonstrated to act as stimultants for cell migration (Mecham et al., 1989; Blood and Zetter, 1989). As well, hyaluronan (HA), a glycosaminoglycan, has been recognized as an important contributor to cell motility (Toole et al., 1984; Turley 1984). Cell surface lectins (Barondes, 1988) and glycosyl-transferases (Shur, 1989) are also vital cellular recognition modulators that affect motility, but due to the limited space, they will not be discussed any further here.

a) Fibronectin (FN)

Fibronectin is a high-molecular-weight glycoprotein present in blood plasma, extracellular matrices, basal lamina and on cell surfaces. It has been implicated in mediating cell migration and remodeling during embryonic development and wound healing by providing a preferred substrate for migration (Dofour et al., 1988; Clarke, 1988). It has been shown that substratum-bound density gradients of fibronectin direct the haptotactic migration of melanoma cells in vitro (McCarthy et al., 1986, 1991). Expression of fibronectin appears to be developmentally regulated and is also modulated by malignant

transformation of cells (Owens et al., 1986; Hynes, 1985, 1986). The tripeptide of Arg-Gly-Asp (RGD) within FN represents a minimum functional unit for it's adhesive function (Pierschbacher & Ruoslahti 1984a and b; and Yamada & Kennedy 1984). Synthetic RGD-containing peptides have been shown to partially inhibit cellular migration on FN substrates (Straus et al., 1989). The RGD motif is not restricted to fibronectin and in fact occurs within more than 100 proteins (D'Souza et al., 1991). However, in some proteins, cell adhesive activity has been ascribed to the RGD sequence, whereas in others the RGD sequence appears to be functionally silent. Deletions of the fibronectin polypeptide show 100-200 fold less activity than the native protein, consistent with the existence of other sites that exhibit a synergistic interaction with RGD. For instance, for efficient cell attachment, spreading and migration (Dufour et al., 1988; Nagai et al., 1991), a synergistic region of 20 KD of polypeptide sequence away from the RGD site is required (Obara et al., 1988; Aota et al., 1991). Other cell adhesion sites of fibronectin include Leu-Asp-Val (LDV) and REDV (Komoriya et al., 1991; Humphries et al., 1986,1987; McCarthy et al., 1986) LDV is present in FNs from a variety of species while REDV is missing from chicken fibronectin (Komoriya et al., 1991; Norton and Hynes, 1987). Synthetic peptides of LDV can partially mimic activity of the intact protein (). A major effect of the multiple binding domains of FN on cell behaviour arrives from a collaboration of ecm domain to promote focal adhesion formation in certain cell types (Burridge et al., 1988; Woods et al., 1986). Other fibronectin domains such as heparin-binding domain II and the CS1 cell adhesive site have also been shown to stimulate or modulate neurite outgrowth and migration of embryonic neural crest cells (Dufour et al., 1988; Humphries et al., 1988).

b) Laminin

The glycoprotein laminin is a prominent constituent of basement membranes and

can serve as an adhesion protein for a variety of cell types, especially epithelial and neuronal cells. It also binds to other components in the matrix (i.e. type IV collagen, heparan sulfate proteoglycan and entactin) and to itself. A number of peptides with attachment activity have also been found within this protein. A unique sequence of five amino acids, YIGSR, from one of the EGF-like repeats in the cysteine-rich region of the B1 short-chain promotes cell adhesion, chemotaxis of several cell types and interacts with the 67 kDa laminin receptor (Davis et al., 1989; Bilozur et al., 1988; Graf et al., 1987). Other laminin peptides including LGTIPG in the B1 chain and IKVAV within the E8 fragment are chemotactic (Perris et al., 1989; Mecham et al., 1989; Tashiro et al., 1989; Bobzon et al., 1989). All the peptides described above exhibit only part of the motility-stimulating activity of intact laminin, implicating that either multiple sites or a specific steric presentation of the individual sequences is bestowed by the intact molecule.

c) Thrombospondin

Thrombospondin, produced by platelets, is synthesized and deposited in extracellular matrices of several cell types (Lawler & Hynes, 1987). In addition to its role in blood coagulation, thrombospondin has been reported to induce chemotaxis of mesangilal cells in dose dependent manner (Taraboletti et al., 1992), as well as the migration of normal epidermal keratinocytes (Nickoloff et al., 1988), granule cells (O'shea et al., 1990) and a variety of tumor cells (Roberts, 1988; Taraboletti et al., 1987). The use of site specific monoclonal-antibodies indicates that the heparin-binding amino-terminal domain mediates chemotaxis, whereas the globular C-terminal domain is responsible for haptotaxis (Taraboletti et al., 1987).

d) Collagen and elastin

Various collagen types have been reported to promote the adhesion and migration of

normal and transformed cells (Aumailley and Timpl, 1986; Dedhar et al., 1987; Herbst et al., 1988; Chelberg et al., 1989). Motility stimulated by type IV collagen is a result of the peptide GVKGDKGNPGWPGAPY (triple-helix domain residues 1263-1277) which represents its RGD-independent, adhesion, spreading, and motility promoting domain. Site directed mutagenesis indicates that the prolyl residues are required for the motile response (Mayo et al., 1991).

The repeated sequence VGVAPG of elastin (Mecham et al., 1989; Blood and Zetter, 1989) as well as other hydrophobic sequences (Long et al., 1988) are associated with this protein chemotaxis-stimulating activity. Adjacent glycyl and prolyl residues are common to the motility-stimulating sequences within laminin, collagen IV, and elastin (see Table 1).

Overall, multivalency in cellular recognition appears to be a general requirement for ecm properties. The current knowledge has been reviewed above, but it remains to be determined whether additional polypeptide sequences stimulating locomotion enhance or synergize with known sequences to provide receptor specificity. Further, some of these cellular adhesive sites may be cryptic or otherwise inactive in the native protein and may therefore require proteolysis of the molecule for function.

e) Proteoglycans/glycosaminoglycans

Proteoglycans are proteins that are covalently conjugated with glycosaminoglycans (linear heteropolysaccharides) and occur at the surface of a variety of cell types as well as in the ecm. The number of glycosaminoglycan chains and their length can vary, as well as their pattern of sulfation. This may result in a diversity of different chain types with different properties and affinity for their respective ligands. The common proteoglycans include the galactosaminoglycans, chondroitin sulfate (CS) and dermatan sulfate (DS), and the glucosaminoglycans, heparan sulfate (HS), heparin, and keratan sulfate (KS).

Hyaluronan (HA) exits as a glycosaminoglycan without protein (see Table 2).

Early evidence of the involvement of cell surface or extracellular proteoglycans in adhesion and motility included the localization of proteoglycan at focal adhesions, the requirement of glycosaminoglycan / proteoglycan binding domains to establish focal adhesions, and the observation that cells lacking glycosaminoglycan synthesis lead to fewer formation of focal adhesions (Lebaron et al., 1988; Woods et al., 1986). Recently, proteoglycans (PGs) and glycoaminoglycans (GAGs) have been shown to regulate protein secretion and gene expression in certain tissues by mechanisms involving both membrane and nuclear events (Rodrigurez-boulan and Nelson, 1989; Reilly et al., 1988; Hadley et al., 1985), including the binding of GAGs to transcription factors (Cardin and Weintraub, 1989; Wright et al., 1989). This may be significant for chronic motility and invasion (Jackson et al., 1991).

It is generally considered that heparan sulfate proteoglycan (HSPG) is associated with a propensity for attachment whereas chondroitin sulfate proteoglycan (CSPG) and HA are associated with weak adhesion and even detachment of cells. Both may affect cell motility by binding or masking the more adhesive ecm molecules such as fibronectin (Ruoslahti, 1988). Synthesis of under-sulfated HSPG or increased expression of CSPG have also been reported to promote tumor cell motiltiy (Iozzo, 1985; Kinsella and Wight, 1986). Alternatively, through interaction with anti-adhesive ecm molecules such as thrombospondin (Murphy and Hook, 1989) and tenascin (Salmivirta et al., 1991), cell surface associated PGs may destablize cell contact points to interfere with cell attachment (Wight et al., 1992).

Membrane-associated proteoglycans of the syndecan family which are HSPGs, are predominantly found on epithelia in mature tissues and contain both transmembrane domains and cytoplasmic tails. Syndecan mediates the binding of cells to fibronectin, thrombospondin, and types I, III, and V collagen via interactions with its HS and CS

chains (Bernfield and Sanderson, 1990; Elenius et al., 1990). The ligand binding to ecm components promotes the association of the membrane domain to actin-rich cytoskeleton. And, when these syndecans at the apical cell surface are cross-linked by antibodies, they assimilate into detergent-resistant, immobile clusters that are subsequently aggregated by the cytoskeleton (Rapraeger et al., 1986). Upon rounding, syndecan is shed from the cell surface by proteolytic cleavage of the core protein at the cell surface, a process that separates the matrix-binding ectodomain from the membrane domain (Jalkanen et al., 1987; Weitzhandler et al., 1988). By these associations / disassociations, syndecan can mediate matrix organization into cellular organization and influence the behaviour of cells.

A subset of proteoglycans are found to be membrane anchored via a covalent linkage with phosphatidylinositol (Drake et al., 1992; Carey and Stahl, 1990; David et al., 1990) and the structure of the glycosylphosphatidyl - inositol anchor has been reviewed (Low, 1989). One advantage of this type of membrane anchor is that it offers a site for cleavage by phospholipases, resulting in the release of the proteoglycan from the membrane.

CD44, also termed Hermes antigen (Jalkanen et al., 1986), Pgp-1 (Hughes et al., 1981), or H-CAM (Culty et al., 1990) is a cell surface glycoprotein, an example of CSPG that participates in cellular adhesion. There are low (80-90 kD) and high (200 kD) molecular forms of CD44. The low molecular weight form without GAG chains attached, is a well known lymphocyte homing receptor. It also serves as a receptor for hyaluronan (Miyake et al., 1990). Multiple forms of CD44 have been identified and are attributed to both alternative splicing and differentiated glycosylation within the extracellular domain (see section HA receptor below). The high molecular weight form containing CS, HS, or both types of GAG chains is believed to take part in cell adhesion through the interaction of GAG chains with ecm ligands such as fibronectin (Brown et al., 1991). The PG forms of CD44 are found to be localized at the filopodia and zones of cell contacts (Brown et al.,

1991). The variations in CD44 glycosaminoglycan composition appear to be tissue-specific (Stamenkovic et al., 1991; Brwon et al., 1991), and may be related to its proposed role in cell adhesion (Hardingham and Fosang, 1992; Faassen et al., 1992; Carter and Wayner, 1988). For example, CD44 expressed as a CSPG on certain melanoma cell types is required for motility and invasion of these cells into collagenous gels (Fassen et al., 1992).

In summary, cell surface proteoglycans may directly function through their adhesive properties or serve as receptors involving signalling events to promote locomotion (see below).

There is also an indirect way for cell-associated proteoglycans to mediate cell adhesion and motility by means of serving as "coreceptors" for other cell adhesion receptors. Many cell adhesion promoting proteins of ecm contain integrin binding domain in close proximity with PG/GAG binding site (Iida et al., 1992; Haugen et al., 1990; McCarthy et al., 1990). For example, specific inhibitors of CSPG synthesis together with anti-$_4$ integrin subunit monoclonal antibodies inhibit melanoma cell adhesion to FN synthetic peptides, demonstrating that human melanoma cell adhesion to the 33 kD carboxyl-terminal heparin binding fragment of FN requires both cell surface CSPG and integrin $_4ß_1$ (Iida et al., 1992). The cell adhesion promoting sites in that fragment must be using CSPG and integrin $_4ß_1$ in a coordinated fashion for recognition events in cell adhesion (Iida et al., 1992).

f) Hyaluronan (HA)

Hyaluronan (formerly termed hyaluronic acid) is the only GAG not associated with a protein core. It is composed of repeating glucuronic acid and N-acetylglucosamine residues that are not sulfated. Excellent reviews on hyaluronan have been published in

Ciba Fdn. symposia (1989). Clinically, HA has been a very intriguing subject for its implication in healing (Laurent et al., 1991; Mast et al., 1991), its implementation in both visosurgery and viscosupplementation for its rheological properties (Ciba Fdn, 1989). Physiologically, apart from its mechanical role as space filler or lubricant between joints (Ciba Fdn, 1989), this simple homopolymer also actively participates in many common biological and pathological situations such as angiogenesis, embryogenesis, repair processes, tumor progression, arthritis, atherosclerosis, and skin disorders (Sampson et al., 1992; Ciba Fdn symposia, 1989; Matsuoka et al., 1987). In particular, evidence suggests that HA regulates cell locomotion. However, the mechanism by which HA regulates cell locomotion has not yet been defined.

Although the manner in which HA exerts its effect is not fully understood, HA is known to be involved in locomotion of fibroblasts (Turley et al., 1991), epithelial cells (Hadden and Lewis, 1991), white cells (Stamenkovic et al., 1991), smooth muscle cells (Boudreaux et al., 1991), sperm (Huszar et al., 1990), ras-transformed cells (Turley et al., 1991) and vascular endothelial cells (Ciba Fdn symposia, 1989). In addition, several studies have shown that onset of embryonic cell migration of neural crest (Derby and Pintar, 1978), corneal fibroblasts (Toole, 1982), heart cushion cells (Markwald et al., 1978) and chondrocytes (Ciba Fdn symposia, 1989); immigration of macrophages and fibroblasts into the wounded area (Bray et al., 1991; Nettelbladt et al., 1989), wound healing (West et al., 1985), tissue remodelling (Ruggiero et al. 1987), tumor invasion (Toole, 1990; Iozzo, 1985; Turley, 1984) and morphorgenesis (reviewed in Toole, 1991) all coincide with a transient and increased production of hyaluronan. Coincidental with cessation of cell migration, HA is apparently degraded by hyaluronidases. This degradation of HA appears to be mainly intracellular and results from receptor-mediated uptake (Bertolami et al., 1992; Raja et al., 1988). HA is necessary for regulation of locomtion by tumor motility factors (Schor et al., 1989) and growth factors such as TGF-ß

(Samuel et al, 1993). Addition of TGF-ß induces increase of HA, RHAMM (one of HA receptors) and cell motility (Samuel et al., 1993).

It has been demonstrated that addition of HA induces a rapid tyrosine phosphorylation and focal adhesion turnover concomitant with HA locomotory response in ras-transformed fibroblasts (Turley, 1989). Moreover, the enhanced phosphotyrosine staining occurs within focal adhesions (Hall et al, 1994). These data suggest that phosphotyrosine is associated with newly formed focal adhesions at the leading lamellae and their loss with reduction of phosphorylation. HA appears to promote cell locomotion via signal transduction pathways involving PTK activity upon focal adhesions. Mechanisms by which HA regulates locomotion are suggested as the following.

1.4 MECHANISMS BY WHICH HA REGULATES CELL LOCOMOTION

HA may regulate cell locomotion both directly and indirectly. HA may affect locomotion indirectly by means of altering connective tissue organization (Toole, 1982). The local increased production of HA, which has a tendency to become hydrated and highly viscous, exerting significant osmotic pressures and causing edematous swelling of resident tissues, may lead to open spaces within tissues to release cells from contact inhibition that then facilitates cell migration. This may also contribute to the invasiveness of tumor cells (Pauli and Knudson, 1988).

HA is a weak adhesion molecule that at relatively high concentration may directly impede cell adhesion and act as a detaching factor influencing cell detachment during the detachment / attachment cycle of cell movement (Culp et al., 1976). Consistent with this proposal, elevated levels of HA are found in the retraction footpads of motile cells (Turley, 1984, 1989).

Furthermore, the colocation of rhodamine labelled-HA with it's receptors RHAMM and CD44 (Turley, 1984) raises additional possibilities that HA-stimulated locomotion may act directly by signalling via one or both of its receptors (Turley, 1991). This RHAMM, for instance, signalling via a protein tyrosine phosphorylation cascade results in focal adhesion turnover and cytoskeleton reorganization (Turley, 1989)(see HA receptors below).

1.5 ECM RECEPTORS INVOLVED IN ECM-MEDIATED MOTILITY

a) Integrins

Cell-matrix interaction is mediated in part via a superfamily of cell surface receptors termed integrins. Cell translocation also partly depends on adhesive interactions mediated by integrins: when adhesion-perturbing anti-$ß_1$ integrin antibodies are applied to preattached cells, retraction of the lamellae occurs and movement ceases (Regen and Horwitz, 1992). In addition, migration of normal fibroblasts is slightly stimulated by an anti-$_5$ antibody, an effect that correlates with a loss of focal contacts (Akiyama et al., 1989).

Integrins are divalent cation-dependent transmembrane heterodimeric glycoproteins containing and ß subunits in noncovalent association and capable of transducing signals from the extracellular environment to the cell interior. Fibronectin is bound by at least two distinct classes of receptors: membrane-associated proteoglycans and the superfamily of integrins, including the "classical" fibronectin receptor $\alpha_5ß_1$ and several other integrin receptors (i.e. $\alpha_3ß_1$, $\alpha_4ß_1$, $\alpha_{IIb}ß_3$, $\alpha_Vß_X$ and a novel neutrophil integrin), potentially permitting a wide diversity of function and regulation of fibronectin (Cheresh et al., 1989; Gresham et al., 1989; Ruoslahti and Piersxhbacher, 1987).

Integrins also serve as receptors for other ecm components such as laminin, tenascin, vitronectin, collagens, many serum proteins (thrombospondin, fibrinogen, von Willebrand factor, complement fragment C3bi) and cell surface immunglobulin superfamily receptor (VCAM, ICAMs) (Mould et al., 1990). The majority of ligands recognized by integrins occurs via the RGD sequence which have been implicated in binding of $_5ß_1$, GPIIb/IIIa, $_Vß_3$ and $_Vß_5$ integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1991).

The binding specificities of any particular heterodimeric of integrins varies from cell type to cell type. The same binding domain is functional in one cell type while it is non active in another. It is plausible that the functional discrepancy may be modulated by an existence of cell type specific conformational features attributable to the post-translational modification i.e. glycosylation or association with specific glycolipids on the plasma membrane. Similar to the multivalent properties of ecm proteins, one specific type of integrins contains a multitude of binding sites and may bind more than one ecm component permitting the assembly of macromolecular aggregates in vivo.

Multivalency is a generalized feature of ecm-integrin interactions. Clustering of

individual receptor also increases multivalency, which yields high binding avidity for ligands on either side of the membrane. The binding of FN to its receptor seems to be important for such clustering, since inhibition of binding by a diversity of ways results in both diffuse receptors and disruption of cytoskeletal elements (Stickel and Wang et al., 1988; Roman et al., 1989). Implicit in this "receptor cluster" model of ecm recognition is that specific information transmitted into the cell may be strongly related to the specific combinations of cell surface receptors that come into proximity on the cell surface as a result of closely spaced cellular recognition domains within the ecm. Cell type-specific differences in ecm receptor expression could therefore alter the molecular composition of such complexes, resulting in profound differences in cell type-specific signals transmitted by ecm. Changes in these receptor complexes may generate aberrant signals that may modulate the tumor invasion and metastasis (Iida et al., 1992).

Integrin-cytoskeleton association vary correspondingly with different combinations of the cytoplasmic domains of integrins. In several studies, chimeras and deletion mutants have been generated specifically to address the functional roles of integrin cytoplasmic domains. Experiments with $ß_1$ chimeras have shown that the cytoplasmic and transmembrane domains of $ß_1$ and $ß_3$ are functionally interchangeable with respect to focal contact formation (Solowska et al., 1991). On the other hand, experiments in which the $ß_1$ subunit in association with various cytoplasmic tails of $_2$, $_4$, or $_5$ were switched and expressed in recipient cells demonstrate markedly differing roles for particular cytoplasmic domains in post-ligand binding events (Chan et al., 1992). Certain integrins also have alternative spliced cytoplasmic tails (Hynes, 1992; Ruoslahti, 1991) and may only be expressed in a cell-type and developmental-stage specific manner.

Many integrins in cells of hematopoietic origin (such as platelets, monocytes, and lymphocytes) require activation for their recognition and adhesion mechanisms to become functional (Hynes, 1992; Ruoslahti, 1991; Albelda and Buck, 1990). For example, it is inferred that the activation of platelets by $ß_3$ monoclonal antibodies causes changes in the conformation of cytoplasmic tail of $ß_3$ which then modulates the affinity of the $_{IIb}ß_3$ integrin for its ligand (Manning and Brass, 1991; O'Toole et al., 1990). The activation of integrins can also be produced by conformationally induced changes on the outside of the cell through the binding of ecm related ligands (Wayner and Kovach, 1992; Du et al., 1991). This modulation of affinity may in general be an important control in mechanism for regulating cell motility.

b) Other ECM receptors

Non-integrin receptors involved in cell motility on ecm components have been identified for laminin, elastin and hyaluronan. A 67 kD high affinity laminin receptor (Mecham et al., 1989) has been described to mediate the migration of some tumor cells on laminin (Wewer et al., 1987) and neurite outgrowth on laminin (Kleinman et al, 1988). As well, a galactosyl-transferase (Runyan et al., 1988) which is thought to bind specific N-linked oligosacharides within the laminin molecule promotes neurite outgrowth and mesenchymal cell migration. There are also non-integrin cell surface proteins involved in mediating chemotactic response to elastin peptide VGVAPG (Mecham et al., 1989). It has been illustrated for elastin-stimulated motility that an increase in chemotactic responsiveness is accompanied by an increase in affinity of cell surface VGVAPG receptors (Blood and Zetter, 1989). It may be a generalized feature of regulation for other ecm-stimulated motility. Two major receptors for HA, CD44 and RHAMM, are involved in cell locomotion and are now considered.

1.6 HA RECEPTORS

Kinetic analysis indicate that HA binds with high affinity to specific cell sites of both myocytes (Angello and Hauschka, 1979) and fibroblasts (Bertolami et al., 1992). A number of putative HA binding proteins have been isolated and characterized (for review see Turley, 1991; Toole, 1990). One class of HA binding protein commonly include link protein (Goetinck et al., 1987; Neame et al., 1986), versican (Krusius et al., 1987), hyaluronectin (Delpech and Halavent, 1981; Perides et al., 1989), aggrecan (Doege et al., 1987) and CD44 (Wolffe et al., 1990; Idzerda et al., 1989; Goldstein et al., 1989; Nottenburg et al., 1989; Stamenkovic et al., 1989; Zhou et al., 1989) and are classified by their link protein homology region. It is presumed that HA binding of this subset proteins is mediated through the single tandem repeat loop created by disulphide bond at the NH_2 terminus of the ectodomain (Gallagher, 1989), even though a consensus binding motif has not yet been elucidated. Currently, from deletion studies two discrete HA binding domains have been identified in the HA receptor RHAMM (see below); suggesting that either there are several unrelated HA binding motifs within two classes of HA-binding proteins or an HA binding motif is based on properties other than the primary amino acid sequence (Yang et al., 1993). Another hyaluronan binding protein found on endothelial cells (Toole, 1990) is also proposed to be involved in locomotion. Its relationship with the other receptors of

HA awaits its further characterization.

a) Endocytic Receptor for HA

Internalization and degradation of HA is mediated through endocytosis have been demonstrated in culture (McGuire et al., 1987; Erickson et al., 1983). The rate of endocytosis is concentration-dependent and the receptors are recycled after the endocytosis of HA. It is also believed that HA endocytosis is using coated-pit pathway since hyperosmolarity inhibits endocytosis and receptor recycling in the low-density-lipoprotein or asialoglycoprotein receptor systems by means of disrupting the coated-pit pathway. Nevertheless, this HABP has not been identified (McGary et al., 1989).

b) CD44

CD44 exists in a variety of molecular forms. It exhibits structural and functional diversity such as lymphocyte and progenitor cell homing, lymphopoiesis and T cell activation, as well as fibroblast adhesion and migration (Miyake et al., 1990; Jalkanen et al., 1986; Lesley et al,. 1985; Jacobson et al., 1984) seen in CD44 molecule are attributed to alternative splicing and to glycosylation differences. A number of alternatively spliced variants of CD44 are selectively expressed on metastatic cells (Gunthert, et al., 1991). However, the ligand for this form of CD44 has not been determined. Recently, the genomic structure of CD44 has been determined (Screaton et al., 1992) and the CD44 gene contains at least 19 exons spanning 50 kb of DNA with at least 10 alternatively spliced exons within extracellular domain and 2 alternatively spliced exons within cytoplasmic domain. Not all CD44 expressing cells bind HA (Hardingham, 1992; Miyake and Murakami, unpublished data), and different CD44 expressing cells have been shown to have different affinities for HA (Murakami et al., 1991).

c) RHAMM

A novel HA receptor, termed RHAMM (**R**eceptor for **HA** **M**ediated **M**otility) is a glycoprotein (Hardwick et al., 1992) that is unrelated to the proteoglycan class of HA binding proteins. It is a part of an HA-receptor complex (HARC) which accumulates in the processes and ruffles of locomoting cells and its expression correlates with rapid locomotion occurring immediately after subculture (Turley, 1985 and 1989a), TGF-ß treatment (Samuel et al.,1993) and ras-transformation. As locomotion decreases and contact inhibition increases, HARC is lost from the cell surface. HARC contains several

protein components of molecular weight 72, 68, 60 and 56-58 kDa. The latter is RHAMM which functions as the critical HA receptor in both TGF-$ß_1$ promoting locomotion (Samuel et al.,1993) and HA-dependent locomotion of ras-transformed cells (Hardwick et al., 1992; Turley, 1991).

RHAMM exists at the cell surface, in the extracellular compartment and cytosol (unpublished observation). These RHAMM isoforms may result from alternative splicing of a single gene or different transcriptive products of a gene family. The cDNA encoding RHAMM has been isolated and contains neither a transmembrane domain nor a signal sequence. Its mode of association with the cell surface is therefore unknown but may resemble animal lectins (Barondes, 1988), the elastin-laminin receptor complex and the high affinity laminin receptor (Rao et al., 1989; Yow et al., 1988). Overexpression of RHAMM cDNA in fibroblasts alters cell mophology and causes loss of contact inhibition, increase of invasive ability and formation of foci (Yang et al., 1993). Antibodies specific to RHAMM block ras-induced locomotion in ras-transformed fibroblasts (Hardwick et al., 1992). Expression of RHAMM is also detectable in terminally differentiated B cells from multiple myeloma patients, non-Hodgkin's lymphomas, maligant hairy cell leukemia B cells, bone marrow and thymic B cells. The locomotion of hairy cells in response to HA is also blocked by monoclonal antibody to RHAMM as detected by video-time-lapse cinemicrography (Turley et al., 1993). All of these data illustrate the significant roles of RHAMM in cell behaviour and locomotion. The molecular mechanism by which RHAMM contributes to both cell behaviour and locomotion in response to HA however,remains elusive.

1.7 MECHANISMS OF ECM-REGULATED LOCOMOTION

In most model systems of motility including AMF-stimulated motility on melanoma cells (Nabi et al., 1992), chemotactic response of Dictyostelium to cAMP (Condeelis et al., 1992; Caterian and Devreotes, 1992), or chemotaxsis of leukocytes in response to bacterial products such as N-formylated peptides and paracine factors such as leukotrienes (Devreotes and Zigmond, 1988), transmembrane signalling is mediated by pertussis toxin sensitive G-proteins that transduce signals of such messenger mechanism as protein phosphorylation, calcium influxes and DAG production (Lester and McCarthy 1992). All of these mechanisms could profoundly influence cytoskeletal assembly in cortex.

Recent studies have investigated the signal transduction mechanisms that govern

matrix-induced cell motility (Kornberg and Juliano, 1992). One of the first clearly defined examples of a signal initiated by the adhesion of cells to a substratum was provided by the important experiment of Wright et al.(1983). They showed that when macrophages spread on a surface of fibronectin, engagement of a β_i integrin results in a signal that enables the phagocytosis by two distinctly different complement receptors CR-1 and CR-3. Blood platelet activation by contact with the substratum also involves signalling (Parise, 1989, Andrews and Fox, 1990). The response includes tyrosine-specific protein phosphorylation apparently regulated by glycoprotein IIb-IIIa (Ferrell and Martin, 1989; Golden et al., 1990) whose activated form has been implicated in platelet Na+/H+ exchange. HA also induces protein kinase activity (see below).

A number of protein kinases such as isoform of protein kinase C, a 125-kD focal adhesion kinase (pp125^{FAK}), pp60^{src} (Schaller et al., 1992; Guan and Shalloway, 1992; Hanks et al., 1992) and several viral tyrosine protein kinases (Ferrell and Martin, 1989) have been found to be localized with the fibroblast integrin complex in adhesion plaques. Ecm receptors themselves are not protein kinases and may rely on the associated kinases such as the src family to transduce a signal. Hirst et al. (1986) has suggested that functional connections between integrins and tyrosine kinases exists and changes in protein phosphorylation apparently regulate the structure and function of focal adhesions in vivo. They have demonstrated that integrins in focal contacts are tyrosine-phosphorylated in RSV-transformed chicken cells and sebsequently these receptors distributed diffusely over the cell surface. This effect of tyrosine phosphorylation appears to be important for dissolution of stress-fiber bundles, decreses of cellular adhesiveness and the resulting cell locomotion. Tyrosine phosphorylation may be responsible for the activation of Na+/H+ channels as well (Golden et al., 1990; Ferrell and Martin, 1989). Receptor clustering of integrins is an essential pre-requisite for signalling via tyrosine phosphorylation. Simple binding of FN to integrins only results in a sequel of phosphoinositide metabolism, arachidonic acid release and calcium fluxes (Ng-Sikorski et al., 1991; Murphy et al., 1990), while, both attachment to FN and clustering of integrins promotes a cascade of tyrosine phosphorylation which affects several different sets of proteins in sequence (Kornberg and Juliano, 1992). These results illustate that several messenger mechanisms are stimulated by FN recognition and the stimulatory effect of FN is relatively slow compared with growth factor stimulation which occurs within seconds. The involvement of several signalling systems may be needed for the control of individual components in cell motility.

Other ECM molecules such as hyaluronan and laminin induce IP_3/DAG, serine/threonine as well as tyrosine protein phosphorylation (Plantefaber and Lander, 1990; Turley, 1989b). Different from the effect of FN, the HA triggered phosphorylation event involving $pp60^{src}$ is rapid, occuring within seconds after addition of HA (Turley, 1989a) and transient; and has been demonstrated as an essential for motility in response to HA. Addition of HA also induces the transient tyrosine phosphorylation and cellular redistribution of $pp125^{FAK}$, concomitant with the disappearance of vinculin from focal contacts (Hall et al., 1994).

1.8 CYTOMECHANICS OF MOTILITY IN LIVING CELLS

a) Actin

A role for the actin cytoskeleton has been implicated in many cellular functions including motility, chemotaxis, cell division, endocytosis and secretion (Devreotes and Zigmond, 1988; Bretscher, 1991). Actin, a highly conserved globular molecule, can exist in a variety of configurations: as linear bundles, two-dimensional networks, and three-dimensional gels. These different assemblies occur simultaneously with a diverse collection of actin binding proteins. Changes in the ratios of monomeric to filamentous actin (G-actin and F-actin, respectively) occur in many cells undergoing changes in shape and motility and is a strong evidence for the importance of actin polymerization/depolymerization cycles in cell motility (Cooper, 1991; Cunningham et al., 1991). Further kinetic analyses of F-actin assembly shows that actin filaments in lamellipodium of fibroblast are undergoing net assembly at their distal, membrane-associated barbed ends and net disassembly at their poximal pointed ends (Cooper, 1991).

The use of inhibitors which restrain actin assembly, dramatically influences cell motility. For instances, cytochalasin, which binds to the barbed end of actin filaments and slows monomer addition (Bonder and Mooseeker, 1986), inhibits cell motility. Botulinum toxin, which caps the barbed end of actin filaments (Aktories and Wegner, 1989) causes cell rounding and loss of motility. Latrunculin, which binds to and sequesters actin monomers in vitro (Coue et al., 1987), also causes cell rounding and loss of motility (Spector et al., 1989). All of these results confirm that actin filaments are a necessary structural component required for motility.

Disassembly of actin filaments appears to be as important to motility as actin

assembly. Actin depolymerization gives rise to an increase in osmolarity. Fluid from other parts of the cell migrates into the region of actin breakdown. This process could result in a formation of a pseudopod in phagocytes by pushing against the plasma membrane and distending it (Stossel, 1989). Of relevant significance to locomotion is the observation that genetic overexpression of gelsolin (the actin severing protein) in mouse kidney fibroblast results in increased rates of locomotion (Cunningham et al., 1991). Regulation of assembly and disassembly of actin filaments involves the cooperative and competitive interactions among a large set of actin binding proteins (especially those that interact with sites on actin polymer ends and those that bind to monomers and regulate monomer access) in response to various signals in the cortex (see below).

It seems likely that an essential target of chemoattractant / motogen ecm receptor elicited signals is the polymerization of pseudopodial actin. Lysates of leukocytes catalyze the polymerization of pyrene-actin in a chemoatrractant-specific manner, whereas inhibition of actin polymerization by cytochalasin completely blocks chemotactic responsiveness (Devreotes and Zigmond, 1988). Removal of the chemoattractant also accelerates the depolymerization of pseudopodial but not cortical actin in leukocytes (Cassimeris et al., 1990).

b) Myosin I and II

In randomly moving cells, myosin is thought to participate in the retraction of lamellipodia. Whereas, in an activated, polarized cell, bundles of actin filaments are aligned in the same polarity and it is thought that myosin molecules are moving along in one direction from minus to plus end to make a sliding of actin filaments in one direction (Bray, 1992).

Studies involving colocalization of actin with the normal double-headed myosin II in Dictyostelium (also other motile cells) together with ATP-induced contraction of isolated cytoskeletons suggested that actomyosin contraction powered the molecular motor of cell locomotion (Clarke and Baron, 1987). However, recent genetic abaltion studies (Manstein et al., 1989; Wessels et al., 1988; De Lozanne and Spudich, 1987; Knecht and Loomis , 1987) showed that Dictyostelium that lacked myosin II still locomoted without major impediment. Futhermore, Dictyostelium amoebae devoid of myosin II produced a normal chemotactic response to cAMP and showed the normal rise in F-actin association with the cytoskeleton (Peters et al., 1988). On the other hand, experiments on macrophages by monoclonal antibody and phosphoprotein phosphatase inhibition surprisingly showed that

both a decrease and increase of MLC_{20} phosphorylation in myosin II inhibit motility. The increase of MLC_{20} phosphorylation coincided with an increase in the amount of myosin associated with the cytoskeleton (Wilson et al., 1992). Maintainence of MLC_{20} phosphorylation within narrow limits may be crucial for translational motiltiy by mammialian cells. The above studies also implicate that myosin II must not function alone during motile events on a solid substrate. Rather, myosin I (discussed below), or other undefined proteins are required to coordinate with myosin II for a normal motility response. In conclusion, myosin II is required for optimal cell movement and may be responsible for cortical contractions that pull the rear of the cell forward (Luna and Hitt, 1992).

Myosin I is a diverse collection of single-headed myosin containing ATP and actin binding sites which are similar to myosin II. To date, all myosin-Is that have been investigated have a C-terminal extension of the heavy chain with lipid-binding properties. Numerous studies with a variety of antibodies have established that myosin-I isoforms are associated with plasma membranes; and are concentrated at the leading edge of advancing pseudopodia and phagocytic cups of Dictyostelium (Fukui et al., 1989) and Acanthamoeba (Yonemura and Pollard, 1990; Baines and Korn, 1989). Genetic studies that deplete myosin I in amoebae show only partial impairment of phagocytosis and do not have an effect on motility. However, there are several loci coding for myosin I and inactivation of one locus or its RNA transcript would not necessarily cause complete cellular depletion of myosin I.

The possible myosin I-based movements in vitro have been suggested by Pollard et al. (1991). The myosin-Is may function: 1) through binding to acidic phospholipids to move membrane vesicles toward the barbed ends of actin filaments; 2) to mediate the movement of freely diffusing membrane receptors toward the leading edge (Sheetz et al., 1992); 3) by anchoring to the substratum through transmembrane linkages to pull actin filaments rearward (Sheetz et al., 1990).

1.9 EVENTS IN LAMELLA FORMATION IN RESPONSE TO SIGNALS

Cell surface protrusions at the leading edge appear to be critical for the movement of most cells (Bray, 1992; Amos and Amos, 1991). In order to advance forward, a cell must extend, make contact on a new spot of substratum, and be anchored there. The spreading of protusions involves the formation of new focal contacts which presumbly provide

traction for the migratory cells (Izzard and Lochner, 1980). Although the mechanisms by which these protusions are generated are not understood, both membrane-cytoskeleton interactions and actin accessory proteins in response to intracellular messengers appear to be involved.

The events occuring immediately after ligand recognition may fall into two categories. Firstly, signal transduction may occur involving second messengers such as cAMP, cGMP, IP^3 or DAG influence cytoskeletal assembly in cortex by protein phosphorylation and calcium dependent processes which are discussed below. Another event involves a direct influence of ecm receptors on cytoskeletal organization at the cytoplasmic face of the adherent membrane. Evidence has already been given that integrin cytoplasmic domains interact with cytoskeletal components (Otey et al., 1990), and that sites of integrin-mediated adhesion to the ecm may serve as nucleation sites for cytoskeletal organization (Burridge et al., 1988).

A number of authors infer that phosphorylation is the first identifiable step of the signalling process (Brickell, 1992; Paolini et al., 1991) involved in cell locomotion. Ecm receptors, ß-integrins, and an HA receptor complex are reported to be tyrosine phosphorylated (Burridge and Turner, 1991; Turley, 1989). Other phosphorylated substrates include talin, paxillin, and vinculin (Luna and Hitt, 1992). This phosphorylation could reduce fibronectin binding as well as loss of binding to the cytoskeletal protein talin (Hirst et al., 1986). $pp60^{c\text{-}src}$ may phosphorylate a vinculin binding protein, paxillin and $pp125^{FAK}$ (Guan and Shalloway, 1992). Both the HA receptor complex and integrin aggregate are associated with $pp60^{c\text{-}src}$ (Burridge and Turner, 1991; Turley, 1989). This may provide a mechanism to promote the breakdown of focal adhesions and consequently of stress fibers. The cytoskeletal assembly may also be affected by serine/threonie, protein kinase C. Protein kinase C has also be documented to be associated with focal adhesion and be the major contributor of myosin phosphorylation (Woods and Couchman, 1992; Omann, 1987) which may promote cell motility as previously mentioned above.

Another major signal transduction is G-proteins based systems including ras-related GTP-binding proteins, rho and rac. Rho is required specifically for the formation of focal adhesions and stress fibers, but not for the membrane ruffles in response to growth factors. When the rho proteins is ADP-ribosylated, cells lose their actin stress fibers and round up (Ridley and Hall, 1992). Rac proteins are involved in regulating growth factor-induced membrane ruffling. It is suggested that growth factors act through rac to stimulate rho-dependent response (Ridley et al., 1992).

The activated G protein causes subunit to dissociate and activate the phospholipase C that cleaves the PIP_2 resulting in the liberation of IP_3 and DAG. The former releases calcium internal storage vesicles filling the peripheral cytoplasm. There, the calcium activates a number of molecules, including gelsolin. Activated gelsolin breaks down the actin network in the resting cell. At the same time, profilin is attached to individual actin molecules, which prevents the formation of new actin filaments. In addition, gelsolin is attached to the preferred end of existing filaments, which also precludes filament elongation.

After these events reach a climax, the severed actin filaments with gelsolin molecules at their barbed ends diffuse to the plasma membrane. Binding to phosphatidylinositol, which may be restored from rephosphorylation of the reaction product of IP_3 and DAG, somehow break the gelsolin- or profilin-actin complex.

Meanwhile, the DAG may activate C-kinase to reduce the calcium level in cytosol. In addition, once actin is freed from inhibitory action of profilin, molecules can aggregate to form nuclei and the filaments are also free to grow through the addition of newly released actin molecules. On the other hand, DAG may also promote actin nucleation at the plasma membrane in a way that is independent of its effect on protein kinase C (Shariff and Luna, 1992). Filament organization at the leading edge is controlled by actin accessory proteins (Bretscher, 1991) including ABP-280, ABP-120, MARCKS (a myristoylated alanine-rich C kinase substrate), spectrin, -actinin, filament bundling proteins (caldesmon, fimbrin, p30a. and villin), and proteins that stabilize filaments (caldesmon and tropomyosin). Some of these proteins may also help link actin to the membrane. For examples, ABP-280, spectrin, and -actinin bind integral membrane proteins, and dephosphorylated MARCKS binds to an unknown membrane site (Thelen et al., 1991). These reactions proceed at the PIP_2-rich plasma membrane domains where the initial extracellular stimulation begin, therefore keeping the actin assembly site specific (Stossel, 1990).

In summary, the molecular mechanisms by which cell motility is regulated are complex. ECM receptors, particularly the HA receptor RHAMM have been demonstrated to be key in this process. Although the cDNA encoding RHAMM has been recently published (Hardwick et al, 1992), it's genomic structure is unknown. Here, I describe the cloning and characterization by restriction mapping of the mouse gene structure of RHAMM. Enclosed is a paper describing the isoforms generated by this gene that was a direct result of this work.

EXPERIMENTAL METHODS

2.1 Cell lines

NIH 3T3 cell lines were maintained in filter sterilized Dulbecco's Modified Engles Medium (Gibco) with 0.2 M Hepes (Sigma), supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah), in a 37^{0}C, 5% CO_2, humidified atmosphere. When cells reached 80% confluency, they were either passaged by mild trypsin (Gibco) digestion (0.25%) or harvested for DNA extraction.

The 10T1/2 cells (P_8F_6) were the kind gift of Dr. Greenberg (Manitoba Institute of Cell Biology). Cells were thawed and maintained in the same above supplemented medium with addition of 100 U/ml penicillin and 100 ug/ml streptomycin. The cells were passaged every 3 days or frozen stocks of low passage cell lines were maintained. Cell lines were not used past passage 10.

Stocks of cells were maintained as following. After trypsinization, appoximately 5 x 10^4 cells were resupended into one ml DMEM containing 20% fetal bovine serum, 0.02% Hepes and 10% DMSO. Cells were transferred to a freezing vial and frozen in liquid nitrogen.

2.2 Extraction of mammalian genomic DNA

Five 150 mm plates of either 3T3 or 10T1/2 cells were grown to confluence for DNA isolation. The monolayers were rinsed twice with Tris-buffered saline (TBS) and scraped from the growth surface with a rubber policeman. After centrifugation at 1000g for 5 min, the cell pellet was washed once with ice cold TBS. Ten milliliter of extraction buffer (10 mM Tris.HCl [pH 8.0], 0.1 M EDTA [pH 8.0], 20ug/ml pancreatic RNAse, 0.5% SDS) was added for each milliliter of cell suspension in TBS. The methodology outlined in Sambrook et al. (1989) was followed with minor modifications. The DNA was extracted by mixing the cell lysate once with Tris-saturated phenol (pH 8.0), and extracting with the aqueous layer twice with Tris-saturated phenol : chloroform (pH 8.0). Usually, the yield of DNA was 30-40 ug/plate of cells.

2.3 Southern blot analysis

Purified genomic DNA obtained above was digested with restriction enzymes (Eco RI, Bgl II, Bam HI, and Sac I) and appropriate buffers overnight. The digested samples were loaded and electrophoresed on a 0.7% agarose gel containing 0.5 ug/mL ethidium

bromide in 1x TBE at 40 volts for 20 hours. The agarose gel was treated with 0.25 M HCl for ten minute to depurinate the DNA nick the DNA and denature it for transfer to Hybond-N plus membrane filter. The gel was re-checked under the UV light to insure that all DNA was transferred. The membrane was briefly rinsed by immersion in 2X SSPE with gentle agitation. The blots then directly underwent the pre-hybridization, hybridization and washing as described in the later section.

2.4 The 3T3 genomic DNA library

The lambda FIX NIH 3T3 genomic library (Stratagene, California, Los Angelas) was prepared by cloning 9-22 kb fragments isolated from 3T3 genomic DNA partially cut by Sau 3AI enzyme into Not I site of lambda FIX. The library was amplified once to 2 x 10^{10} pfu/ml and was stored as a glycerol stock at -80^{0}C.

2.5 Screening the genomic library

The host bacterium SRB(P2) was inoculated into 50 ml LB medium containing 10 mM $MgSO_4$ and 0.2% maltose. The medium was grown overnight with vigorous shaking at 30^{0}C to ensure that the cells would not overgrow. This overnight culture was then centrifuged at 1000g for 10 min and resuspended into 0.4 original volume of 10 mM $MgSO_4$.

35 µl of a 10^{-4} dilution of genomic library phage was used to infect each 600 ul aliquot of the above bacteria (OD_{600} = 0.5) to yield about 10000 plaques in one 150 mm diameter petri dish. This mixture was gently shaked and incubated for 20 minutes at 37^{0}C to allow the bacteriophage particles to absorb to the bacteria. Then, 6.5 ml melted (50^{0}C) 0.7% NZY agarose was added into each tube and the mixture was gently swirled and immediately poured into prewarmed (37^{0}C) NZY agar plate. Once the top agarose was hardened, the plates were incubated until the plaque size reached to 1 mm diameter or the plaques barely touched one another (about 8 hours). Afterwards, the plates were chilled at 4^{0}C for at least one hour to allow the top agarose to harden that can prevent the top agarose from peeling off easily when removing plaque filter blot (see below).

The number of colonies that were theoretically needed to be screened to obtained a RHAMM positive plaque were calculated as follows. The number of independent clones, N, that must be screened to isolate a particular sequence with probability P is given by the equation below, where I is the size of the average cloned fragment and G is the size of the target genome.

$$*N = \ln (1 - P) / \ln [1 - (I / G)]$$

There is a 99% chance of isolating RHAMM ($I = 2 \times 10^4$ bases, and $G = 3 \times 10^9$ bases), when

$$N = \text{In } (1 - 99) / \text{In } [1 - (2 \times 10^4 / 3 \times 10^9)]$$
$$= 690{,}000 \text{ clones}$$

According to mathmatical calculation, about 69 plates were needed to be done in order to screen the complete genome once.

2.6 Plaque blotting

The Hybond-C membrane filter was placed on the top agarose for 5 min. The membrane position in relation to the plate was marked by stabbing a needle with indian ink through both the membrane and the plate at appropriate positions. With the colony side of membrane facing up, the membrane filter was placed on the 3 MM Whatmann paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min and was neutralized by placing the membrane on the neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM EDTA) for 5 min. This was repeated once using a fresh pad soaked in neutralizing solution for 5 min. The filter was then gently washed in 4X SSPE for about one minute, air-dried, and baked for 2 hours at 80^{0}C oven.

Before the prehybridization, the plaque filter blots were prewashed with solution containing 5X SSPE, 0.5% SDS and 1 mM EDTA at 42^{0}C to wash off agarose residues.

2.7 Labelling of DNA probes

A 1.7 kb RHAMM cDNA (see fig 1) was labelled by the random priming method (Pharmacia). Briefly, about 50 ng of the cDNA was mixed with 50 uCi of ^{32}P labelled dCTP, Klenow fragment, and the appropiate volume of reaction buffer from Pharmica kit. The mixture was left at room temperature for at least 3 hours. The unincorporated nucleotides were separated from labelled probe by passing the solution through a Sephadex-G-50 column equilibated with TE (pH 8.0).

2.8 The prehybridization / hybridization / washing

Both the genomic southern blot and plaque blot filters were treated the same in the following manner. They were incubated at 42^{0}C for at least 6 hours in prehybridization solution (50% Foramide, 6X SSPE, 0.5% Blotto, 0.5% SDS with 200 ug/ml denatured

sheared salmon sperm DNA). At the end of prehybridization, the solution was drained and replaced with a hybridization solution containing 50% Foramide, 4X SSPE, 5% Dextran Sulfate, 0.5% Blotto, 0.5% SDS, 200 ug/ml denatured salmon sperm DNA, and ^{32}P labelled 1.7 kb cDNA with a specific activity of 2 x 10^5 cpm/ml. Hybridization was conducted for at least 12 hours.

The washing conditions for different blots are described in table 3. After the washings listed in the table 3 were conducted, the blots were monitored. If the background signal was still high, another 15 min or longer time of washing with 0.1X SSPE/0.5% SDS solution at 65^{0}C was conducted; if not, the membrane filters were rinsed with 2X SSPE briefly, then wrapped with Saran-wrap. The blots were then exposed to the X-OMAT AR X-ray film for at least one week at -80^{0}C prior to the development.

2.9 Picking plaques and rescreening

The putative positive clones identified from the plaque blots were isolated with a wide pore pasteur pipet and kept in phage SM buffer at 4^{0}C with 3% chloroform, to prevent bacterial contamination. Clones were then successively rescreened until all plaques showing on the filters were positive. This required at least two more times of rescreenings.

2.10 Large scale preparation of bacteriophage

About 10^{10} bacterial cells (20 ml overnight culture in LB medium containing 10 mM $MgSO_4$ and 0.2% maltose) were taken to be infected by 10^8 pfu/ml lambda phage. After 8 hours of incubation, 3% chloroform was added and the mixture was vigorously shaked at 37^{0}C to lyse all the cells. All the chloroform containing bacterial debris were then pipetted out. Large chromosomal DNA and RNA were digested at room temperature by incubating with DNase I and RNase to the final concentration of 1 ug/ml. To promote the dissociation of bacteriophage particles from bacterial debris, solid NaCl was added to the final concentration of 1 M. The resulting medium was kept on ice for at least an hour; then centrifugated at 5000g for 10 min to remove the bacterial debris. The supernatant was then brought up to 10% PEG 8000 to extract phage particles. The pellet was collected by centrifugation at 10,000g for 20 min at 4^{0}C and resupended into the phage (SM) buffer. After extraction with equal volume of chloroform, 0.75g of solid CsCl/ml to the aqueous phase. After the CsCl was dissolved, the phage suspension was transferred to an

ultracentrifuge tube which fitted into a Beckman Ti70 rotor and was centrifuged at 40,000 rpm for 20 hrs at 4^{0}C.

2.11 Extraction of Bacteriophage DNA

The lambda DNA was extracted according to methods described in Sambrook et al. (1989). The virus particles were collected by puncturing the side of the tube using an 18-gauge needle. The elute was then transferred into a cellulose dialysis sac (molecular weight cut off size: 12,000 - 14,000) and dialyzed twice to remove CsCl against buffer containing 10 mM NaCl, 50 mM Tris.Cl (pH 8.0) ,and 10 mM $MgCl_2$ at room temperature for one hour . The phage capsules were digested by incubating the phage suspension in 20 mM EDTA, 50 ug/mL proteinase K, and 0.5% SDS at 56^{0}C for an hour. The phage DNA was then extracted once with equal volume of phenol, phenol:chloroform, and chloroform. The aqueous phase was again transferred into the dialysis sac and dialyzed against 1000-fold volumes of TE (pH 8.0) overnight at 4^{0}C. The purified phage DNA in TE was stored at -20^{0}C.

2.12 Characterization of positive clones

All phage DNAs of positive clones were cut with the restriction enzymes (Sac I, Eco RI, Bam HI, and Bgl II). A triple set of Southern blots was prepared for the digested products of individual clones under the same above conditions (see Southern blot analysis) and separately hybridized to 1.1 kb, 0.6 kb, and 1.2 kb cDNA probes of RHAMM (see fig 2). Finally, before development, the blots were exposed to the X-OMAT AR X-RAY film overnight at room temperature.

2.13 Subcloning

The mouse RHAMM DNA insert was cut out of the lambda FIX vector arms by using the restriction enzyme Sac I. The DNA fragments, which had been isolated and cleaned with GENECLEAN (Biorad), were mixed with similarly digested plasmid Bluescript Sk- vector in 3:1 ratio; and were ligated together by incubating the enzyme T4 DNA ligase overnight at 15^{0}C. The ligation product was then used to transform competent XL1-blue cells by calcium chloride heat shock technique as described in Sambrook et al (1989). Transformants were selected on the LB-ampicillin plates (100 ug/ml) spread with the chromophore X-Gal (250 ug/plate) and the inducer isopropylthio-ß-D-galactoside (0.5 mmol/plate). After overnight incubation at 37^{0}C, white colonies were selected and

transferred onto fresh LB-AMP-X-GAL/IPTG plates for another overnight incubation to ensure that the picked colonies were true recombinants (the true white colonies). The plasmid DNA of the recombinants were then isolated using a minipreparation of plasmid DNA method described in Sambrook et al. (1989); and were verified by agarose gel electrophoresis.

The DNA plasmids of the true subclones were amplified, collected using alkali lysis method as described in Sambrook et al. (1989), and were purified by the PEG 8000 precipitation method.

2.14 Restriction mapping

The DNA was restricted using the appropiate restriction enzyme and its corresponding buffer according to manufacture's instruction. The choice of which enzymes to be used was based on the RHAMM cDNA map. The DNA was restricted with excess enzyme at 37^{0}C for at least 3 hours. The reaction was quenched by boiling at 65^{0}C for 20 min. The restricted products and 1 kb DNA marker (Gibco) were loaded and electrophoresed at 80-100 volts in a mini agarose gel apparatus (Biorad) for 2 hours. The gels were then photographed. Using the 1 kb DNA ladder, a plot of log molecular weight vs distance migrated was used as a calibration to obtain the molecular weights of the fragments of the RHAMM genomic DNA. The deduction of the order of the fragments in most instances was based on the electrophoretic analysis of the presence or absence of common nucleotide sequences between fragments resulting from digestion of a given DNA with one restriction enzyme and those resulting from cleavage with a second restriction enzyme as well as accurate molecular weight determinations of the resulting fragments.

2.15 Co-transfection

The method used for gene transfer was based on a calcium phosphate precipitation technique developed by Gorman et al. (1983). 10T1/2 cells were grown to 40% confluency/60 mm pertri dish for 24 hours prior to transfection. The DNA to be transfected in a calcium phosphate precipitate was prepared in the following manner. 2.76 ug of the phage DNA containing RHAMM and 0.22 ug of vector DNA of pSV2-neo (a gift from Dr. Mowat), a selectable marker of the drug G418 (Geneticin) were mixed (4 : 1 ratio, a total volume of 120 ul) with 120 ul of buffer A from Pharmacia Transfection kit (0.5 M $CaCl_2$ / 0.1 M HEPES / pH 7.5) and incubated for 10 min at room temperature. Then, 240 ul of buffer B (0.28 M NaCl / 0.05 M HEPES / 0.75 mM NaH_2PO_4 / 0.75 mM

Na_2HPO_4 / pH 7.5) was added into the mixture A and immediately vortexed for a few seconds. The precipitate was incubated for 15 min at room temperature. The control was prepared by only using 3 ug of pSV2-neo DNA in calcium phosphate precipitate form to do the transfection.

The calcium phosphate-DNA precipitates were added to cell cultures and incubated at 37^{0}C for 12 hours to allow the DNA precipitate to be taken up by cells. The preciptate that was not taken up was removed by washing twice with normal DMEM medium. 1.5 ml of 15% glycerol in isotonic HEPES buffer (10 mM HEPES / 150 mM NaCl / pH 7.5) was then added and incubated for 2 min to enhance the entry of the calcium phosphate-DNA precipitate. The cells were then rinsed and replaced with fresh supplemented DMEM medium and grown under normal conditions. After 24 hours, the cells were harvested and resupended into five 100 mm plates with complete medium containing the antibiotic Geneticin (G418).

2.16 Selection for G418 resistance in transfected cells

The medium placed on the transfected cells contained G418 at a concentration of 300 ug/ml. The cells were grown in this antibiotic-containing medium for ten days, by which time G418 resistant colonies had appeared. The resulting colonies were trypsinized in cloning cylinders and plated onto 35 mm plates containing DMEM with 300 ug/ml of G418. Fresh G418 containing medium was placed on the cells every 3 to 4 days.

2.17 Lysing of cells for RHAMM Western

Cells were washed twice with ice cold PBS then 0.6 mL of RIPA buffer containg protease inhibitors (0.1 mL inhibitors/2.0 mL buffer) was added to each plate. Cells were vigorously scraped with scraper and the lysate was aliquoted and transferred into eppendorf tubes which were left on ice for ten minutes before centrifugation at 13000 RPM for 15 mins at 4^{0}C. The supernatants were then transferred into new eppendorf tubes and stored at -80^{0}C until use.

2.18 Western blot analysis

Electrophoretic analysis was conducted on 10% SDS-PAGE stacking gel system using the Biorad miniprotein gel apparatus. Ten micrograms of protein of different lysates quantitated by DC protein assay kit from Biorad, were loaded on the gel and were run at

200 volts for an hour at room temperature. Proteins were then transferred to nitrocellulose membranes (Biorad) using a electrophoretic transfer apparatus (Biorad). Before the two-hour-incubation with the rabbit polyclonal antibodies (anti-fusion proteins) aganist RHAMM at room temperature, non-specific binding sites were blocked with 5% defated milk in TTBS (50 mM Tris.HCl [pH 7.4], 200 mM NaCl, 0.05% Tween 20) at room temperature. The blot was washed with TTBS and was then incubated with the secondary antiobody, a goat anti-rabbit (Horse Radish Peroxidase) HRP conjugate (1 in 5000 dilution using 1% milk/TTBS solution), for an hour at room temperature. After washing with TTBS, detection was completed by using ECL kit from Amersham. That is, equal volume of detection reagents 1 and 2 were mixed and immediately poured onto the blot with one minute incubation. Exposure was done immediately at various time points.

Restriction enzymes, which gave only one cut on the cDNA, were firstly chosen to be used to digest the genomic DNA. The orientation was found out by trying fitting different restriction enzyme sites respectively according to the cDNA map. In the case of confusion, the smaller genomic fragments were isolated before another restriction enzyme digest was done upon it. Sometimes, smaller fragments were cut out from cDNA and used as a probe to check the orientation, or to see if the certain genomic fragments from the digest would contain the encoding region.

RESULTS

3.1 SOUTHERN BLOT ANALYSIS

Agarose gels were prepared of 3T3 chromosomal DNA (10 ug) digested to completion with the restriction enzymes Sac I, Bgl ll, Bam HI and Eco RI (fig 2a). DNA was then blotted in a Southern assay on Hybond N^+ membrane and hybridized with the radiolabelled 1.7 kb cDNA of RHAMM (Hardwick et al, 1992).

The 1.7 kb cDNA probe hybridized with an 8.0 kb, 2.5 kb and 2.3 kb of Sac I digested chromosomal DNA (fig 2b). Digestion with Eco RI generated 9.0 kb and 7.6 kb bands. Digestion with Bam HI also generated two bands of 12.0 kb and 7.6 kb. Digestion with Bgl II generated four major bands ranging from 7.6 kb to 1.1 kb. The simple pattern of this Southern blot predicts that the RHAMM gene is a single copy gene.

PCR analysis, using Mt 830-886 of RHAMM cDNA as a primer, resulted in identical size of fragments using all genomic clones. As well, identical size of fragments were obtained from four out of five isolated genomic clones using Mt 1-100 as a primer suggesting that all genomic clones encode a common nucleotide sequence, indicating they are derived from the same gene .

3.2 SCREENING OF 3T3 GENOMIC DNA LIBRARY

The 3T3 genomic library was screened with 1.7 kb RHAMM cDNA (fig 3A). A total of seven plaques were positive. These clones were rescreened at a plaque density of about 300 pfu/80 mm plate. Five out of seven were again confirmed to be true positives by rescreening. They are designated as FIX(1-5). The occurrance of the true positve plaques, against a background of the negative plaques, are illustrated in fig 3B. These five positives were further purified by a third screening at a density of 0.1 pfu/80mm plate. All plaques remained positive (fig 3C).

3.3 CHARACTERIZATION OF POSITIVE CLONES

Positive plaque clones [FIX(1-5)] were amplified in the SRB strain of E. coli as described in "Experimental methods"; and the phage DNAs were separately collected,

extracted and purified (Sambrook et al., 1989).

To determine which clones were distinct and which contained overlapping sequences, several restriction endonucleases were utilized to digest all isolated genomic clones and the products were electrophoresed on 0.7% agarose gels. Restriction of the genomic clones with Sac I enzyme has allowed the construction of a draft genomic map (fig 4). The sizes of five genomic inserts (FIX1-5) are deduced as 19.4 kb, 12.6 kb, 14.5 kb, 11.4 kb and 18.0 kb respectively. A 5.4 kb Sac I fragment was common to clones FIX1, 2, and 4. A 1.2 kb Sac I fragment was common to clones FIX1 and 2 while a 2.3 kb Sac I fragment was common to clones FIX3 and 5. FIX4, which is the smallest insert, may duplicate part of FIX2. The result shows that genomic clones 1, 2, 3, and 5 appear different but 2 and 4 appear to be identical.

Electrophoresis of Sac I digested genomic clones are documented in fig 5. The banding pattern of FIX1 and 2 are different from FIX3 and 5 suggesting that they do not contain overlapping regions.

Further analyses of the genomic clones involved finer mapping of the clones using RHAMM cDNA as a standard. Southern blots using RHAMM cDNA probes (1.1 kb, 0.6 kb, and 1.2 kb) were conducted to determine whether each phage clone contained the complete open reading frame encoded in RHAMM cDNA. The results of Southern blot analyses are illustrated in fig 6.

λFIX1 and 2 hybridized with the 1.1 kb cDNA probe which covers 710 bases of the 5' end of RHAMM cDNA. Hybridization of the 0.6 kb cDNA were weak in both clones. Hybridization of the 1.2 kb cDNA probe, which covers 130 bases of 3' end of RHAMM cDNA, was also weak in λFIX1 and absent in both λFIX2 and 3. λFIX3 shows only signals for both 0.6 kb and 1.2 kb and may contain only the 3'end of RHAMM gene. In contrast, λFIX5 strongly hybridized with all three probes and therefore likely contains the entire RHAMM cDNA.

3.4 SUBCLONING OF THE INSERTED SEQUENCES OF THE BACTERIOPHAGE

The 18 kb clone (λFIX 5) appeared to encode the entire RHAMM cDNA and was therefore further characterized. This clone was restricted into smaller segments with Sac I then subcloned into XL1-blue plasmid for further analysis. Four resulting fragments of 8.0 kb, 4.6 kb, 3.0kb and 2.3 kb were then generated and were subcloned into XL1-blue plasmid by excision from the bacteriophage with Sac I and ligation to Sac I treated

XL1-Blue plasmid DNA.

3.5 ENDONUCLEASE CLEAVAGE MAP OF RHAMM

A pattern of restriction enzyme digestion, as predicted by the RHAMM cDNA, was initially used. The orientation of the clone was deduced from the restriction sites of the RHAMM cDNA restriction map. To aid in this interpretation, the genomic restriction fragments were isolated from agarose gels and further digested. As an additional apporach, different fragments of RHAMM cDNA were utilized as probes. Sizes of digested DNA fragments were determined from a standard curve of DNA ladder (see "Experimental methods").

A restriction map of the λFIX5 is shown in fig 7. Restriction and gel electrophoresis analyses (not shown) indicate that this clone is 17.9 kb in size and that the RHAMM gene is contained within Sac I fragments and occupies about 10 kb of genomic DNA (fig 7). The 5' end of the gene was located by the occurence of Sac I and Eco RI restriction sites whereas the 3' end of the gene was located by the occurence of Sac I and Acc I restriction sites. A B2 repeat site corresponding to such a site in the 3' non-coding region of the RHAMM cDNA was located at the 3' end of the genomic clone within two Acc I fragments.

3.6 EXPRESSION OF THE FIX 5 GENOMIC CLONE (18 KB)

Since λFIX 5 (18 kb) appears to encode the entire RHAMM cDNA, 10T1/2 cells were chosen for transfection studies because in confluent state 10T1/2 cells are contact-inhibited and their RHAMM expression is down-regulated. In addition, the rate of spontaneous transformation of 10T1/2 is very low. Furthermore, transfection of the cDNA is non-transforming (Curpen, thesis).

The 18 kb genomic clone was transfected together with pSV2-neo. Cells that contained functional altered pSV2-neo or altered pSV2-neo that integrated at a site where they were under control of a cellular transcriptional apparatus will survive through the selective medium contaning G418. G418 resistant colonies of 10T1/2 were picked (see "Experimental methods"). Transfected clones that were grown to confluence exhibited a very different morphology from control cells (see below).

3.7 EXPRESSION OF RHAMM IN TRANSFECTANTS

To determine if the G418-resistant transfectants show an increased expression of RHAMM protein, confluent cultures of control and transfected cells were lysed with RIPA buffer. The lysates were electrophorsed in SDS polyacrylamide gels and analyzed with Western immunoblot assay probing against an anti-RHAMM fusion protein. It is evident that several transfected clones showed higher expression of RHAMM (see fig 8) in comparsion to the endogenous RHAMM protein levels of the parental 10T1/2 cells and vector control. A quantitative measure of RHAMM was obtained with densitometric scanning of Western blots, showing that transfected clones have about one to four fold of increased expression (see fig 9).

FACS analysis also shows an increased of RHAMM staining among most of the transfectants (data not shown). Northern analysis of CW12, control and parental 10T1/2 cell lines confirmed an increase in RHAMM by showing elevated levels of RHAMM mRNA (data not shown).

3.8 GENOMIC DNA ANALYSIS OF TRANSFECTANTS

That RHAMM overexpression resulted from the introduction of RHAMM genomic clone was confirmed in several ways. Firstly, genomic DNA from individual transfectant clones (Control, CW2, CW6, CW7, CW8, CW10, CW11, CW12) and 10T1/2 cells were analyzed by the Southern blot method. Integration of foreign DNA is random with respect to the site of integration. This integration of DNA may therefore contribute to an alteration of banding patterns in the Southern analyses. Thus, it is expected that the presence of exogenous RHAMM genomic sequences should result in the presence of additional DNA fragments with hybridization signals or/and increase of hybridization intensity among orignal hybridized DNA fragments.

Total genomic DNA was extracted from both parental and transfected cells as described in "Experimental methods". 15 ug of DNA was restricted with Sac I to completion and analyzed by hybridization with labelled 1.7 kb cDNA (see fig 10). The Southern blot studies suggested an increase in copy number of RHAMM gene occurred in some of transfected cells (i.e. clone 12) even though bands detected by the Southern blot among transfected clones were the same as those of the control cell lines. An appearance of additional bands in clone 6 and 8 also suggested the insertion of a RHAMM gene.

As an alternative, more sensitive additional approach, the transfected clone 12 was probed with the viral arms of λFIX. Clone 12 was shown to hybridize with viral arms indicating the insertion of the RHAMM gene in clone 12 (data not shown).

3.9 RHAMM GENE OVEREXPRESSION RESULTS IN ALTERAION OF MORPHOLOGY

Transfection of the RHAMM genomic clone resulted in a morphological change from flat and contact inhibited (characteristics typical of 10T1/2 fibroblasts) to spindle shaped and non-contact inhibited, typical of transformed phenotype. The vector control fibroblasts retained their resemblance to untreated 10T1/2 cells (fig 11). Loss of contact inhibition was confirmed by a focus formation assay(data not shown). Furthermore, clone 12 was shown to be tumorigenic while the RHAMM cDNA clone was not, suggesting that the gene encodes additiional sequences to those encoded in the RHAMM cDNA.

DISCUSSION AND CONCLUSIONS

Increased production of HA has been associated with cell motility in morphogenesis of many tissues (Copp and Bernfield, 1988 a and b; Toole et al., 1984; Toole et al., 1989), during wound repair (Toole et al., 1984; Weigel et al., 1986) and tumor invasion (Iozzo, 1985; Pauli et al., 1983; Toole, 1982; Turley, 1984). It is likely that HA mediates cell motility via interaction with its specific cell surface receptors that induce signalling events such as tyrosine phosphorylation which promotes alterations of cytoskeleton and cell attachment sites and indeed both HA receptors of CD44 and RHAMM are linked to cell motility. Focal adhesions, also functioning as signal transduction units, appear to be an essential target of HA signal reception. Rapid assembly and disassmbly of vinculin in focal adhesions after HA addition have been documented (Hall et al, 1994).

RHAMM has been shown to be colocalized with actin in migratory chick heart fibroblasts (Turley et al., 1991) and to promote locomotion in H-ras transformed fibroblasts (Hardwick et al., 1992; Turley et al., 1991), malignant B cells (Turley et al., 1993) and other cell types as well (unpublished data). The RHAMM cDNA does not encode a signal sequence and how it is transported to the cell surface remains elusive. The functions and regulations of its multiple isoforms (Entwistle et al, 1994) existing in relation to locomotion are also unknown. We have obtained recent evidence that isoforms are generated by alternative splicing of the primary transcript of a single gene. Results presented here are consistent with the occurence of a single gene (see below).

Interestingly, transformed cells (H-ras transformed cells and malignant hairy B cells) express increased levels of RHAMM, particularly at the cell surface. In this regard, it is interesting that TGF-ß increases both HA and RHAMM expression (Toole et al., 1989; Heldin et al., 1989; Samuel et al., 1993). TGF-ß is a multifunctional regulator of mesenchymal, endothelial and epithelial cells (Roberts ans Sporn, 1989; Barnard et al., 1990; Massagrue, 1990; Roberts et al., 1990). Its action is tighly regulated through transcriptional control. It has been proposed that tumors may use TGF-ß in an autocrine manner which influences the invasion of tumor cells through the ability of TGF-ß to enhance cell motility (Postlethwaite et al., 1987; Wahl et al., 1987) and regulate protease activity (reviewed in Massague, 1990). The ability of TGF-ß to enhance cell motility requires increased expression of HA and RHAMM (Samuel et al., 1993). The above results predict that RHAMM will be a critical molecule in events requiring cell motility.
Isolation of the RHAMM gene is essential to discuss the structural differences of RHAMM

isoforms and to assess it's regulatory mechanisms. Assuming that all isoforms are expressed by one gene, isolation of the genomic clone will also allow overexpression transgenic studies to probe the functions of each RHAMM isoform.

The simple pattern of 3T3 genomic Southern analysis is consistent with the occurrence of one gene for RHAMM (fig 1). The hybridization patterns of 3T3 genomic DNA digested with both Eco RI and Sac I are closely matched with Southern patterns of FIX5 genomic DNA digested with Eco RI and Sac I hybridized with the same probe. In PCR studies, it was also found that an identical size of segments were generated from all five genomic clones (data not shown), illustrating that all clones contained a common sequence, again consistent with the occurence of one gene for RHAMM.

Apparently the RHAMM gene also exists for CD44. The size of the CD44 gene is 50 kb long, which is relatively too big for a gene encoding a protein size of 85 kDa (Screaton et al., 1992). It contains as much as 12 alternatively spliced exons, contributing to various molecular forms of CD44 existing in many cell lines.

4.1 λFIX5 CONTAINS ONE COMPLETE GENE OF RHAMM

Southern analysis utilizing the RHAMM cDNA as a Standard, indicates that FIX5 encodes the complete cDNA of RHAMM. Overexpression of genomic clone 5 increased all forms of RHAMM expression (fig 8), suggesting that all forms of RHAMM were encoded in one gene. When 1.7 kb of RHAMM cDNA is used as a probe, the hybridization pattern showing two hybridized bands of 8.0 kb and 2.3 kb is most closely matched to the hybridization pattern of 3T3 genomic Southern (fig 1). The restriction map of FIX5 (fig 7) is also matched with the restriction enzyme map of the RHAMM cDNA and even exhibits a B2 repeat within Acc I restricted fragments found in cDNA RHAMM. FIX5 has been partly sequenced and it is confirmed that it includes both start and stop sites of cDNA (data not shown). All of the above evidence supports that FIX5 encodes the known sequences of RHAMM predicted by it's cDNA.

FACS analysis indicates increased expression at the cell surface and Northern blot analysis confirms an increase in RHAMM translational machinery. Further, the overexpression of RHAMM coincides with the increased motility and altered morphology among the transfected clones (see below).

4.2 RHAMM MEDIATES LOCOMOTION AND ALTERED MORPHOLOGY OF CELLS

CW12 showed almost a double rate of motility and a three to four fold increase of HA-stimulated motility (data not shown). CW12 has been injected into mice to see if tumors would be formed. CW12 was found to be tumorgeneic.

From co-transfection studies, RHAMM appears to be important in regulating cell contact behaviour as well. The increased expression of RHAMM coincides with the morphologic transformation and with loss of contact inhibition as documentated by foci formation (data not shown). This event is not the result of spontaneous transformation since several clones exhibited the same effect. The effect was related to the level of RHAMM expressed and vector controls did not transform.How RHAMM regulates contact behaviour is not understood.Further, since the RHAMM cDNA is not transforming when overexpressed, it is predicted that the RHAMM gene represented by Fix 5 contains an additional sequence that confers transforming capability.

The selection for transfectants relies on cell killing by an aminoglycoside antibiotic, G418 which interferes with the function of 80S ribosomes and blocks protein synthesis in eukaryotic cells (Davies and Jiminez, 1980). The response time for cell killing appears to correlate with growth rate, since the most rapidly growing cells are killed in the shortest intervals. Even though at lower concentrations of G418 (100 ug/ml) there is a significant delay, the cells are killed eventually (Southern and Berg, 1982). Studies show that most of tested cell lines can be killed within a week at concentration of 400 ug/ml and at high cell density, cells may require longer time (10-14 days) before the cell killing can be observed (Southern and Berg, 1982). Studies show that transfection of a wide variety of mammalian cell lines with pSV-neo yields stable transfectants that are resistant to G418 at a frequency of one transfectants per 10^4-10^5 cells. The efficiency is subjected to change for co-transfection (Southern and Berg, 1982).

In the co-transfection studies discussed here, a small number of transfected clones (seven) were collected and examined and the transfection frequency was relatively low. This low frequency could have been due to the addition of antibiotic G418 for selection after 24 hrs of transfection (see "Experimental methods") which might not permit cell growth for the expression of neo gene. It has been reported that a significant reduction in the transfection frequency occurred if G418 was added before 48 hrs (Southern and Berg, 1982).

After transferring a vector into a recipient cell, a vector molecule may undergo

end-modification, and fragmentation as well as recombination with other vector molecules and host molcules before integration (Brouillette and Chartrand, 1987; Wake et al., 1985; Anderson et al., 1982). Both homologous and nonhomologous recombination can occur between transfected vector molecules and between vector molecules and host DNA. Most of this recombination has been shown to be nonhomologous (Brouillette and Chartrand, 1987; Roth et al., 1985). Additional DNA fragments were detected by cDNA probe in two transfectants (fig 10), and these possibly derived from such nonhomologous recombination events.

The DNA transfected by the calcium phosphate technique will traverse the cytoplasm and the nucleoplasm where it is subject to attack from nucleases and to recombinational events with filler or cDNA. Specifically, in this co-transfection studies carrier DNA was not used. Carrier DNA acts to protect the transfecting DNA from cellular attack simply by the fact that carrier DNA is in excess and therefore takes the brunt of the attack from nucleases. Without the carrier DNA there is no shield except for the calcium-phosphate complex. Since no carrier DNA was used, it is more likely that the eventual integrants would have been subject to more enzymatic attack than would DNA transfected along with carrier DNA. This might contribute to underestimated copies of DNA integrating into genome. The promotion of RHAMM expression in the transfected cells suggests that λFIX 5 clone also contains promoter elements.

4.4 CONCLUSION

The full length genomic clone of RHAMM has been isolated. The overexpression of RHAMM results in an altered morphology, increased motility and transformation. These results support a role for RHAMM in cell motility; and transformation, as well as suggest a role for RHAMM in contact behavior. In the future, further work such as CAT assay and sequencing will be conducted to identify regulating elements within the RHAMM receptor. The results of this thesis have been incorporated into a paper describing the structure of the RHAMM gene that accompanies this thesis in Appendix I.

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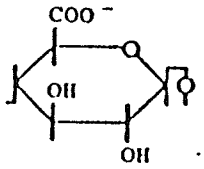
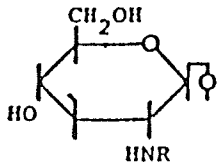
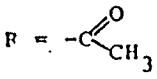
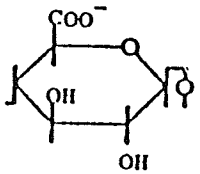
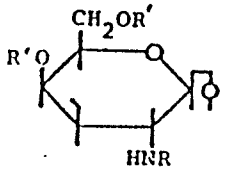
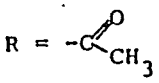
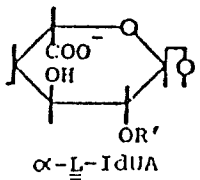
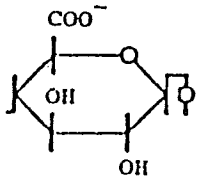
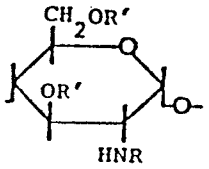
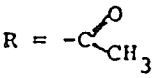
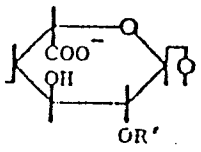
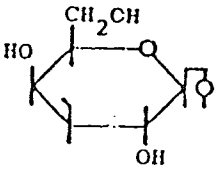
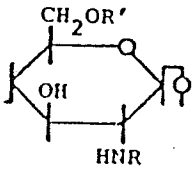
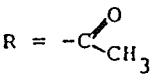
Table 1: Peptide motifs involved in ECM-stimulated motility

Matrix Molecule	Peptide Motif
Fibronectin	RGDS
Laminin	YIGSRT IKVAV LGTIPG
Collagen	GP GPA GVKGDKGNPGWPGAP
Elastin	VGVAPG AGVPGPGVG GPGVGAGVP
Thrombospondin	Unknown
Vitronectin	Unknown

Table 2 Structure of glycosaminoglycans

Polysaccharide	Monosaccharide units* A	B	Substituents
Hyaluronate	 β-D-GlcUA	 β-D-GlcN	R = 
Chondroitin sulfates Dermatan sulfate	 β-D-GlcUA	 β-D-GalN	R =  R′= -H or $-SO_3^-$
	 α-L-IdUA		
Heparan sulfate and Heparin	 β-D-GlcUA	 α-D-GlcN	R =  or $-SO_3^-$ R′= -H or $-SO_3$
	 α-L-IdUA		
Keratan sulfate	 β-D-Gal	 β-D-GlcN	R =  R′= -H or $-SO_3$

*The polysaccharides are depicted as linear polymers of alternating A and B monosaccharide units. Abbreviations: GlcUA, glucuronic acid; IdUA, iduronic acid; GlcN, galactosamine; Gal, galactose.

Table 3: Post-hybridization washes of Hybond N+ and Hybond C

Hybond N±

	Solution	Temp (°C)	Time (minutes)
1.	2x SSPE/0.1% SDS	25	briefly
2.	2x SSPE/0.1% SDS	25	15 with shaking
3.	2x SSPE/0.1% SDS	25	15 with shaking
4.	1x SSPE/0.5% SDS	65	15 with shaking
5.	0.1x SSPE/0.5% SDS	65	15 with shaking

Hybond C

	Solution	Temp (°C)	Time (minutes)
1.	2x SSPE/0.1% SDS	25	briefly
2.	2x SSPE/0.1% SDS	25	15 with shaking
3.	2x SSPE/0.1% SDS	25	15 with shaking
4.	0.1x SSPE/0.5% SDS	42	15 with shaking

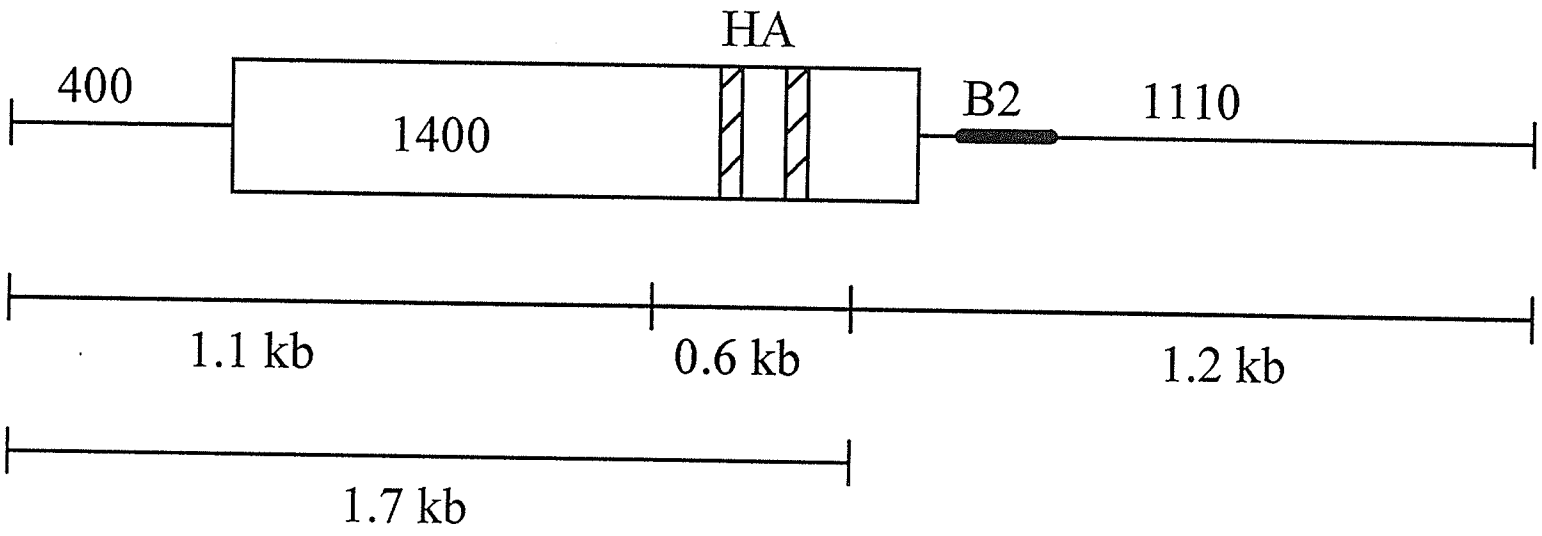
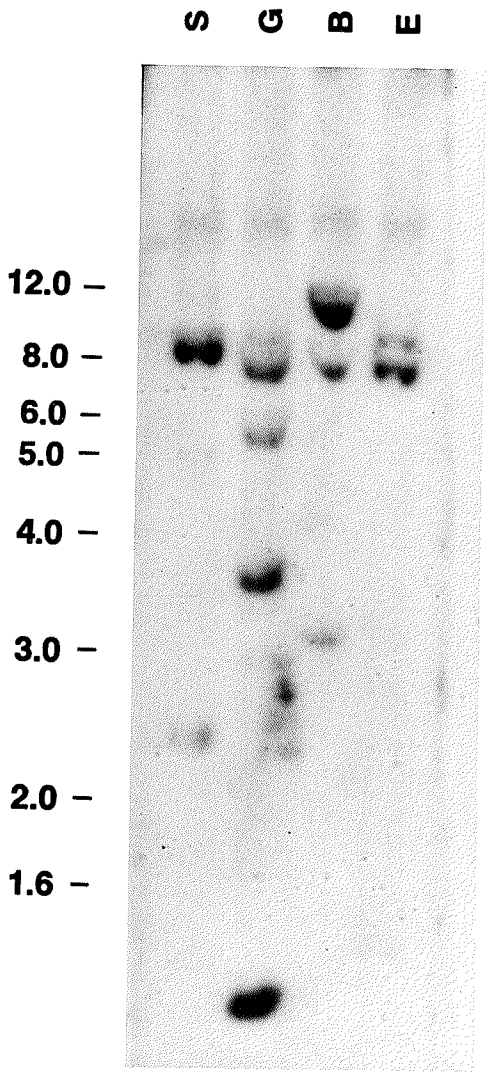


Fig **1** The cDNA map of RHAMM. Two distinct HA binding domains are indicated in ▨ areas.

Figure 2: Agarose gel and Southern Blot Analysis of 3T3 Genomic DNA

3T3 genomic DNA was extracted as described in "Experimental Methods". 10 μg of DNA was digested to completion with the restriction enzyme *Sac*I (S), *Bgl*II (G), *Bam*HI (B) and *Eco*RI (E). The digested DNA was electrophoresed on 0.7% agarose gel overnight, Southern blotted onto Hybond N+ and hybridized with the random primer labelled 1.7 kb (1-1300 bases) of RHAMM. Both hybridization and washing were performed at high stringency. Molecular size are as indicated x 10^3 kilobases.


S
G
B
E
12.0
8.0
6.0
5.0
4.0
3.0
2.0
1.6

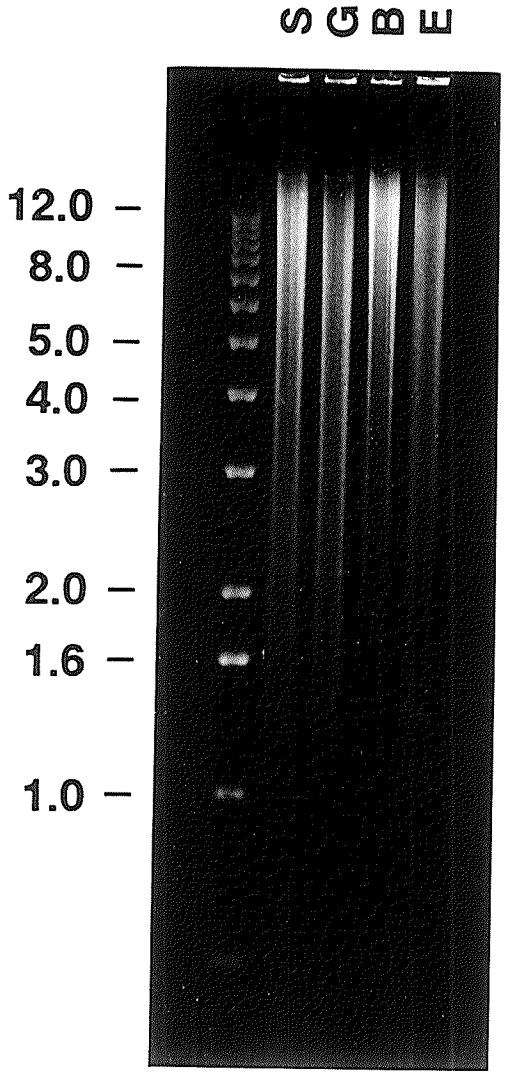

S
G
B
E
12.0
8.0
5.0
4.0
3.0
2.0
1.6
1.0

Figure 3: Genomic DNA Library Screening

The commercial 3T3 NIH mouse genomic library was plated and screened with ^{32}P-labelled 1.7 kb cDNA of RHAMM by methods stated in "Experimental Methods". A putative positive clone from primary screening was confirmed by secondary screening at a lower density of plaque forming units per plate and was finally purified by tertiary screening. A: primary screening showing one positive clone; **B:** secondary screening showing true positives against the negative background; **C:** tertiary screening showing all positives.

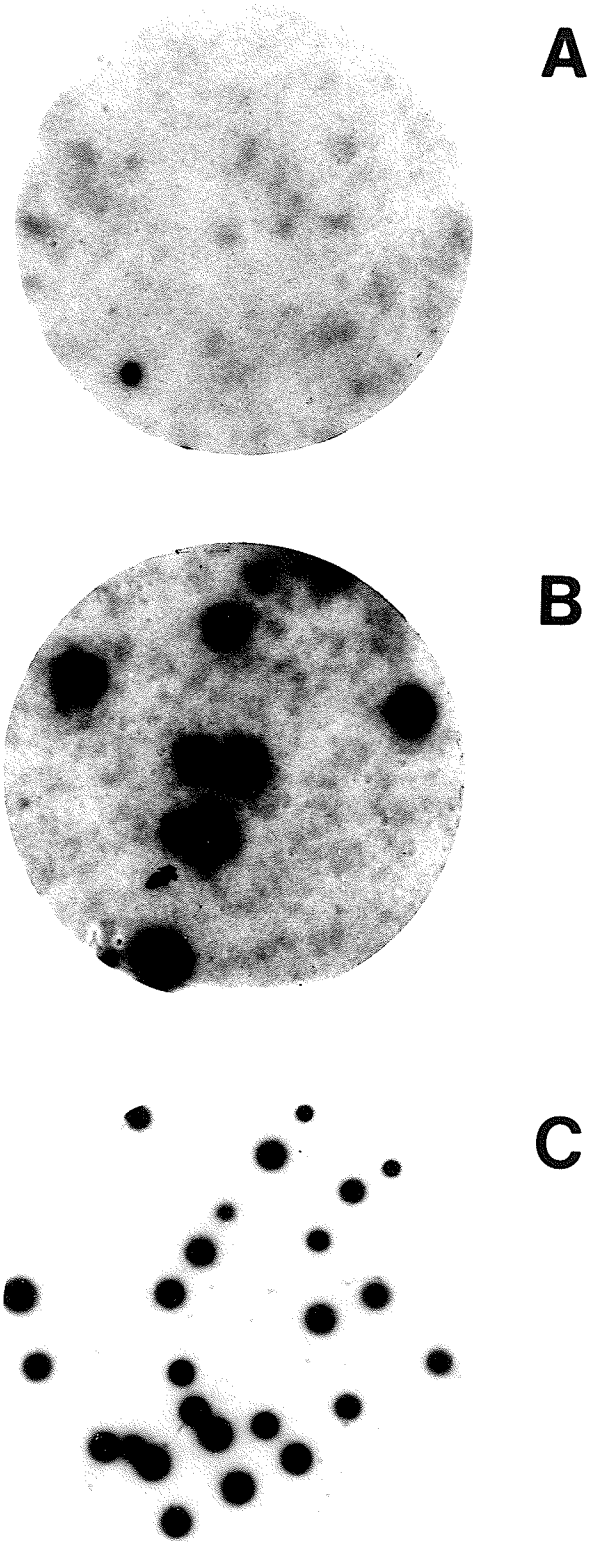

A
B
C

Figure 4: Restriction Mapping of Genomic DNA Inserts

Draft restriction maps of λFIX 1-5 and how they are related to each other as illustrated. It shows that λFIX1 and 2 have an overlapped region of 1.2 kb and 5.4 kb. λFIX 2 and 4 have overlapped regions of 5.2 kb and 6.0 kb. λFIX 3 and 5 have a common region of 2.3 kb.

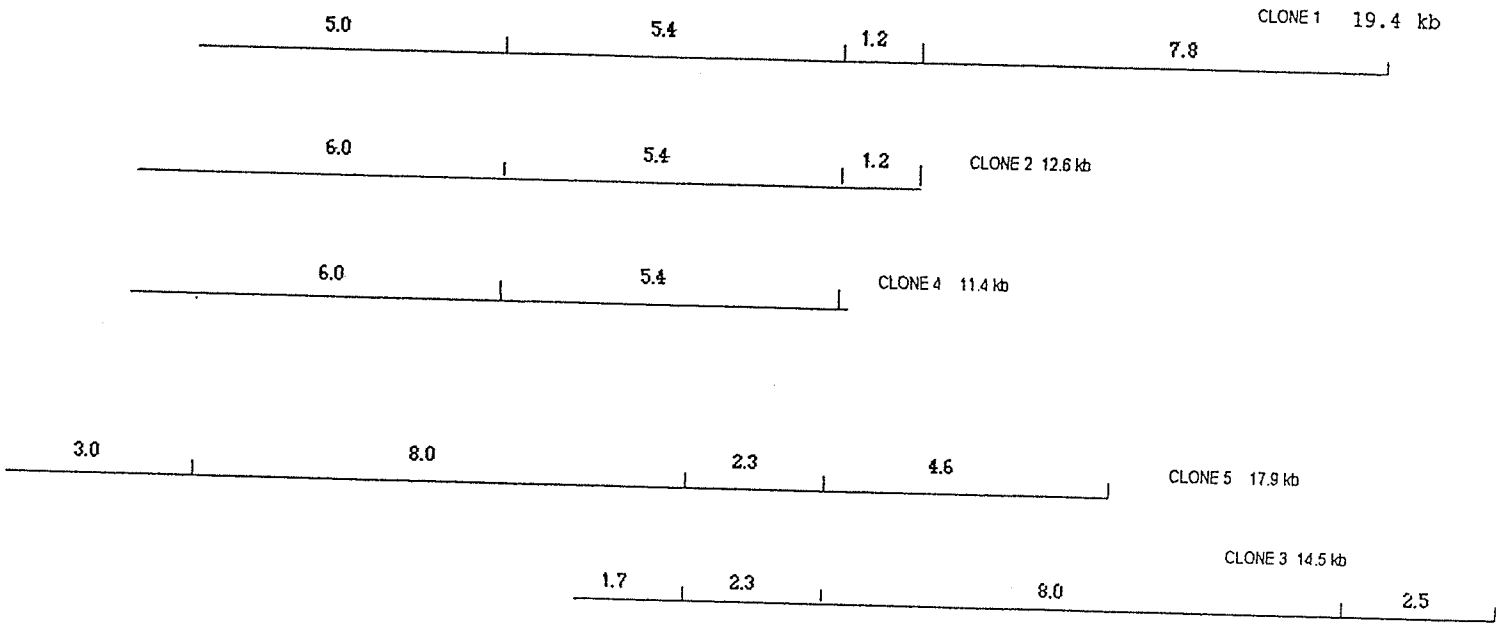

5.0
5.4
1.2
7.8
CLONE 1 19.4 kb
6.0
5.4
1.2
CLONE 2 12.6 kb
6.0
5.4
CLONE 4 11.4 kb
3.0
8.0
2.3
4.6
CLONE 5 17.9 kb
CLONE 3 14.5 kb
1.7
2.3
8.0
2.5

Figure 5: Agarose Gel of Genomic DNA Inserts

Genomic inserts of λFIX 1, 2, 3 and 5 were digested with *Sac*I enzyme and electrophoresed in a 0.7% agarose gel. The results show different patterns of restriction digested products. It also demonstrates that λFIX 1 and 2 are not related to λFIX 3 and 5 since there is no common region between these two sets of clones.

Lane 1, 5, 9	λFIX 1
Lane 2, 6, 10	λFIX 2
Lane 3, 7, 11	λFIX 3
Lane 4, 8, 12	λFIX 4
Lane 5, 9, 13	λFIX 5

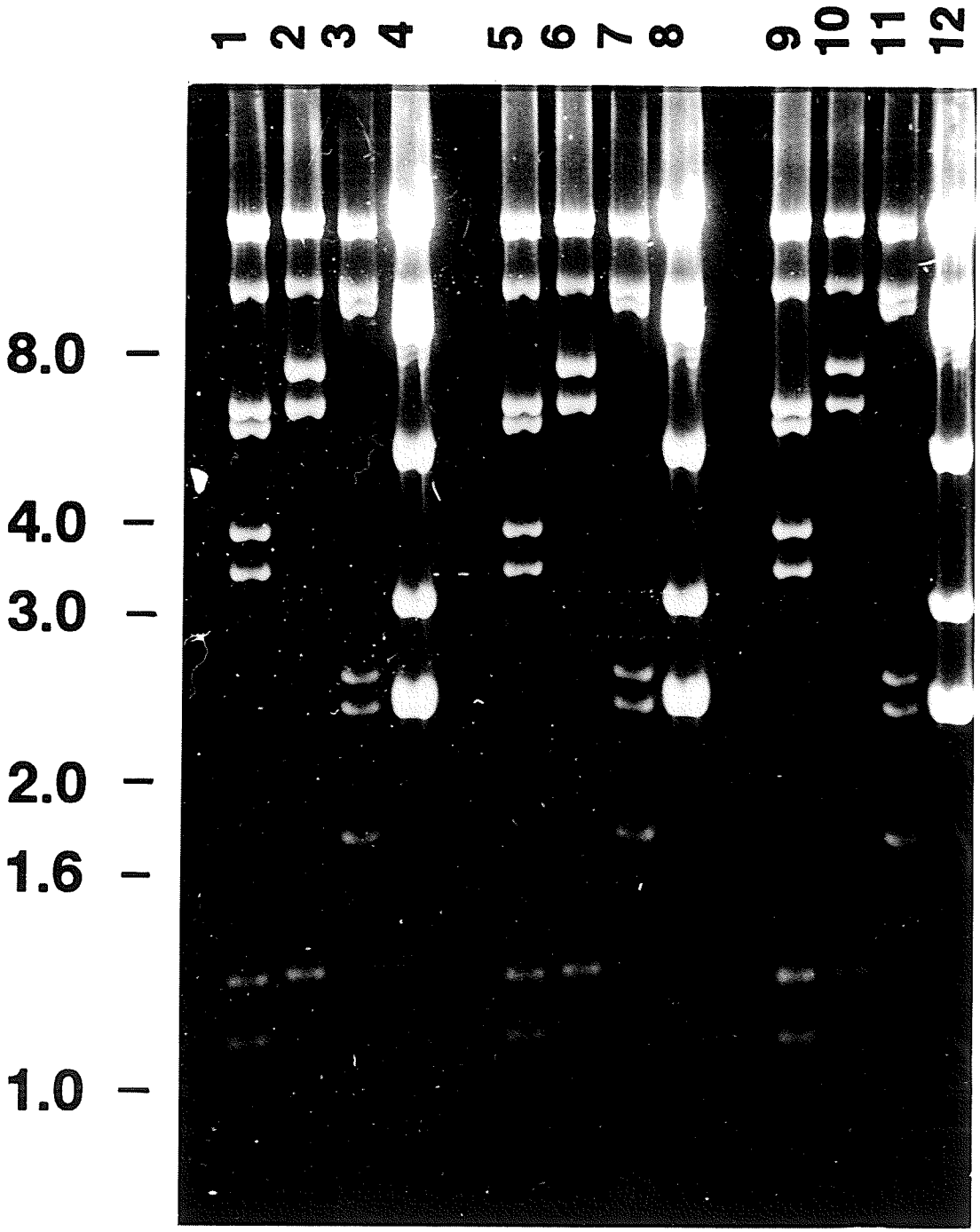

1 2 3 4 5 6 7 8 9 10 11 12
8.0
4.0
3.0
2.0
1.6
1.0

Figure 6: Southern Blot Analysis of Genomic Clones

The *Sac*I digested genomic DNA inserts were Southern blotted and hybridized to various radiolabelled regions of a complete RHAMM cDNA.

A: a blot probed with 1.1 kb cDNA (encodes 1-710 bases of the 5' end of RHAMM) showing that λFIX 1, 2 and 5 hybridized strongly to 1.1 kb cDNA

B: a blot probed with 0.6 kb showing that all four lambda clones containing 0.6 kb cDNA (encodes 710-1300 bases of RHAMM) at various degrees

C: a blot probed with 1.2 kb (encodes 1300-1430 bases of RHAMM) showing that λFIX 1, 3 and 5 contain 5' end of RHAMM cDNA

Molecular sizes are as indicated x 10^3 kilobases

Lane 1, 5, 9	λFIX 1
Lane 2, 6, 10	λFIX 2
Lane 3, 7, 11	λFIX 3
Lane 4, 8, 12	λFIX 5

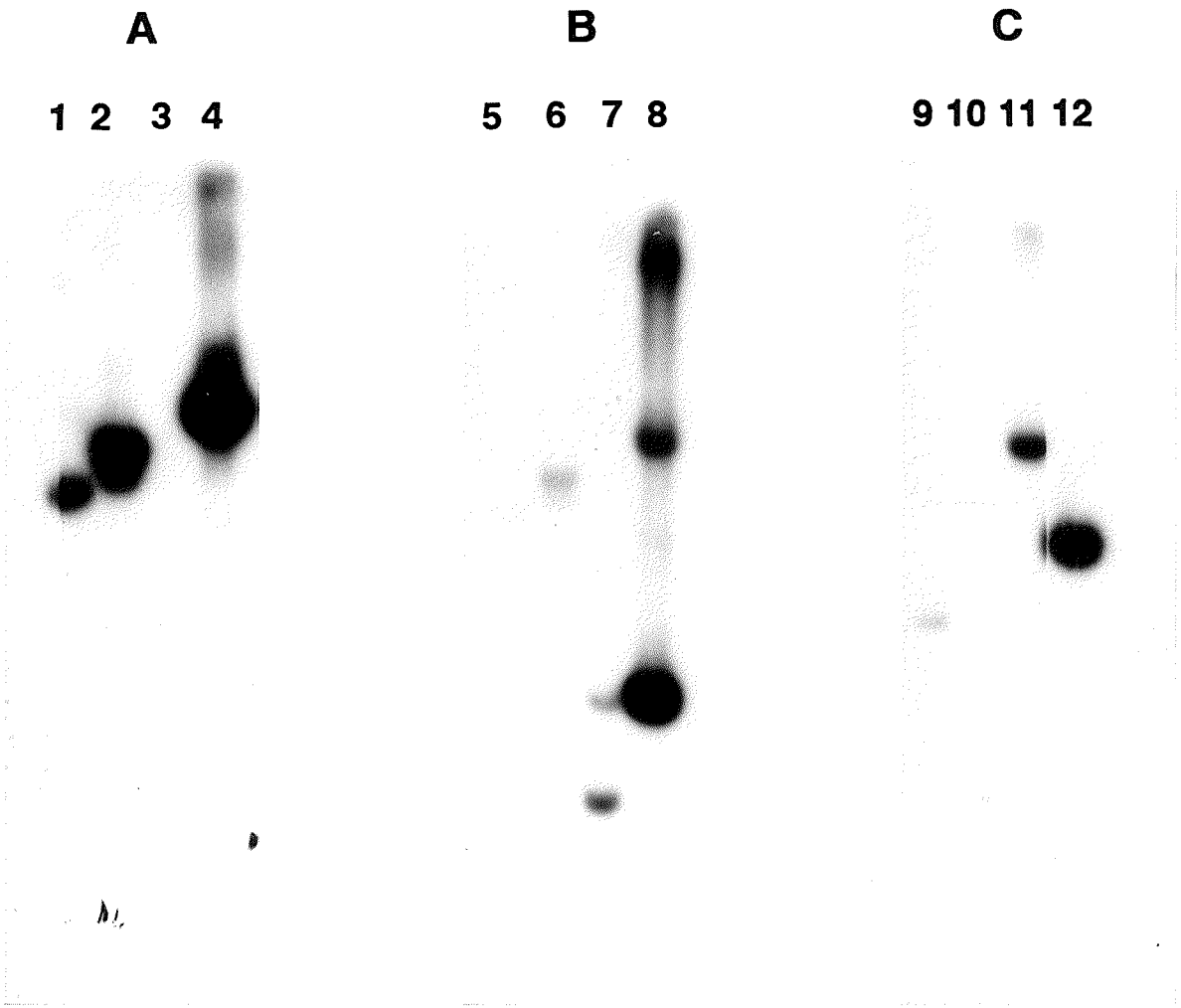

A
1 2 3 4
B
5 6 7 8
C
9 10 11 12

Figure 7: Endonuclease Cleavage Map of Genomic RHAMM Clone (λFIX 5)

A detailed restriction map delineates an alignment between genomic RHAMM clone and the cDNA of RHAMM. The 18.0 kb insert present in the λFIX vector. A, B, C and D: the four *Sac*I fragments of the insert, were subcloned into XL1-Blue. Open box represents the open reading frame of RHAMM cDNA. Restriction enzymes are abbreviated as follows:

*Acc*I (C), *Afl*II (A), *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Nco*I (N), *BstE* II (T), *Afl* III (F) and *Sac*I (S).

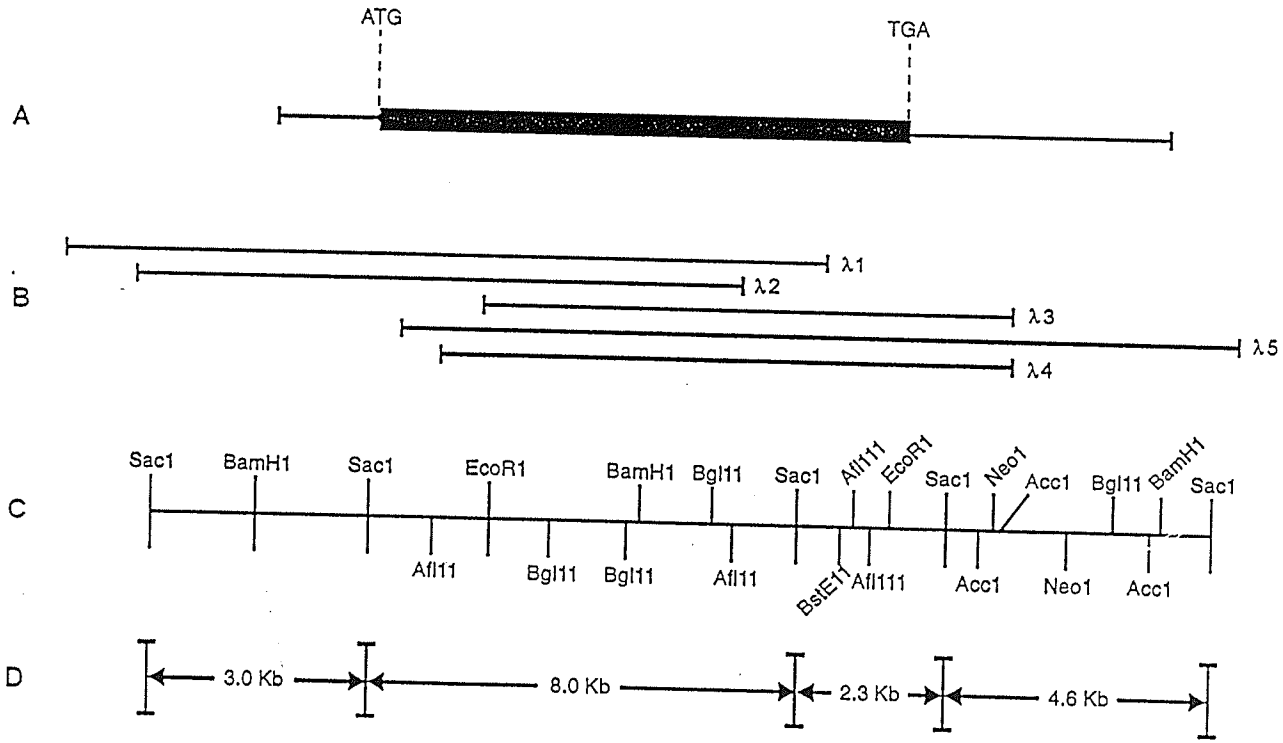

A
ATG
TGA
B
λ1
λ2
λ3
λ5
λ4
C
Sac1
BamH1
Sac1
EcoR1
BamH1
Bgl11
Sac1
Afl111
EcoR1
Sac1
Neo1
Acc1
Bgl11
BamH1
Sac1
Afl11
Bgl11
Bgl11
Afl11
BstE11
Afl111
Acc1
Neo1
Acc1
D
3.0 Kb
8.0 Kb
2.3 Kb
4.6 Kb

Figure 8: Western Blot Analysis of Parental and Transfected Cell Lysates, Probed with Anti-RHAMM Fusion Protein

Cell lysates of both normal and transfected cell lines were analyzed using Western blots with polyclonal antibody to the RHAMM fusion protein (1:1000 dilution) as a probe. Increased expression of the 58 kDa RHAMM is indicated by an arrow. Molecular weights are as indicated x 10^3 daltons.

Lane 1	10T1/2 (parent cell line)
Lane 2	control vector (10T1/2 + pSV2-neo)
Lane 3	CW2 (co-transfected cell line)
Lane 4	CW 6
Lane 5	CW 7
Lane 6	CW 8
Lane 7	CW 10
Lane 8	CW 11
Lane 9	CW 12

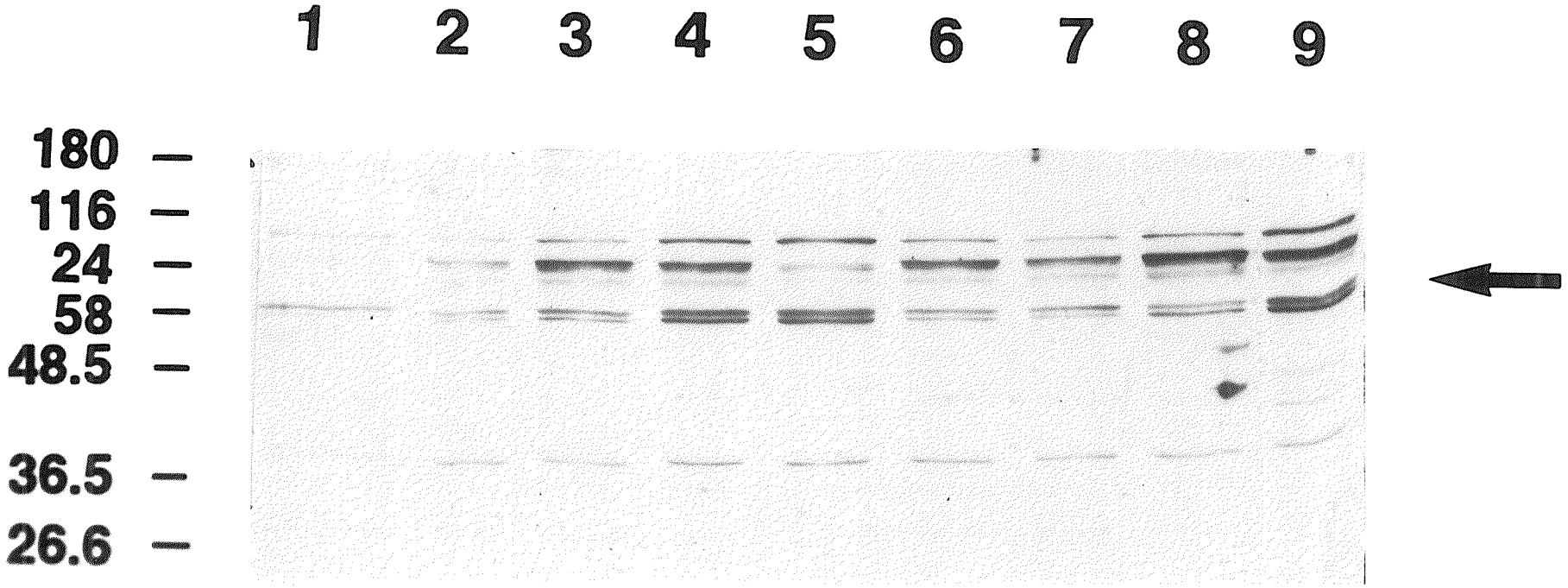

1
2
3
4
5
6
7
8
9
180
116
24
58
48.5
36.5
26.6

Fig.9 A quantitative measure of RHAMM by densitometric scanning

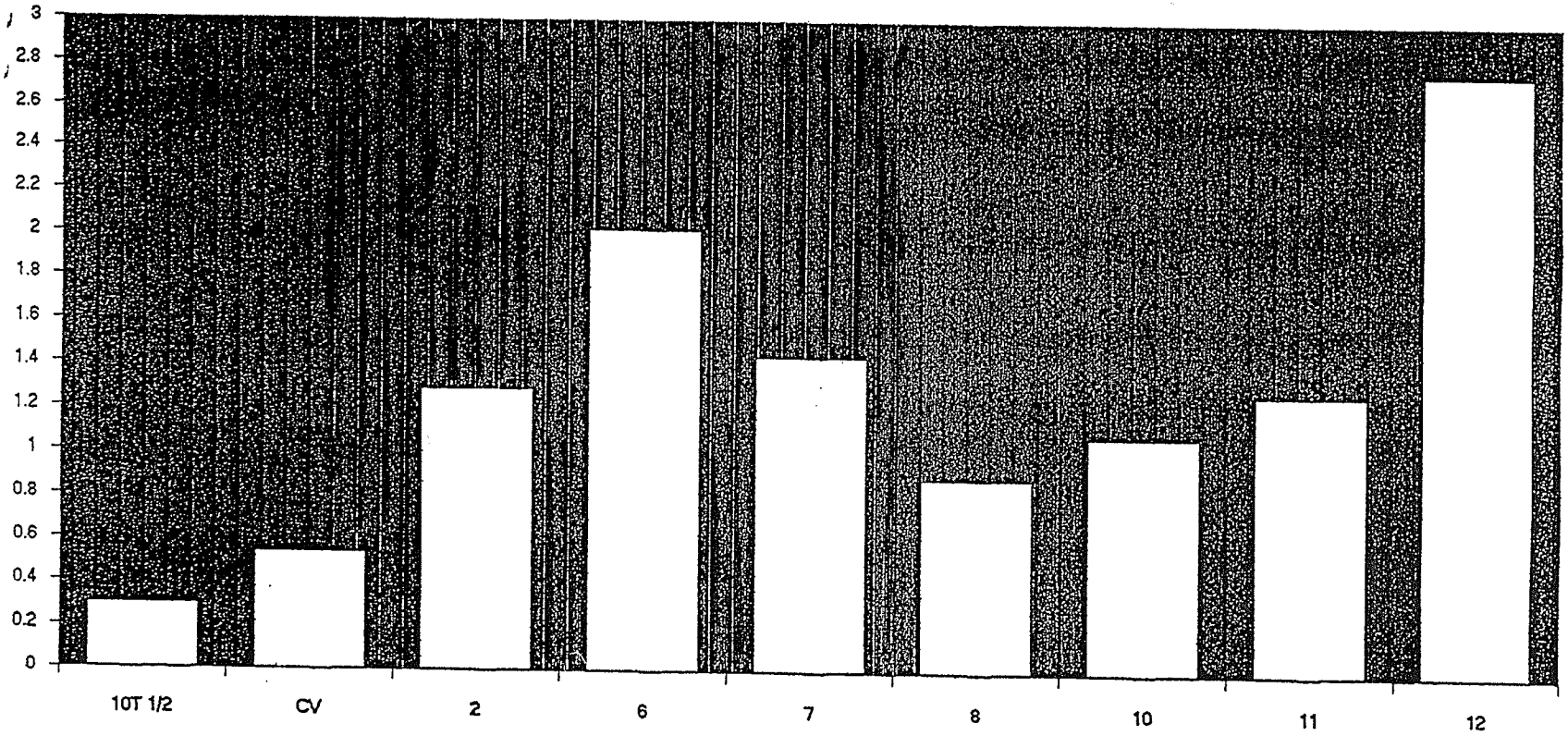


Figure 10: Southern Blot Analysis of Parental and Transfected Cell Lysates, Probed with 1.7 kb cDNA of RHAMM

The extra bands are indicated by the arrows. Both hybridization and washings were performed at high stringency conditions. Molecular sizes are as indicated x 10^3 bases.

Lane 1	10T1/2 (parent cell line)
Lane 2	control vector (10T1/2 + pSV2-neo)
Lane 3	CW2 (co-transfected cell line)
Lane 4	CW 6
Lane 5	CW 7
Lane 6	CW 8
Lane 7	CW 10
Lane 8	CW 11
Lane 9	CW 12

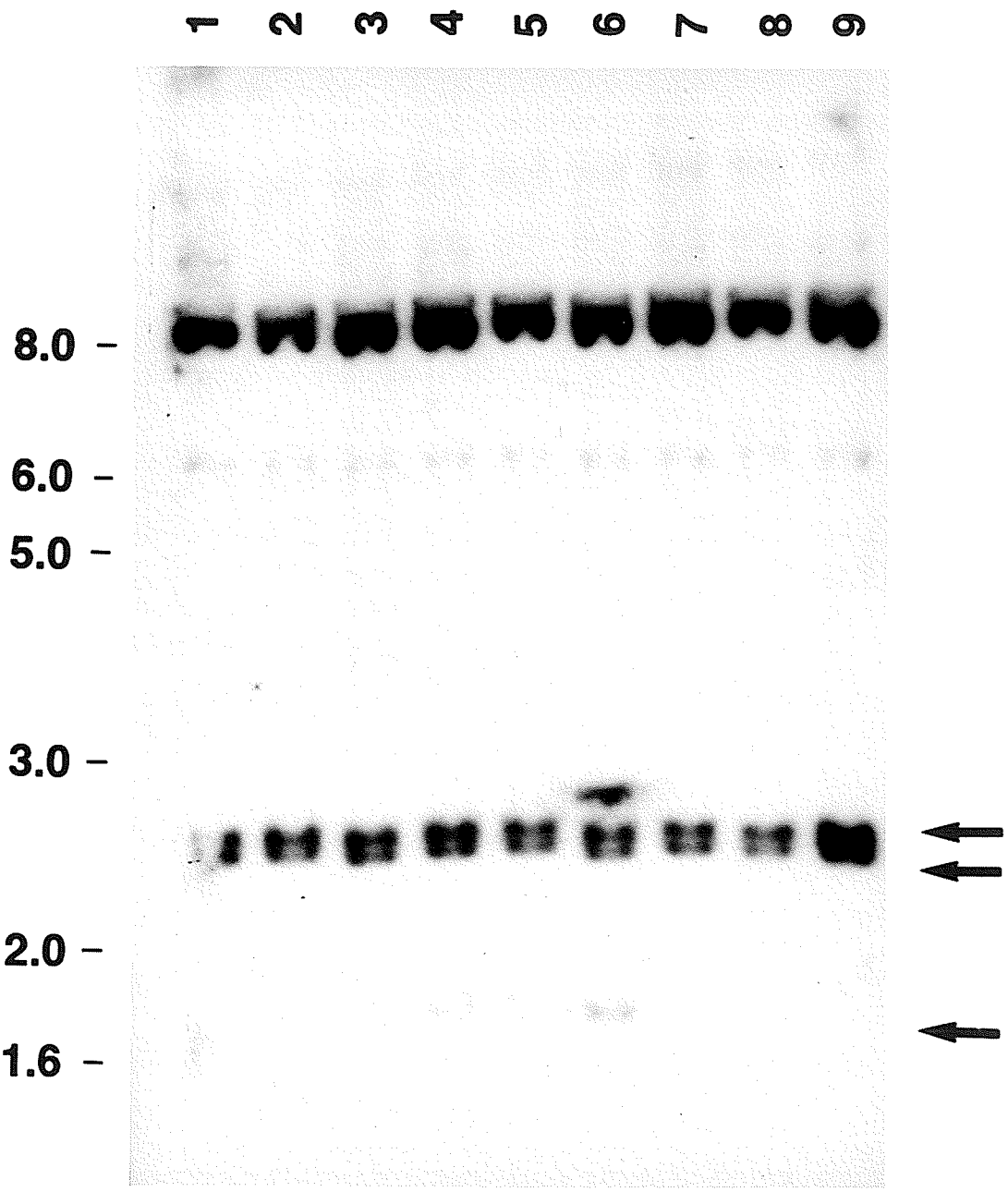
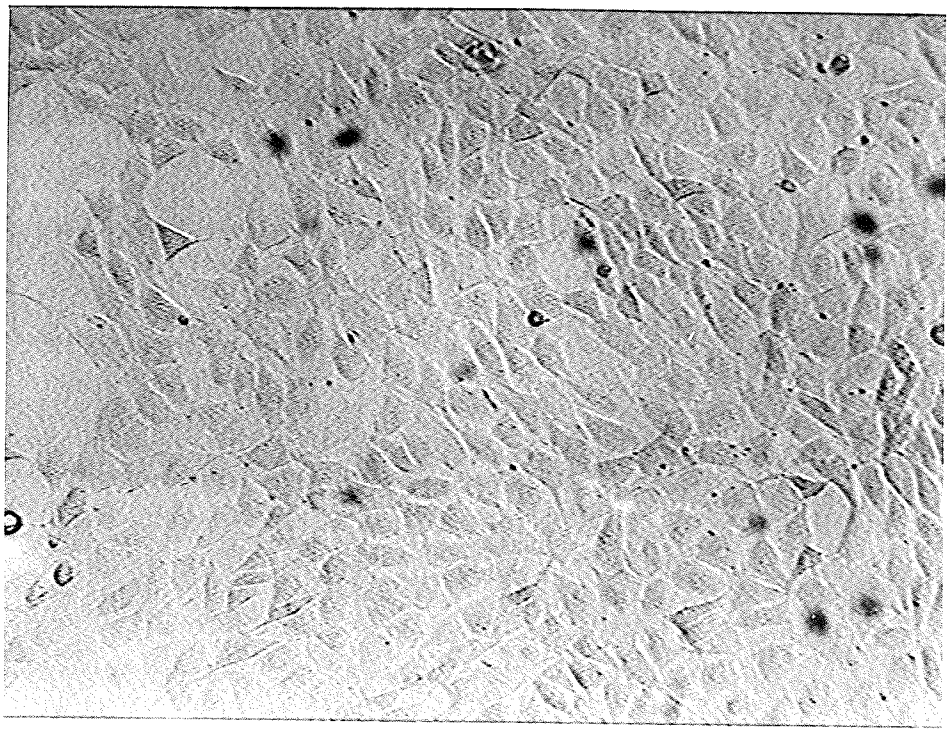

1
2
3
4
5
6
7
8
9
8.0
6.0
5.0
3.0
2.0
1.6

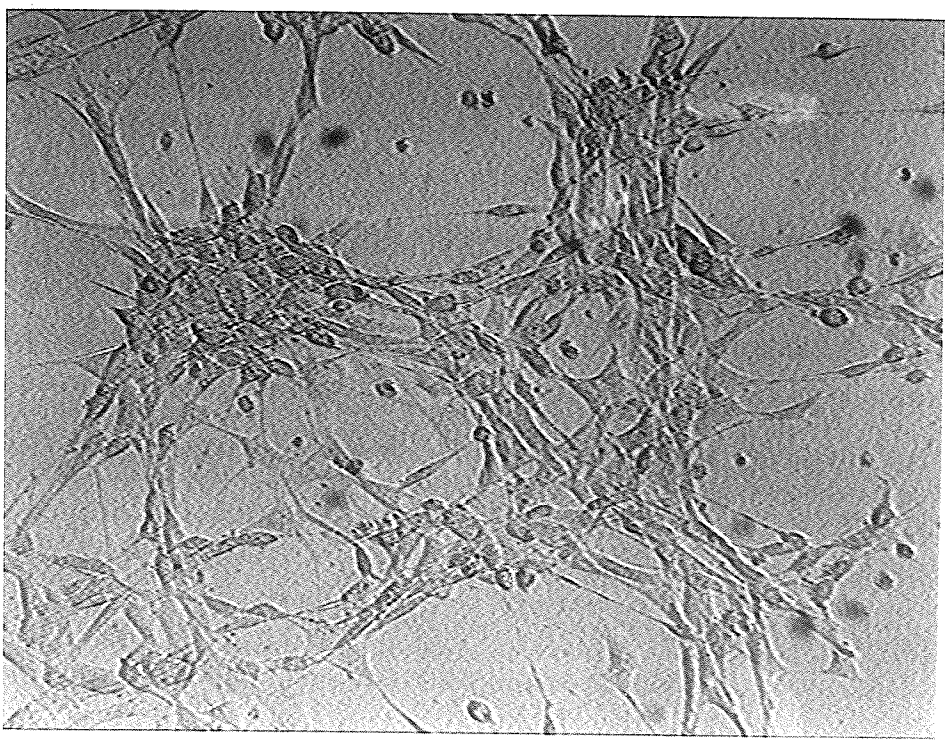
Figure 11: Phase Contrast Micrographs of Transfected Cells

Morphology of transfected 10T1/2 cells in completed medium containing G418: **A).** control vector, clone obtained after transfection of pSV2-neo plasmid; **B).** CW12, clone obtained after transfection of pSV2-neo plasmid and RHAMM cDNA in phage vector. Pictures were taken after three weeks of culturing.

A



B



THE STRUCTURAL ORGANIZATION OF THE GENE ENCODING THE HYALURONAN RECEPTOR, RHAMM, AND THE IDENTIFICATION OF A SECRETED ISOFORM

(hyaluronan receptor, cell locomotion, protein isoforms, alternative splicing)

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ABSTRACT

We describe the isolation and characterization of the murine gene encoding RHAMM, a hyaluronan receptor which is required for motility of *ras*-transformed cells and leukocytes. Southern blot analysis indicated that RHAMM occurs as a single copy gene. The gene spans 26 kilobases and comprises 14 exons ranging in size from 60 to 1099 base pairs. Twelve exons encode RHAMM I, with a deduced amino acid sequence of 79 kDa, which utilizes AUG 1 for translation. An alternately spliced form of RHAMM I (namely RHAMM I-2a) encoding an extra 25 amino acids is also described and two alternate 5' non coding regions of RHAMM I (designated RHAMM IA and RHAMM IB) were also identified. The transcripts of RHAMM IA and RHAMM IB isoforms were synthesized from different start sites, separated by 1.8kb. The two mRNAs appeared to diverge their 5' non coding regions due to alternate splicing of exon 1B and alternate exon usage. Exons 4 to 12 encode the previously described RHAMM II, with a deduced amino acid sequence of 55 kDa, which uses AUG 2 for translation. Primer extension studies indicated the transcript start point (tsp) for RHAMM I is in position -31. The previously described RHAMM II is a truncated form of RHAMM I with multiple tsps initiating between AUG 1 and AUG 2. Northern blot analyses of mouse fibroblast RNA using RHAMM cDNA probes identified at least two hybridizing species and *in vitro* translation of mRNA yielded proteins of 70 and 55 kDa. Transfection and overexpression of the RHAMM II isoform indicated that it accumulated as a cellular 65 kDa protein. Similar transfection studies with the RHAMM I-2a isoform showed the overexpression of a 70 kDa protein in cell lysates and the accumulation of a 100 kDa secreted protein in the supernatant media. The complex structure of the gene therefore appears to allow multiple levels of expression and compartmentalization of the RHAMM protein.

INTRODUCTION

Hyaluronan is a large glycosaminoglycan that is ubiquitous in the extracellular matrix and whose synthesis has been linked to cell migration, growth and transformation (Turley 1984; Toole et al., 1984; Iozzo 1985; Boudreaux et al., 1991). This glycosaminoglycan interacts with cell surfaces via specific protein receptors that mediate many of its biological effects (Turley 1992). Three distinct hyaluronan receptors, CD44 (Dalchau et al., 1980; Aruffo et al., 1990; Stamankovic et al., 1991), RHAMM (Hardwick et al., 1992; Yang et al., 1993), and HARLEC (Forsberg and Gustafson 1991; Rampyari et al., 1988) have been isolated, and characterized. These receptors regulate cell locomotion and have been implicated in malignant transformation.

RHAMM is a glycoprotein that is critical to cytokine, oncogene and injury-regulated locomotion: RHAMM is required for cell locomotion in response to TGF-β_1, the activated *ras* oncogene, bleomycin-induced lung injury and culture wounding, as demonstrated by antibody blockade and peptide mimicry studies (Khalil et al., 1989; Turley et al., 1991; Samuel et al., 1993). Expression of cell surface RHAMM is tightly regulated, being absent in most normal tissues, but increasing upon tissue injury in macrophages, epithelium, fibroblasts and smooth muscle cell populations (Savani et al., submitted). *In vitro* cell surface RHAMM expression is density- and transformation-dependent so that it is only expressed for a few days after the subculture of normal cells, but is elevated upon exposure to cytokines (Samuel et al., 1993) or to oncogenes (Turley et al., 1991). Collectively, these results predict a critical role of RHAMM in cell locomotion, particularly during injury and oncogenesis.

RHAMM is expressed on the cell surface, as determined by surface labelling, FACS analysis, subcellular fractionation and sensitivity of RHAMM to light protease treatment. Fluorescence

studies and subcellular fractionation also suggest that it occurs as an intracellular protein and biochemical analysis indicates that a form of this protein is secreted (Turley et al., 1987). The presence of RHAMM in multiple cell compartments is similar to the heterogeneous subcellular distribution of other receptors such as CD44 (Stamenkovic et al., 1991), several growth factor receptors (Mosley et al., 1989; Flanagan et al., 1991; Johnson et al., 1990; Rorsman and Betsholtz, 1992), bcl proteins (Oltvai et al., 1993; Boise et al., 1993) and CAM proteins (Probstmeier et al., 1989; Gearing et al., 1992). However, the molecular basis for RHAMM compartmentalization is currently not known. Indeed, in spite of the compartmentalization of RHAMM, the RHAMM II cDNA previously reported does not encode a signal sequence or a transmembrane domain (Hardwick et al., 1992). We sequenced the RHAMM gene to determine if the heterogeneity and compartmentalization of this protein are generated by expression of protein isoforms that result from differential RNA splicing, alternate promoter usage or the presence of several related genes. In this report, we describe the isolation and structure of the entire RHAMM gene and show by Southern blot analysis that RHAMM occurs in a single copy. We provide evidence that the gene encodes multiple isoforms, two of which accumulate intracellularly and one of which is secreted. In addition, we show that two distinct mRNAs encoding the RHAMM I protein are transcribed having different 5' non coding regions.

MATERIALS AND METHODS

Isolation of Genomic Clones and Sequencing

RHAMM genomic clones were isolated by screening a mouse fibroblast genomic library in the lambda Fix II vector with the RHAMM II cDNA clone (Hardwick et al., 1992) as a probe. The

probe was labelled with ^{32}P using the random oligonucleotide primer kit (Pharmacia, Pixaroway, N.J.). Filters were hybridized overnight in 5xSSPE (1xSSPE is 150 mM NaCl, 1 mM NaH_2PO_4 (pH 8.3) and 1 mM EDTA (pH 7.5), 5x Denhardt's solution (1x Denhardt solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin, 0.2% sodium dodecyl sulphate) and 100 µg/ml denatured salmon DNA for 16 h at 65°C. Membranes were washed at 65°C with 2 X SSC, then 1 x SSC and 0.1 x SSC for a total of 90 minutes (1xSSC is 150 mM NaCl, 15mM sodium citrate pH 7.0 and 0.1% SDS) and autoradiographed at -80°C. Positively hybridizing recombinant bacteriophage were purified and phage DNA was prepared as described previously (Sambrook et al., 1989).

Restriction Endonuclease Mapping of the Genomic Clones

Phage clones were digested with restriction endonucleases and the resulting fragments were separated on 0.9% agarose gels. After denaturing they were transferred to nylon membrane for Southern blotting. Filters were hybridized with the randomly labelled RHAMM II cDNA as a probe. Hybridization and washing conditions were as previously described. Detailed restriction maps of each lambda clone were made and selected restriction fragments were then either subcloned and sequenced using the T7 sequencing kit (Pharmacia, Piscataway, N.J.) or the cycle sequencing kit (Gibco BRL Gaithersburg, M.D.) following the strategy depicted in Fig. 2F. Sequencing primers were made using RHAMM II cDNA as a reference. Introns were sized either by sequencing or by using exon\intron flanking sequence to amplify the introns, using the polymerase chain reaction (PCR), followed by sizing on 1% agarose gels. PCR reactions were carried out as described in the 5' rapid amplification of cDNA ends (RACE) section except that lambda clones were used as a DNA template and 30 cycles of amplification were used.

Isolation of DNA and Southern Blots

High molecular weight DNA was isolated from 3T3 fibroblast cells (Sambrook et al., 1989). Approximately 5 x 10^6 cells were washed twice with Tris-buffered saline, removed from the culture dish by scraping and centrifuged at 1000 g for 5 min. DNA was isolated by proteinase D-SDS as previously described (Sambrook et al., 1989). DNA samples were incubated with restriction endonucleases and electrophoresed through a 0.7% agarose gel and transferred onto a nitrocellulose membrane. The membrane was hybridized with randomly labelled RHAMM II cDNA. Hybridization and washing conditions were exactly as described in the isolation of genomic clones section.

Cloning RHAMM I cDNA

Two ug of cytoplasmic RNA from 3T3 fibroblasts was subjected to first strand cDNA synthesis as described in the cDNA first stand synthesis kit (Clontech Palo Alto, CA). A primer from the start of exon 1 5' GCGGTCGACATGAGAGCTCTAAGCCTGGAA 3' and one from the 3' non coding region of RHAMM II 5' CGCGGATCCCCTTTGGTGATGAACAGCAG 3' were used to amplify a 2.2kb fragment. The primers contained a Sal 1 and an BamH1 restriction site respectively to facilitate cloning into the expression vector (pH βAPr-1-neo) (Gunning et al., 1987) which contained a neomycin resistance gene for selection. The plasmid construct was sequenced and subsequently stably transfected into 10T1\2 fibroblasts using the lipofection kit (Gibco, BRL, Gaithersburg, M.D.). Selected clones were analysed by Western analysis for protein size and elevated RHAMM expression, at confluence, using the anti-RHAMM antibody R.3.2 (Hardwick et al., 1992).

Rapid Amplification of cDNA Ends (RACE)

The 5' sequence of the RHAMM transcript was cloned using the rapid amplification of cDNA ends (RACE) technique. Briefly, an oligonucleotide complementary to residues 490-469 of the existing RHAMM II cDNA was used to prime cDNA synthesis from 3T3 fibroblast mRNA, as described in the 5' amplifinder RACE kit protocol (Clontech, Palo Alto, CA). A single stranded oligonucleotide anchor was then ligated directly to the 3' end of the first strand cDNA. Following ligation, the cDNA template was used for polymerase chain amplification (PCR) using a primer complementary to the anchor and a nested gene specific primer complementary to residues 81-60. PCR cycling parameters were denaturation at 94°C for 4 min, denaturation at 94°C for 45 sec annealing at 55°C for 45 sec and extension at 72°C for 2 min. 35 cycles were used with a final extension time of 7 min. PCR amplification yielded a faint band of 350 bp. Reamplification using the same primers and conditions yielded a strong band of 350bp which was then directly sequenced using cycle sequencing.

Mapping the 5' End by Primer Extension

Primer extension assays were performed with ^{32}P-end-labelled oligonucleotide primers as described (Sambrook et al, 1989), using the following two primers. A 22-mer primer (5'-TTCCAGAGCCAGCCTCTCTGT-3') complementary to the region of RHAMM II located 34-59 bp 3' of AUG 2. A 25-mer primer (5'-TCATCTTTGTCTCTCTCTTATTTCT-3') complementary to the region of RHAMM I located 11-36 bp 3' of AUG 1. Six ng of end labelled primer were hybridized with 15 μg total RNA from C3 cells. The extension reaction was done with 20 units AMV reverse transcriptase (Clontech, Palo Alto, CA) for 1.5 hr at 42°C. The same primer was used

on cloned genomic DNA template to generate dideoxy sequencing ladders for sizing the primer extension initiation sites.

In Vitro Translation

The messenger RNA, obtained from 3T3 cells with Pharmacia Quick-Prep mRNA Purification kit, was translated into protein using rabbit reticulocyte lysate (Amersham Oakville, ON) and S^{35}-L-methionine-S^{35}-L-cysteine mix, (TRAN^{35}S-label ICN). Tobacco mosaic virus was used as a positive control. One µl RNA guard (Pharmacia 27-0815-01) was used to protect the 50 µl reaction mixture during incubation at 30°C for 90 min. 1 µl was precipitated onto a glass filter and counted in scintillation fluid for the confirmation of Trans ^{35}S-label incorporation. The remaining reaction mixture was immunoprecipitated with normal rabbit IgG or the anti-RHAMM peptide antibody as described (Hardwick et al., 1992; Yang et al., 1993). The resulting samples were run on a mini-gel, fixed and subject to fluorography.

Northern Blot Analysis

Total RNA was extracted from 90% confluent 3T3 fibroblasts using the guanidinium thiocyanate method (Sambrook et al., 1989). Eighty µg of total RNA was electrophoresed in a 1.3% agarose gel, transblotted onto Hybond N^+ membrane and hybridized with RHAMM II cDNA as a probe (Hardwick *et al*, 1992). The hybridization was carried out in 5x Denhardt's solution, 10% (w/v) Dextran solution, 50% formamide and 50 µg/ml denatured Salmon sperm DNA at 42°C overnight (Sambrook et al., 1989). Washing conditions were 2xSSC and 0.1% SDS, then 1xSSC and 0.1% SDS and finally 0.1xSSC and 0.1% SDS at 42°C for a total of 30 min. The blot was exposed to Kodak X-Omat film at -80°C for 1-2 days.

Western Blot Analysis

Eighty percent confluent cultures were lysed in RIPA buffer (Sambrook et al., 1989) and solublized protein was quantified with a protein assay kit (Biorad Mississauga, ON). Ten μg of protein was electrophoresed in SDS-PAGE and transferred electrically to nitrocellulose membranes. Remaining protein binding sites on the membrane were blocked with 5% defatted milk and the membranes were incubated with 1:1000 dilution anti-RHAMM antibody, R.3.2 (Hardwick *et al.*, 1992). Membranes were washed in Tween (0.05%)-Tris-buffered saline (TTBS); probed with the second antibody (goat anti-rabbit-HRP) and visualized with the chemiluminescence method (Amersham Oakville, ON).

Transfection of RHAMM I-2a and RHAMM II cDNA

The RHAMM I-2a and RHAMM II cDNAs were inserted into the pH βAPr-1-neo vector (Gunning et al., 1987) containing a neomycin resistance gene for a selection marker. 10T½ fibroblasts were transfected with the plasmid using a lipofectin kit (Gibco BRL, Gaithersburg, M.D.). Cells were selected in G418 and cloned. Clones were analysed for increased RHAMM expression at confluence by Western transblot analysis using the anti-RHAMM peptide antibody (Hardwick et al., 1992). 15 clones of each transfection were obtained that overexpressed RHAMM by 2-3 fold as determined by densitometric analysis of Western blots. Three clones of each transfection were then characterized in detail.

Isolation of RHAMM from the Supernatant Media

3T3 fibroblast and 10T½ transfected cells were grown to confluence. 10ml of supernatant media was collected and concentrated to 2ml and desalted using centripreps (Amicon, Oakville, ON) and Affi-gel Blue buffer (0,1M K_2HPO_4, 0.15M NaCL pH 7.25). The 2 ml concentrate was further

concentrated to 1 ml using centricons (Amicon, Oakville, ON) and protease inhibitor was added. The 1.0 ml concentrated sample was then added to a 1.0 ml Affi-gel Blue (Biorad) column to remove BSA. Fifteen mg of protein were used for Western analysis, using 1:1000 dilution anti-RHAMM peptide antibody (Hardwick et al., 1992).

RESULTS

A. Southern analysis of 3T3 DNA using the RHAMM cDNA as a probe yields a simple hybridization pattern

To assess RHAMM gene copy number, Southern analysis was carried out on DNA from NIH 3T3 cells as shown in Fig. 1. Digested genomic DNA was hybridized using randomly labelled RHAMM II cDNA clone as a probe. The simple restriction pattern obtained (Fig. 1) is consistent with RHAMM being a single copy gene.

B. Isolation and organization of the RHAMM gene

Five genomic clones were isolated from the mouse 3T3 fibroblast DNA library using the RHAMM II cDNA as a probe. Restriction digests indicated that the clones were overlapping (Fig. 2E). PCR analysis of these clones using a primer at the start of AUG 2 (5' ATGCAGATCCTGACAGAGAGG 3') and a primer complementary to the end of intron 3 (5' CTGCATTCAGACAGGTAAGCA 3') produced one fragment of the same size for each clone, confirming that they contained overlapping regions. Sequencing the PCR products confirmed that a core fragment was identical in each clone. Clones (1) and (4) contained the entire RHAMM gene (Fig. 2E). The PCR products and nucleotide sequencing strategy used to determine the RHAMM gene structure, the sequence of the entire coding regions and exon\intron boundaries are shown in Figures 2, 3 and 4.

Using the RHAMM II cDNA clone for reference, the gene was determined to be organized into 14 exons interrupted by 13 introns spanning 26 kilobases of DNA (Fig. 2). The exons range in

size from 60 bp (exon 1) to 1.1 kb (exon 12) (Table 1). The intron sizes vary considerably ranging from 90 bp (intron 1) to 6500 bp (intron 2) (Table 1).

The deduced amino acid $AA^{124-229}$ sequence encoded in the RHAMM protein (Hardwick et al., 1992) which comprises five perfect repeats of 21 amino acids occurs in one exon (exon 6). The two HA binding domains (Yang *et al.*, 1993) are encoded in exons 10 and 11 separated by a 2.2 kb intron (Fig. 2D).

C. The RHAMM gene encodes several protein isoforms

The genomic structure of RHAMM is more complex than was predicted by the previously reported cDNA (Hardwick et al., 1992). Exons 4 through 12 correspond to the previously isolated RHAMM cDNA (RHAMM II), its deduced amino acid sequence predicts a 55 kDa protein. However, sequencing the genomic clones revealed that there were a further three exons of open reading frame (exons 1 to 3) upstream of exons 4-12. The deduced amino acid sequence of exons 1 to 12 predict a 70 kDa protein (RHAMM I). The two proteins overlap such that the deduced amino acid sequence of RHAMM I included the RHAMM II sequence plus an additional 129 amino acids at the amino terminal end (Fig. 2, 3 and 4).

RT-PCR, sequencing and Western analysis were used to demonstrate the existence of the RHAMM 1 isoform and to confirm that exons 1-3 were coding exons. In RT-PCR analysis, 1st strand cDNA, derived from reverse transcribed cytoplasmic 3T3 RNA, was used as a template. Two primers, one at the start of exon 1 and one in the 3' non-coding region of RHAMM II, were used for amplification. The resulting 2.2 kb fragment was sequenced and shown to be identical with exons 1-12. RT-PCR, using the same two primers also identified an alternately spliced exon of RHAMM

I, namely exon 2A which has a 25 amino acid insert in the amino terminal end (Fig. 5), this isoform was designated RHAMM I-2a.

Transfection and overexpression of RHAMM II resulted in an overexpression of a 65 kDa protein in cell lysates (Fig. 6A). RHAMM II protein did not accumulate in supernatant mediums. Endoglycosidase treatment of the 65 kDa protein reduced it to 55 kDa species (data not shown). A corresponding band of 55 kDa was seen when mRNA from 3T3 fibroblasts was translated *in-vitro* (Fig. 7). Transfection and overexpression of RHAMM I-2a produced two proteins of MW 70 kDa and 100 kDa. The 70 kDa protein was overexpressed in transfected cell lysates (Fig. 6B). The 100 kDa protein was overexpressed and accumulated in the supernatant media (Fig. 6C) of transfected cells. *Ras*-transformed cells also accumulated the 100 kDa RHAMM protein in the supernatant media. Endoglycosidase treatment of the 100 kDa protein reduced it to a 70 kDa form (data not shown) and a 70 kDa protein was observed from *in vitro* translation of mRNA (Fig. 7). Preabsorption of the anti-RHAMM peptide antibody with RHAMM fusion protein indicated the specificity of the 100 kDa RHAMM bands (Fig. 6c). The proteins encoded by RHAMM I, RHAMM I-2a and RHAMM II did not have transmembrane domains. Possible weak signal sequences exist in the proteins encoded in RHAMM I and I-2a isoforms. Neither protein has homology to other protein sequences recorded in the data banks.

D. RHAMM Contains Internal transcription start points (tsps)

Primer extension assays predicted the occurrence of two classes of transcript and identified the transcript start points (tsps) that would theoretically yield two classes of RHAMM isoforms (Fig. 8A and 8B). The tsp of RHAMM I was identified as a G residue -31 bases upstream of the

AUG 1 codon (Fig. 8A). There were six tsps for the RHAMM II isoform which would initiate internally between AUG 1 and AUG 2 (Fig. 8B) in positions 83, 126, 129, 134, 290 and 383 nt upstream of AUG 2. Products were sequenced for confirmation. Of the 6 tsps observed, three were strong bands and three were weak, (Fig. 8B). Transcript start points in positions 83, 126, 129, 134 and 290 use AUG 2 for initiation of translation. It is unknown whether tsp 383 would use AUG 2 given that there are other possible initiators prior to AUG 2. The results suggest the presence of a family of RHAMM mRNAs with 5' ends starting downstream of AUG 1 which will produce the truncated RHAMM II. Although these results are reproducible we cannot totally exclude that some of the weaker tsps were due to secondary structure interference in the primer extension assays. It is possible that a single transcript is made and that the RHAMM I and RHAMM II isoforms are produced by alternate initiation of translation. However, our data are consistent with production of alternate transcripts.

E. Several RHAMM mRNA Transcripts are Transcribed

There are multiple RHAMM transcripts which are consistent with the presence of several RHAMM proteins found both in cell lysates and in the *in vitro* translation of mRNA. Northern blots of 3T3 fibroblast total RNA, using cDNA derived probes, identified two hybridizing species of 4.2kb and 1.7kb. The 4.2kb band is very broad spanning 3.9kb to 4.2kb and represents several message populations (Fig. 9). Indeed early exposures of Northern blots indicate several distinct bands in this region (data not shown). The origin of the 1.7kb species is unknown. Given that two distinct 5' non-coding regions of RHAMM I have been identified (see below), the alternately spliced exon 2A

and the existence of the truncated RHAMM II transcripts, the presence of several messages of a similar size is not unexpected.

F. The RHAMM I Isoform has Different 5' Non-coding Regions

To determine the 5' end of the mRNA, the 5' RACE technique was used which yielded a 350bp product that was subsequently sequenced. The sequence of this fragment is shown in (Fig. 10A) and compared to that already known from RT PCR studies on RHAMM I cDNA (Fig. 10A). The sequences are identical for 24nt upstream of AUG 1 then the two sequences diverged. The longer sequence RHAMM IA, generated by the 5' RACE is found in an exon 1.8kb upstream from RHAMM IB. The protein coding sequences for both forms are identical, initiating with AUG 1. However, alternate splicing in exon 1A and alternate exon usage produced two entirely different 5' non-coding regions, see Fig. 10.

DISCUSSION

We describe the structural organization of the M. musculus RHAMM gene. This gene encodes a hyaluronan receptor that promotes cell locomotion and regulates focal adhesion turnover in *ras*-transformed cells (Hardwick et al., 1992; Hall et al., in press). The RHAMM gene spans 26 kilobases and is comprised of fourteen exons. The previously described RHAMM II cDNA is encoded within exons 4 to 12. We also identify and clone two new isoforms of RHAMM, designated RHAMM I and RHAMM I-2a encoded within exons 1 - 12. RHAMM I-2a is identical to RHAMM I but contains the alternatively spliced exon 2A. RHAMM I and RHAMM II are predicted to be colinear isoforms such that RHAMM II is a truncated form of RHAMM I.

Evidence for the existence of RHAMM II is derived from several approaches. A previously published cDNA encoding RHAMM II has been isolated from a 3T3 expression library (Hardwick et al., 1992). The overexpression of the RHAMM II cDNA produces a 65 kDa protein which aligns with the size of a RHAMM protein seen in Western analyses of *ras*-transformed cells. Endoglycosidase treatment reduced the 65 kDa protein to a 55 kDa form which is consistent with the deduced amino acid sequence of RHAMM II and with the size of a protein immunoprecipitated after *in vitro* translation of RHAMM mRNA. Primer extension studies predict multiple potential transcription starts for this isoform, however neither the significance of multiple starts nor direct evidence of their utilization is yet clear.

These data are consistent with the proposal that RHAMM II is generated from distinct transcripts rather than alternative initiation of translation from the longer RHAMM I. However, we cannot entirely rule out that some of the tsps observed for RHAMM II resulted from secondary structures in the RNA creating pausing during the primer extension assays. Further, the biological role of the RHAMM II isoform remains to be determined but our overexpression studies indicate that unlike RHAMM I-2a, it does not accumulate in the medium. We are currently determining whether RHAMM II is targeted to the cell surface or remains as an intracellular protein.

Transfection and overexpression of RHAMM I-2a produced two protein species. One is a 70 kDa protein which accumulates in cell lysates and may represent a nonglycosylated RHAMM I-2a and the other is a 100 kDa protein which accumulates in the supernatant media. The production of two proteins, one cell associated and one secreted, following the transfection and overexpression of a single cDNA is also seen for the type 1 tumor necrosis factor receptor (TNF-R) (Nophar et al., 1990). This may be the case for RHAMM I-2a or possibly the glycosylation mechanism of the cell

has been overwhelmed by the overexpression. Endoglycosidase digestion of the 100 kDa protein reduces it to 70 kDa species, consistent with the presence of nine potential sites for N-glycosylation. However, since the cDNA was not epitope tagged before transfection the possibility remains that overexpression of RHAMM I-2a has resulted in the up regulation of another as yet unidentified variant form that is secreted. Further sequence analysis of introns and use of tagged cDNAs encoding the RHAMM I-2a form will be necessary to discern among these possibilities. Nevertheless, peptide blocking experiments indicated the specificity of the products derived from RHAMM I-2a transfections and clearly demonstrate the existence of a secreted form of RHAMM.

It is noteworthy that the secreted RHAMM I-2a isoform does not contain an obvious signal peptide although amino acids 1-20 may encode a weak signal peptide. We have previously proposed (Hardwick et al., 1992) that RHAMM may resemble certain animal lectins (Barondes, 1988) and the high affinity elastin/laminin receptors (Yow et al., 1988; Rao et al., 1989) in their mode of association with the cell surface. None of these proteins are integral and they do not contain signal sequences. Rather they appear to be transported to the cell surface by carrier proteins and associate with the cell surface via integral docking proteins. Transfection of the RHAMM I isoform will be the subject of future studies and will give an indication as to the functional role of the alternately spliced 25 amino acids seen in the RHAMM I-2a isoform. It is possible that RHAMM I binds to a docking protein producing surface RHAMM and that exon 2A disrupts the binding site to produce a secreted form. The interplay of the two isoforms may be very important in the regulation of cell locomotion.

There are a growing number of cell surface receptors that also exist in soluble form. These include the interleukin-2 receptor (Rubin et al., 1985), the interleukin-7 receptor (Goodwin et al.,

1990), the epidermal growth factor receptor (Petch et al., 1990) the leucocyte tyrosine kinase receptor (Toyoshima et al., 1993), the HA receptor CD44 (Sy et al., 1992) and cell-associated adhesion molecules (Gearing et al., 1992). The soluble forms of these receptors can be produced either by alternate splicing which will eliminate the transmembrane domain, the insertion of an in-frame stop codon prior to the transmembrane domain producing a truncated receptor or by proteolytic cleavage. Occasionally soluble receptor forms are produced by an alternate splicing event which leads to the elimination of a glycolipid tail as seen with the human urokinase plasminogen activator receptor (Pyke et al., 1993).

High expression of surface RHAMM coincides with maximal rate of cell locomotion in *ras*-transformed cells and in cells after stimulation with TGF-β_1 (Samuel et al., 1993). In contrast to the cell associated form, the soluble form of RHAMM (RHAMM I-2a) inhibits cell locomotion (Yang et al., submitted) and chemotaxis (Savani et al., submitted) in ng amounts and significantly more RHAMM I is expressed by normal rather than transformed cells (data not shown). Thus, the regulation of RHAMM induced cell locomotion may be partly achieved by the ratio of RHAMM I:RHAMM I-2a. The mechanisms by which soluble RHAMM inhibits locomotion remains unclear, but requires the presence of hyaluronan binding domains predicting that like the soluble TNF-R receptor, soluble RHAMM may compete with cell associated RHAMM for the hyaluronan ligand.

The significance of the alternate-usage of the two 5' untranslated regions of RHAMM I remains unknown. It is possible that these sequences contain motifs that determine message stability and/or indicate alternate usage of promoters. Either mechanism may be important for the regulation of cell locomotion. Analysis of sequence both in intron 2 and 5' of RHAMM IA and RHAMM IB indicated the presence of several promoter regions (data not shown) predicting that the different

isoforms are alternately regulated. A similar spatial separation of alternate promoters driving the transcription of different isoforms, is seen in the β-galactoside α2,6-sialyl-transferase gene (Wang et al., 1990) and the angiotensin I-converting enzyme (ACE) gene (Hubert et al., 1991).

The heterogeneity manifested by the RHAMM gene has also been observed in another hyaluronan receptor, namely CD44. CD44 is by and large a constitutively expressed protein which occurs as a variety of isoforms that are generated by alternative exon splicing. However, the RHAMM gene appears to differ from that of CD44 in several important ways. Unlike CD44, the apparently more limited diversity of RHAMM is generated by some alternate splicing, alternate promoter usage and the translation of a truncated form which are targeted differently. Alternate splicing appears to be more restricted (data not shown) than that observed in the CD44 gene (Screaton et al., 1992), although this aspect of gene structure has yet to be thoroughly investigated for the RHAMM gene. Although RHAMM and CD44 have both been implicated in cell adhesion, locomotion and tumor transformation (Stamenkovic et al., 1989; Stamenkovic et al., 1991), differences in their regulation predict that they may have distinct roles in these biological processes.

In conclusion, the structure of the RHAMM gene has been presented we have identified three isoforms of RHAMM and demonstrated that RHAMM I-2a is secreted. In addition, we provide preliminary evidence of alternate promoters which would increase the flexibility and control of several isoforms being expressed from a single gene. Future interests will focus on determining the function and subcellular locations of the various isoforms and defining the role of attachment of surface RHAMM.

FIGURE LEGENDS

Fig. 1

Southern blot analysis fo the mouse RHAMM gene

Mouse genomic DNA was digested with the following restriction endonucleases. Lane S:Sac I, Lane G: Bgl II, Lane B: BamHI and Lane E: EcoRI. The filter was hybridized with mouse RHAMM II cDNA.

Fig. 2

Organization of the mouse RHAMM gene: Schematic representation of the two RHAMM isoforms.

A and B: Schematic representations of the organization of the RHAMM I and RHAMM II cDNA clones. Single lines indicate the 5' and 3' non-coding regions. Coding regions are boxed and shaded areas represent the overlapping regions of these clones.

C and D: Alignment of the cDNA clones RHAMM I and RHAMM II with the RHAMM gene. Numbers indicate exon/intron boundaries, AUG 1 being nucleotide 1, AUG 2 is in position 388 and 1821 is the stop codon. Open boxes indicate exons numbered 1 to 12. The diagonally hatched region in exon 6 corresponds to the 21 amino acids repeated five times and the horizontal hatching in exons 10 and 11 correspond to the HA binding domains 1 and 2, respectively

E: Overlapping lambda clones that were isolated and found to cover the entaire RHAMM gene.

F: Arrows indicate the sequencing strategy.

Fig. 3

Nucleotide and the deduced amino acid sequence for the RHAMM gene

The cDNA sequence is in uppercase letters, intron boundaries are in lowercase letters. Twelve nucleotides are presented for each intron/exon boundary. The translation initiation of AUG 1 (+1) and AUG 2 (+388) are boxed. The termination codon is indicated by an asterisk. The transcript start point (tsp) for RHAMM I is indicated by an arrow in position -31. Numbers on the left and right correspond to the amino acid sequence.

Fig. 4

Complete nucleotide and deduced amino acid sequence of RHAMM I cDNA

The translation initiation codons for RHAMM I (AUG 1) is boxed, the termination codon is indicated by an asterisk. The initiation codon for RHAMM II (AUG 2) is indicated by a hatched box. Numbers on the left correspond to the nucleotide sequence beginning with the first methionine of the open reading frame (AUG 1). Potential glycosylation sites from N-linked glycans are underlined.

Fig. 5

Schematic representation, nucleotide and deduced amino acid sequence of the alternately spliced exon 2A.

A: Diagrammatic representation of alternately spliced exon 2A.

B: Intron/exon boundaries of exon 2A.

C: Nucleotide and deduced amino acid sequence of exon 2A.

Fig. 6

Western analysis of cells transfected with RHAMM I-2a and RHAMM II cDNAs

Transfection and overexpression of the RHAMM I-2a and RHAMM II cDNAs, in 10T½ fibroblasts, using the pH β APr-1-neo vector. Overexpression was detected at confluence by Western blotting using the RHAMM peptide antibody. Transfections using the vector (pH β APr-1-neo) alone were used as a control. Fifteen μg of protein were loaded per lane.

A. Overexpression of the RHAMM II isoform in cell lysates. Lane 1: vector control, lane 2: vector containing RHAMM II cDNA.

B. Overexpression of RHAMM I-2a in cell lysates. Lane 1: vector control, lane 2: vector containing RHAMM I-2a cDNA.

C. Overexpression of RHAMM I-2a in supernatant media. Lane 1: vector control, lane 2: vector containing RHAMM I-2a insert, Lanes 3 and 4 are the vector control (lane 3) and RHAMM I-2a containing insert (lane 4) probed with the anti-RHAMM peptide antibody after preabsorption with RHAMM fusion protein. RHAMM II did not accumulate in the supernatant media.

Fig. 7

In-vitro translation

mRNA from 3T3 fibroblasts was translated with [^{35}S]-methionine\[^{35}S]-cysteine using the rabbit reticulocyte system. Immunoprecipitation was carried out using the RHAMM antibody R3.2. Lane 1: No mRNA, lane 2: mRNA from 3T3 fibroblasts.

Fig. 8

Primer extension analyses of RHAMM I and RHAMM II

Primers complementary to 5'-untranslated regions of RHAMM I and RHAMM II were radiolabelled at their 5' terminii, hybridized to total RNA isolated from 80% confluent cultures and extended with reverse transcriptase. Products were separated on 6% sequencing gels adjacent to sequencing reactions (lanes a,c,g and t) for sizing. Major 5' terminii are indicated by arrows.

A: 5' terminus of the RHAMM I mRNA transcript.

B: 5' terminii of the RHAMM II mRNA transcripts.

Fig. 9

Northern blot analysis of RHAMM expression in 3T3 fibroblasts

Total RNA was isolated from 80% confluent cultures of 3T3 fibroblasts. The RNA was fractionated on 1% formaldehyde-agarose gels, transferred to a nitrocellulose membrane and hybridized with ^{32}P-labelled RHAMM II cDNA as a probe.

Fig. 10

Alternate 5' non-coding regions of the RHAMM I cDNA

A: Nucleotide and deduced amino acid sequence of the 5' non-coding regions and first coding exon of RHAMM IA and RHAMM IB, respectively. Stop codons in frame with the initiation codons are boxed. The point of divergence of the two sequences is indicated by a diagonal line.

B: Schematic representation of RHAMM IA and RHAMM IB respectively. Shaded boxes represent the protein coding regions; open boxes indicate the 5' and 3' untranlsated regions which are identical. The hatched and shaded boxes indicate where the two 5' untranslated regions diverge. Single lines are introns.

TABLE 1

Identification of Introns

Nucleotide sequence of the intron-exon junctions in the mouse RHAMM gene.

Exon No/Size (bp)	cDNA Position of exons in RHAMM I	Intron No.	Donor	Acceptor	Intron Size (kb)	Method
1(60)	1 - 60	I	GG/gtgagt	gtgcag/AG	0.09	sequencing
2(101)	61 - 161	II	CT/catgtg	atacag/CT	6.50	PCR
3(176)	162 - 337	III	AG/gtactg	ttacag/AC	0.38	sequencing
4(149)	338 - 486	IV	AG/gtagct	atgcag/GA	0.52	sequencing
5(216)	487 - 701	V	GA/gtttgt	ctttag/GA	0.80	PCR
6(368)	702 -1070	VI	AG/gtatt	tataag/CT	1.80	PCR
7(147)	1071 -1217	VII	AG/gtgagt	ctaagg/GA	0.29	sequencing
8(153)	1218 -1370	VIII	AG/gtaagt	atacag/AA	0.12	sequencing
9(97)	1371 -1470	IX	AG/gtttgt	ttccag/CA	1.30	PCR
10(179)	1471 -1647	X	CG/gtttgt	tcacag/GA	2.20	PCR
11(152)	1648 -1801	XI	AG/gtaaaa	cttcag/GC	0.90	PCR
12(1099)	1802 -2904	XII				

Consensus splice sites: Donor: AG/gtat/gt; Acceptor: ttacag

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Fig. 1

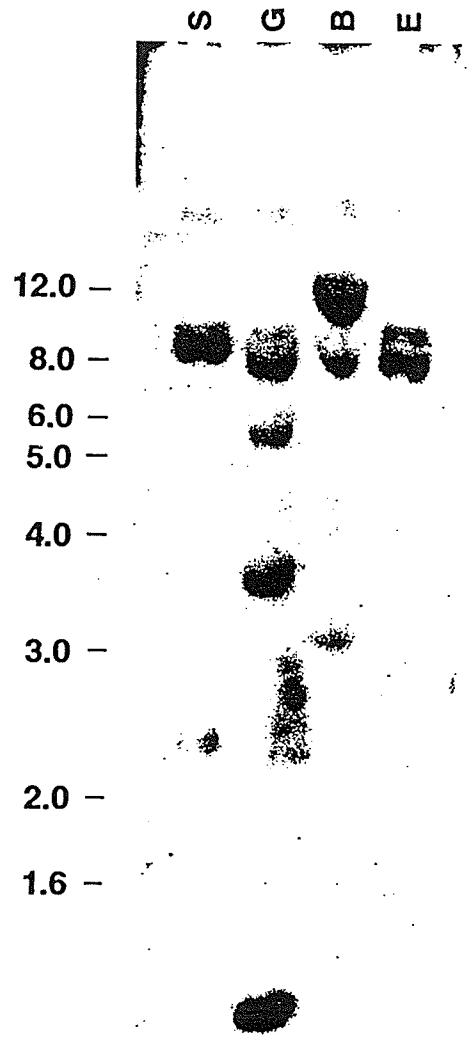
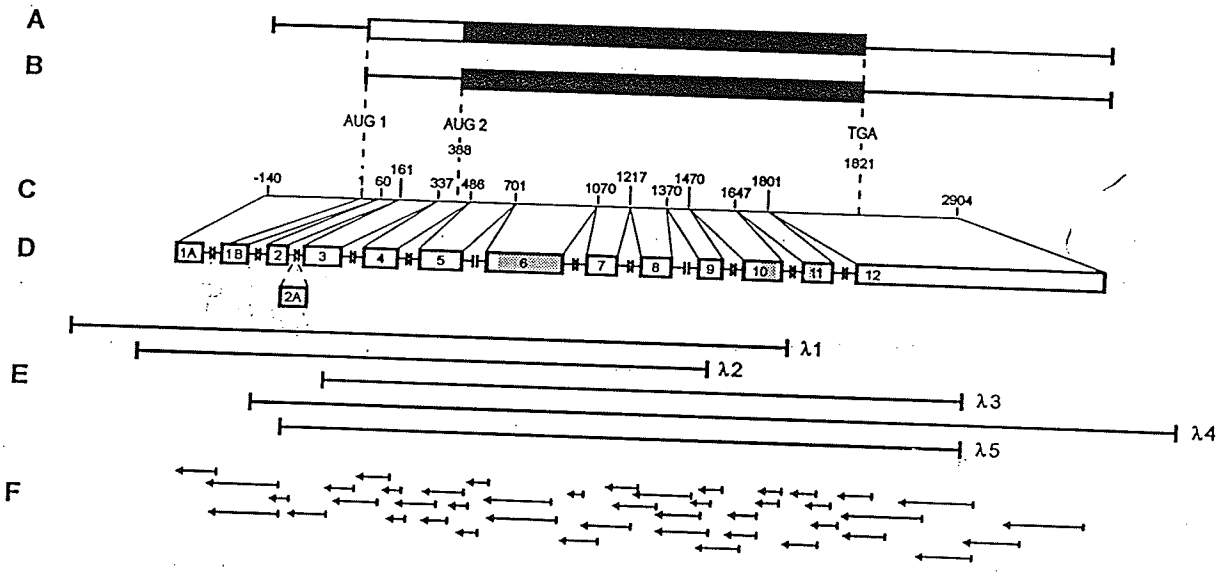


FIG. 2.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2904
D
1A
1B
2
2A
3
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11
12
E
λ1
λ2
λ3
λ4
λ5
F

FIG. 3

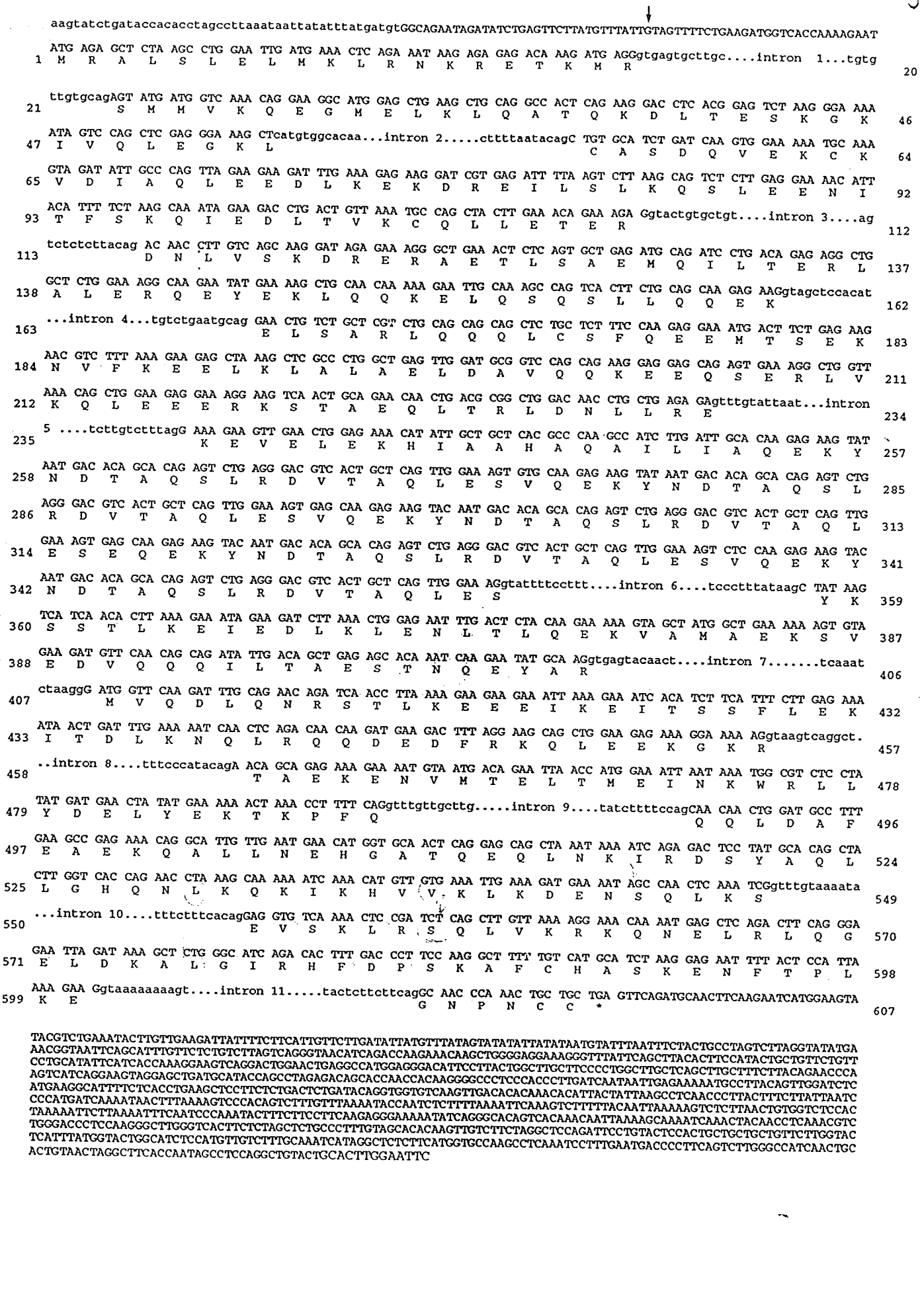
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Fig. 4

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    M   R   A   L   S   L   E   L   M   K   L   R   N   K   R   E   T   K   M   R   S   M   M   V   K   Q   E   G

85  ATG GAG CTG AAG CTG CAG GCC ACT CAG AAG GAC CTC ACG GAG TCT AAG GGA AAA ATA GTC CAG CTG GAG GGA AAG CTC TGT GCA
    M   E   L   K   L   Q   A   T   Q   K   D   L   T   E   S   K   G   K   I   V   Q   L   E   G   K   L   C   A

169 TCT GAT CAA GTG GAA AAA TGC AAA GTA GAT ATT GCC CAG TTA GAA GAA GAT TTG AAA GAG AAG GAT CGT GAG ATT TTA AGT CTT
    S   D   Q   V   E   K   C   K   V   D   I   A   Q   L   E   E   D   L   K   E   K   D   R   E   I   L   S   L

253 AAG CAG TCT CTT GAG GAA AAC ATT ACA TTT TCT AAG CAA ATA GAA GAC CTG ACT GTT AAA TGC CAG CTA CTT GAA ACA GAA AGA
    K   Q   S   L   E   E   N   I   T   F   S   K   Q   I   E   D   L   T   V   K   C   Q   L   L   E   T   E   R

337 GAC AAC CTT GTC AGC AAG GAT AGA GAA AGG GCT GAA ACT CTC AGT GCT GAG ATG CAG ATC CTG ACA GAG AGG CTG GCT CTG GAA
    D   N   L   V   S   K   D   R   E   R   A   E   T   L   S   A   E   M   Q   I   L   T   E   R   L   A   L   E

421 AGG CAA GAA TAT GAA AAG CTG CAA CAA AAA GAA TTG CAA AGC CAG TCA CTT CTG CAG CAA GAG AAG GAA CTG TCT GCT CGT CTG
    R   Q   E   Y   E   K   L   Q   Q   K   E   L   Q   S   Q   S   L   L   Q   Q   E   K   E   L   S   A   R   L

505 CAG CAG CAG CTC TGC TCT TTC CAA GAG GAA ATG ACT TCT GAG AAG AAC GTC TTT AAA GAA GAG CTA AAG CTC GCC CTG GCT GAG
    Q   Q   Q   L   C   S   F   Q   E   E   M   T   S   E   K   N   V   F   K   E   E   L   K   L   A   L   A   E

589 TTG GAT GCG GTC CAG CAG AAG GAG GAG CAG AGT GAA AGG CTG GTT AAA CAG CTG GAA GAG GAA AGG AAG TCA ACT GCA GAA CAA
    L   D   A   V   Q   Q   K   E   E   Q   S   E   R   L   V   K   Q   L   E   E   E   R   K   S   T   A   E   Q

673 CTG ACG CGG CTG GAC AAC CTG CTG AGA GAG AAA GAA GTT GAA CTG GAG AAA CAT ATT GCT GCT CAC GCC CAA GCC ATC TTG ATT
    L   T   R   L   D   N   L   L   R   E   K   E   V   E   L   E   K   H   I   A   A   H   A   Q   A   I   L   I

757 GCA CAA GAG AAG TAT AAT GAC ACA GCA CAG AGT CTG AGG GAC GTC ACT GCT CAG TTG GAA AGT GTG CAA GAG AAG TAT AAT GAC
    A   Q   E   K   Y   N   D   T   A   Q   S   L   R   D   V   T   A   Q   L   E   S   V   Q   E   K   Y   N   D

841 ACA GCA CAG AGT CTG AGG GAC GTC ACT GCT CAG TTG GAA AGT GAG CAA GAG AAG TAC AAT GAC ACA GCA CAG AGT CTG AGG GAC
    T   A   Q   S   L   R   D   V   T   A   Q   L   E   S   E   Q   E   K   Y   N   D   T   A   Q   S   L   R   D

925 GTC ACT GCT CAG TTG GAA AGT GAG CAA GAG AAG TAC AAT GAC ACA GCA CAG AGT CTG AGG GAC GTC ACT GCT CAG TTG GAA AGT
    V   T   A   Q   L   E   S   E   Q   E   K   Y   N   D   T   A   Q   S   L   R   D   V   T   A   Q   L   E   S

1009 GTG CAA GAG AAG TAC AAT GAC ACA GCA CAG AGT CTG AGG GAC GTC ACT GCT CAG TTG GAA AGC TAT AAG TCA TCA ACA CTT AAA
     V   Q   E   K   Y   N   D   T   A   Q   S   L   R   D   V   T   A   Q   L   E   S   Y   K   S   S   T   L   K

1093 GAA ATA GAA GAT CTT AAA CTG GAG AAT TTG ACT CTA CAA GAA AAA GTA GCT ATG GCT GAA AAA AGT GTA GAA GAT GTT CAA CAG
     E   I   E   D   L   K   L   E   N   L   T   L   Q   E   K   V   A   M   A   E   K   S   V   E   D   V   Q   Q

1177 CAG ATA TTG ACA GCT GAG AGC ACA AAT CAA GAA TAT GCA AGG ATG GTT CAA GAT TTG CAG AAC AGA TCA ACC TTA AAA GAA GAA
     Q   I   L   T   A   E   S   T   N   Q   E   Y   A   R   M   V   Q   D   L   Q   N   R   S   T   L   K   E   E

1261 GAA ATT AAA GAA ATC ACA TCT TCA TTT CTT GAG AAA ATA ACT GAT TTG AAA AAT CAA CTC AGA CAA CAA GAT GAA GAC TTT AGG
     E   I   K   E   I   T   S   S   F   L   E   K   I   T   D   L   K   N   Q   L   R   Q   Q   D   E   D   F   R

1345 AAG CAG CTG GAA GAG AAA GGA AAA AGA ACA GCA GAG AAA GAA AAT GTA ATG ACA GAA TTA ACC ATG GAA ATT AAT AAA TGG CGT
     K   Q   L   E   E   K   G   K   R   T   A   E   K   E   N   V   M   T   E   L   T   M   E   I   N   K   W   R

1429 CTC CTA TAT GAT GAA CTA TAT GAA AAA ACT AAA CCT TTT CAG CAA CAA CTG GAT GCC TTT GAA GCC GAG AAA CAG GCA TTG TTG
     L   L   Y   D   E   L   Y   E   K   T   K   P   F   Q   Q   Q   L   D   A   F   E   A   E   K   Q   A   L   L

1513 AAT GAA CAT GGT GCA ACT CAG GAG CAG CTA AAT AAA ATC AGA GAC TCC TAT GCA CAG CTA CTT GGT CAC CAG AAC CTA AAG CAA
     N   E   H   G   A   T   Q   E   Q   L   N   K   I   R   D   S   Y   A   Q   L   L   G   H   Q   N   L   K   Q

1597 AAA ATC AAA CAT GTT GTG AAA TTG AAA GAT GAA AAT AGC CAA CTC AAA TCG GAG GTG TCA AAA CTC CGA TCT CAG CTT GTT AAA
     K   I   K   H   V   V   K   L   K   D   E   N   S   Q   L   K   S   E   V   S   K   L   R   S   Q   L   V   K

1681 AGG AAA CAA AAT GAG CTC AGA CTT CAG GGA GAA TTA GAT AAA GCT CTG GGC ATC AGA CAC TTT GAC CCT TCC AAG GCT TTT TGT
     R   K   Q   N   E   L   R   L   Q   G   E   L   D   K   A   L   G   I   R   H   F   D   P   S   K   A   F   C

1765 CAT GCA TCT AAG GAG AAT TTT ACT CCA TTA AAA GAA GGC AAC CCA AAC TGC TGC TGA
     H   A   S   K   E   N   F   T   P   L   K   E   G   N   P   N   C   C   *
```

FIG 5.

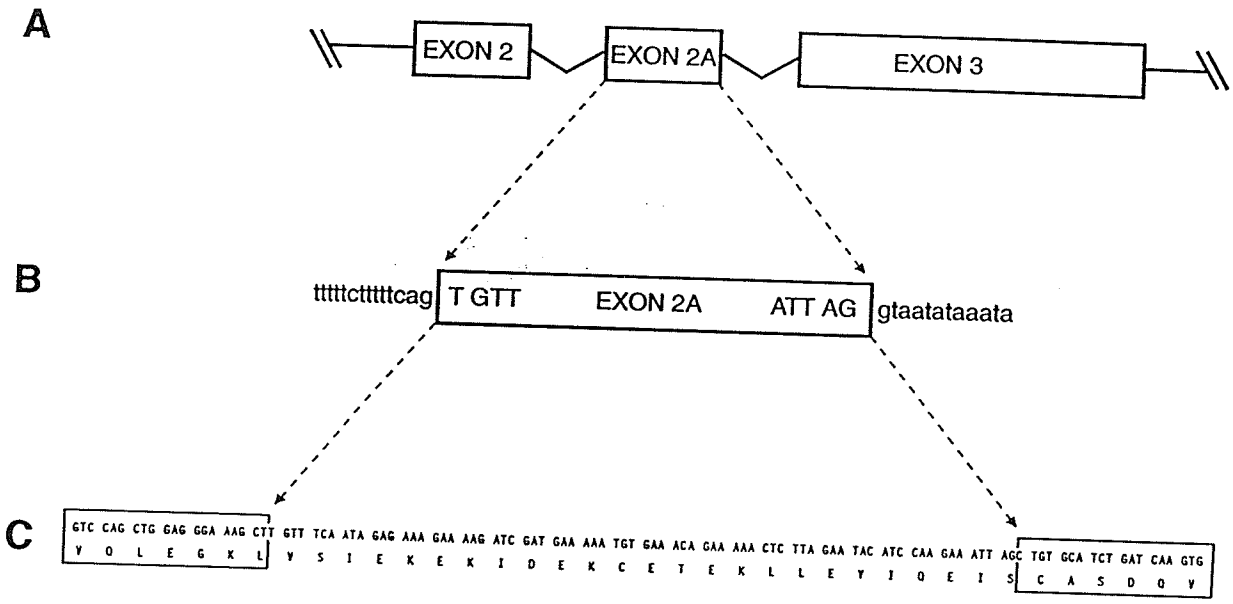

A
EXON 2
EXON 2A
EXON 3
B
ttttctttttcag
T GTT
EXON 2A
ATT AG
gtaatataaata
C
GTC CAG CTG GAG GGA AAG CTT GTT TCA ATA GAG AAA GAA AAG ATC GAT GAA AAA TGT GAA ACA GAA AAA CTC TTA GAA TAC ATC CAA GAA ATT AGC TGT GCA TCT GAT CAA GTG
V Q L E G K L V S I E K E K I D E K C E T E K L L E Y I Q E I S C A S D Q V

Fig 1

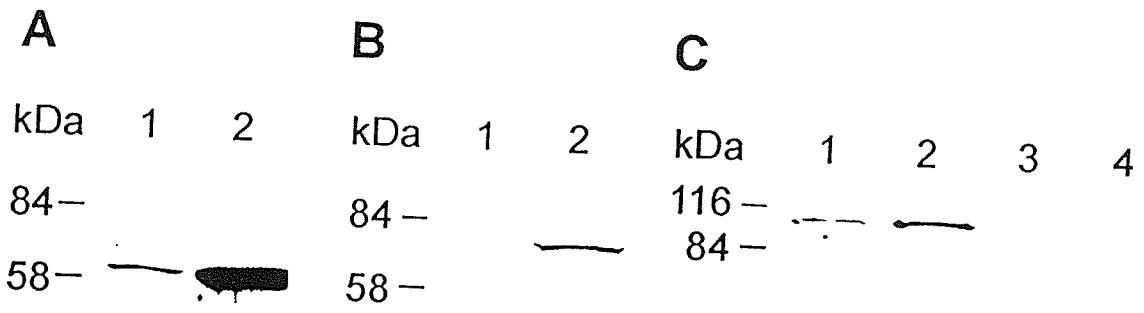


Fig. 7.

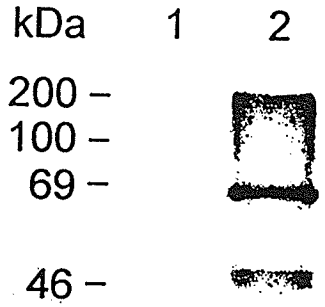


Fig. 8A

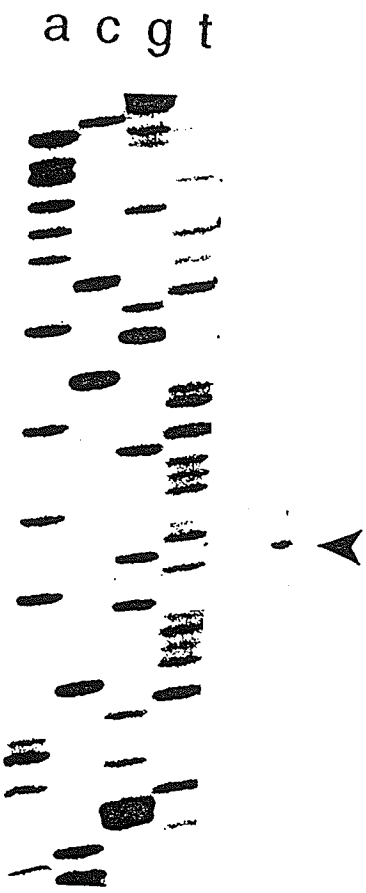


Fig. 8B.

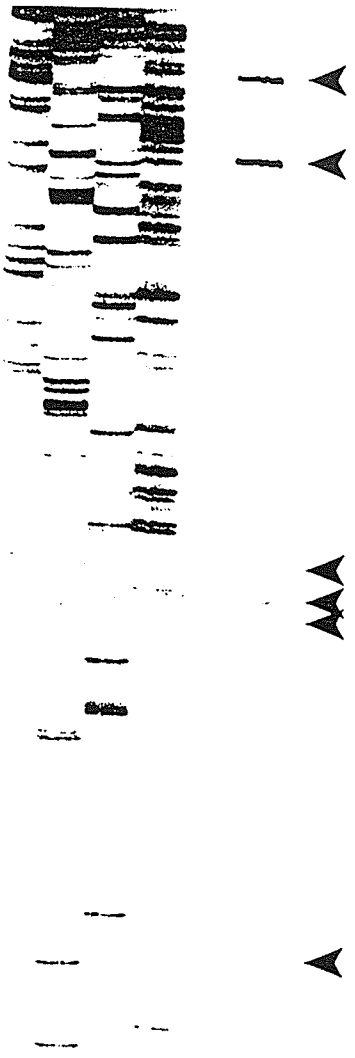


Fig 9.

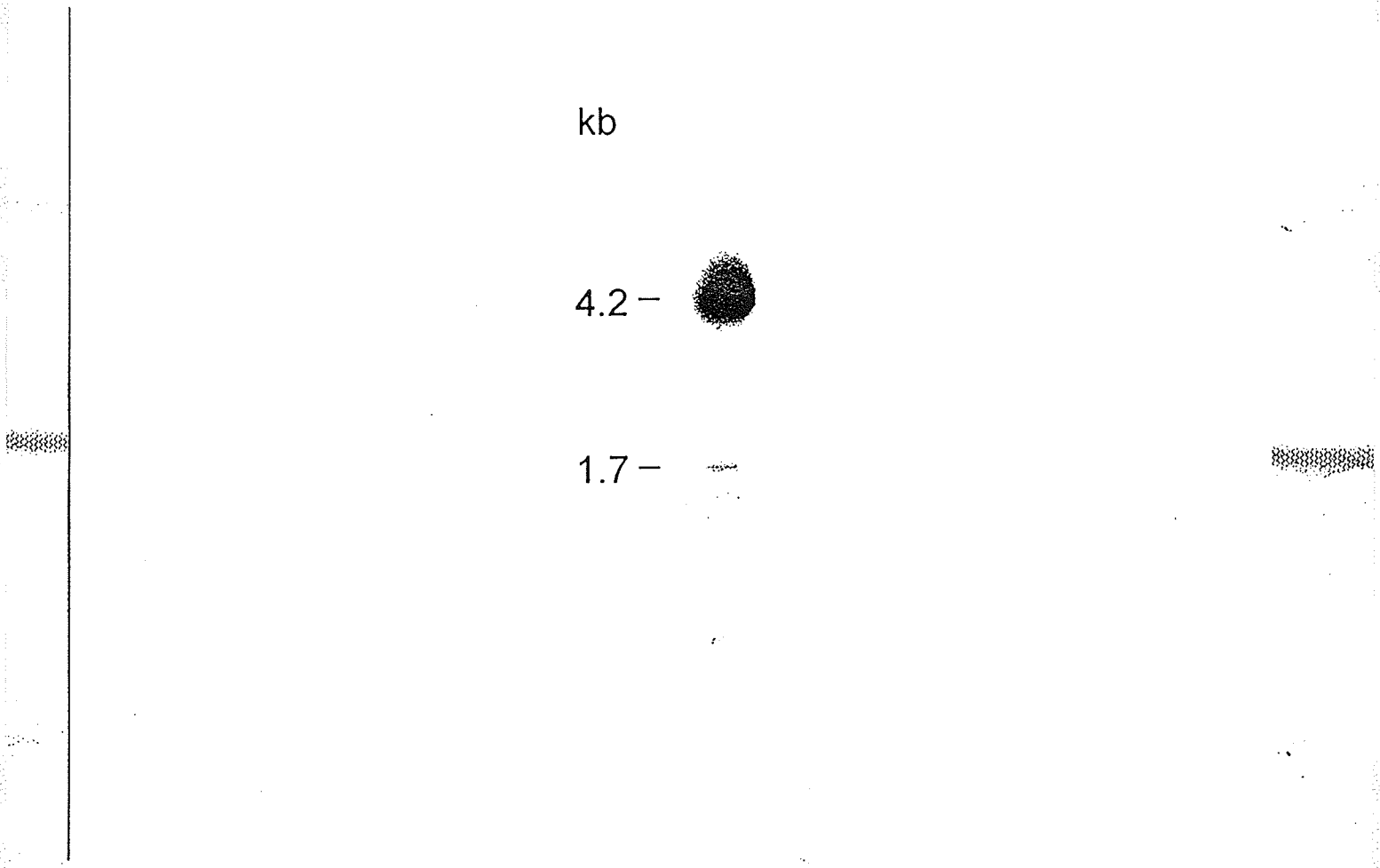


Fig. 10

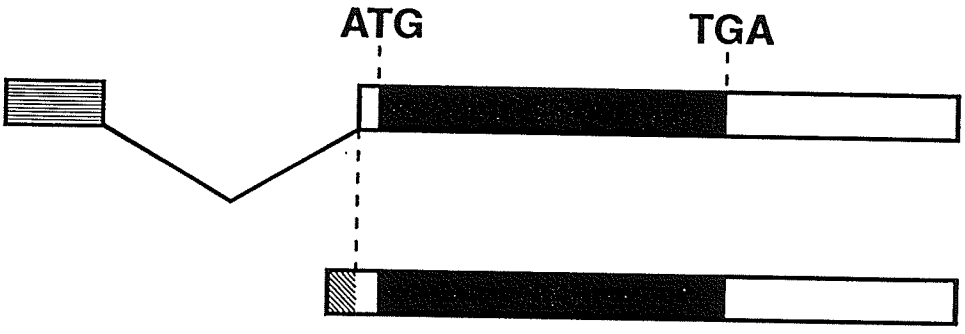
A

RHAMM IA AGGCCTTAGGTCCAGGAAGGAGGAAAAACCATCTTCTTCTCTGCGAGTAATGCTTCACTGGTAAA
RHAMM IB ·····

AACGGCTTACTGAATTAACCAGAGCCAACGAGCTACTAAAAGGCTAAAGGA
·····GGCAGAATAGATATCTGAGTTCTTATGTTTATTGTAGTTT TCTGAAGATGGTCACCAAAAGAAT

ATG AGA GCT CTA AGC CTG GAA TTG ATG AAA CTC AGA AAT AAG AGA GAG ACA AAG ATG AGG
M R A L S L E L M K L R N K R E T K M R

B



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University of Manitoba

23 September 1994

To Whom it may concern:

We the undersigned, the authors of the manuscript, "The Structural Organization of the Gene Encoding the Hyaluronan Receptor, RHAMM, and the Identification of a Secreted Isoform", agree to allow Carol (Lai Man) Wong to include this manuscript as an appendix to her MSc. thesis entitled "Isolation and Characterization of RHAMM Genomic Clones".

Joycelyn Entwistle

Shiwen Zhang

Baihua Yang

Genevieve Curpen

Michael Mowat

Arnold H. Greenberg

Eva A. Turley