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Molecular mechanisms that regulate hyaluronan synthesis

Review Article

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Summary

Hyaluronan is an ubiquitous glycosaminoglycan found in almost all tissues of the body in vertebrates and in the extracellular capsule of certain pathogenic bacteria. Hyaluronan is biologically active and affects cell migration and proliferation. The amount of hyaluronan in the extracellular matrix increases during inflammation, wound healing and certain forms of cancer. Hyaluronan is synthesized by a membrane-bound enzyme which uses UDP-sugar nucleotides precursors (UDP-glucuronic acid and UDP-N-acetylglucosamine). Three mammalian hyaluronan synthase genes (HAS1, HAS2 and HAS3) have been identified. The encoded proteins share 56-71% sequence similarities. The synthesis of hyaluronan is carefully regulated in a cell specific manner. Certain growth factors and cytokines lead to an increased activity of existing synthase molecules as well as induce the synthesis of new enzyme molecules.

I. Introduction

Hyaluronan was first isolated by Meyer and Palmer (1934) from the vitreous body of the eye and therefore named hyaluronic acid (from the Greek word *hyalos*, which means glass). Its architectural construction of repeating disaccharide units [D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine(1-β-4)]$_n$ was established twenty years later by Weissmann and Meyer (1954). Hyaluronan is an unbranched linear polysaccharide with a molecular mass ranging from about 200 kDa in blood to 5000 kDa in synovial fluid. The largest molecules have a chain length in the average of 10 µm. The chains form in solution kinked coils which immobilize water within their domains (Laurent and Fraser, 1992; Fraser and Laurent, 1996) (**Figure 1**). Hyaluronan is found virtually in every tissue and body fluid in vertebrates and in the capsules of certain bacterial pathogens, *e.g* Gram-positive group A and C streptococci and type A *Pasteurella multocida* (Carter and Annau, 1953; Laurent and Reed, 1991). Excessive amounts of hyaluronan are found during the first stage in the generation of extracellular matrix. This stage is followed by a decrease in the hyaluronan content as cell growth and differentiation ensues. In normal healthy tissues the amount of hyaluronan is maintained in equilibrium by a balance between synthesis and degradation. However, during inflammation, wound healing and certain forms of cancer the amount of hyaluronan increases and hyaluronan becomes the dominant glycosaminoglycan in the extracellular matrix. Accumulation of hyaluronan leads to retention of fluid which most likely contributes to the swelling of tissues characterizing inflammation (Laurent, 1998; Knudson, 1998). However, during restoration of damaged tissue hyaluronan-rich matrix has been shown to have beneficial effects; increased amounts of hyaluronan in the matrix forms a favorable environment which promotes cell migration and proliferation. For example, treatment with

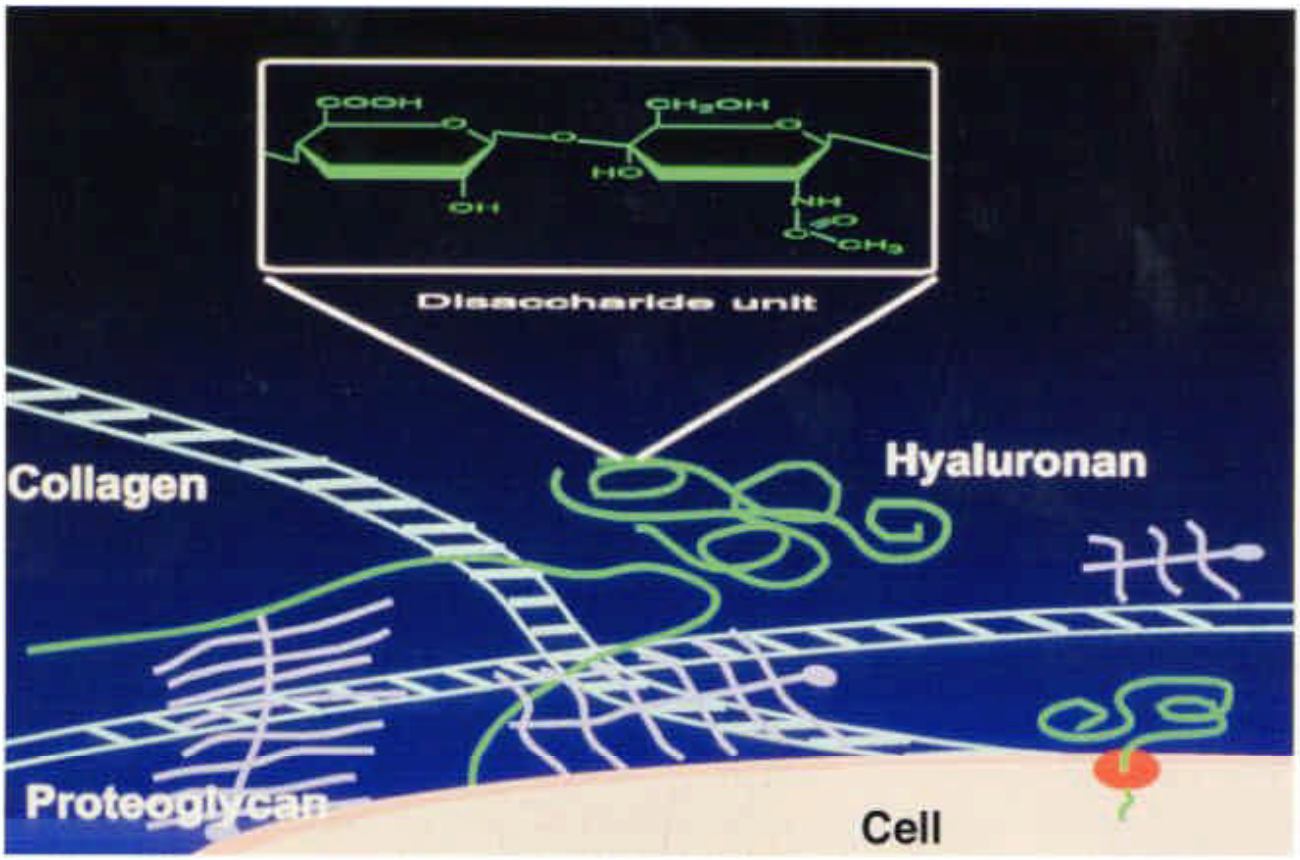
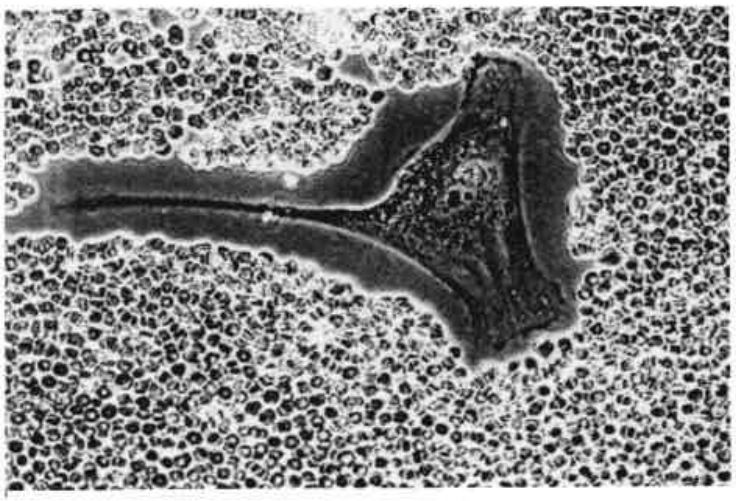

COOH
CH2OH
HO
OH
NH
CH3
Disaccharide unit
Collagen
Hyaluronan
Proteoglycan
Cell

Figure 1. Schematic drawing of cell surface associated hyaluronan chains and hyaluronan chains asociated with extracelular matrix proteins.

Figure 2. Hyaluronan containing coat surrounding a normal human mesothelial cell. The coat is visualized by the exclusion of formalin fixed erythrocytes. The picture was kindly provided by Dr. Håkan Pertoft (Uppsala, Sweden).



hyaluronan gel led to improved healing with less scar formation of damaged tendons and the tympanic membrane of the ear (Balazs and Denlinger, 1989; Laurent et al, 1986). The biological effects of hyaluronan in tumor tissues have not yet been clarified. Hyaluronan may facilitate tumor invasion (Lesley et al, 1997; Knudson, 1998) or may be involved in a host defense response (Teder et al, 1995).

Hyaluronan stabilizes the architecture of extracellular matrix through its interactions with specific matrix proteins and receptors, collectively referred to as hyaladherins (Toole, 1990). Moreover, it is involved in many biological processes modulating cell behavior during embryogenesis, inflammation and tumorigenesis (Knudson and Knudson, 1993; Sherman et al, 1994). These effects may be mediated through hyaluronan containing coats which constitute the immediate cellular environment (**Figure 2**). These coats are three-dimensional gels formed around some cells, and can be visualized *in vitro* by their exclusion of formalin-fixed erythrocytes (Clarris and Fraser, 1968; Knudson et al, 1993; Heldin and Pertoft, 1993). The most thoroughly studied hyaladherins are the link protein and aggrecan of cartilage (Hardingham and Muir, 1972) which have analogues in other tissues such as versican in connective tissues (Zimmermann and Ruoslahti, 1989), and hyaluronectin in the brain (Delpech et al, 1989). Hyaladherins are also found in certain body fluids, *e.g*. tumor necrosis factor (TNF)-stimulated gene-6 (TSG-6) found in synovial fluids of patients with arthritis and in sera of patients with different inflammatory or autoimmune disorders. TSG-6 forms a complex with the plasma protein inter-α-trypsin inhibitor that is also a hyaluronan binding protein (Day and Parkar, 1998). Cell-associated hyaluronan receptors are not only confined to connective tissue cells. Hyaluronan binding to cells of non mesodermal origin was demonstrated by the aggregation of lymphoma cells but not normal lymphocytes after addition of the polysaccharide (Wasteson et al, 1973). A putative hyaluronan receptor is the lymphocyte homing receptor $gp90^{Hermes}$ (CD44). The interaction between CD44 and hyaluronan leads to internalization of hyaluronan by lung macrophages and chondrocytes, adhesion of lymphocytes to the endothelium, formation of pericellular matrices around chondrocytes and increased invasion of certain tumors (Knudson and Knudson, 1993; Sherman et al, 1994). Another hyaluronan receptor is the receptor for hyaluronan-mediated motility (RHAMM) which affects cell locomotion and proliferation (Hall et al, 1994, 1995; Savani et al, 1995). Furthermore, the liver endothelial cells carry specific receptors for clearance of hyaluronan from the blood (Smedsrod et al, 1990). The non-immunogenic character of hyaluronan and its ability to bind specifically to cell surface receptors has led to the use of hyaluronan-based products in areas such as drug delivery and tissue repair (Balazs and Laurent, 1998).

Hyaluronan forms a capsule around group A and group C Streptococci. This is a perfect camouflage which allows these bacteria to be more successful pathogens by evading host defense (Schmidt et al, 1996; Husmann et al, 1997). Acapsular mutants of group A Streptococci exhibit considerable losses in virulence (Wessels et al, 1994).

II. Hyaluronan biosynthesis

The molecular mechanisms that regulate the biosynthetic pathway of hyaluronan are yet unclear. Hyaluronan biosynthesis in mammalian cells differs from that of other glycosaminoglycans which are synthesized in the endoplasmic reticulum/Golgi and then transported to the cell surface. The key enzymes in hyaluronan biosynthesis are hyaluronan synthases (HAS), a family of enzymes located in plasma membrane, which have beeen identified both in bacteria and vertebrates (Sugahara et al, 1979; Prehm, 1984; Philipson and Schwartz, 1984). During the synthesis process the hyaluronan chains are extruded into the intercellular space. This unique route of biosynthesis may be facilitated through interactions between the hydrophobic patches of hyaluronan chains and plasma membrane phospholipids (Ghosh et al, 1994); continuous extrusion through the membrane may be necessary because of the large size of hyaluronan chains. Hyaluronan synthesis requires intracellular sources of UDP-glucuronic acid (UDP-GlcA) and UDP-N-acetyl-glucosamine (UDP-GlcNAc), which are generated in the glycolytic pathway (O´Regan et al, 1994).

A. Growth factor and cytokine regulation of hyaluronan biosynthesis

The tissue hyaluronan content is increased during inflammation, wound healing, certain malignancies, *e.g*. mesotheliomas and Wilm´s tumor, and in other destructive injuries such as reumatoid arthritis. Some pathways on the molecular mechanisms of regulation of hyaluronan synthesis during cancer cell metastasis, wound healing and inflammation have recently been deciphered. Initial studies by us and other laboratories revealed that exogenously added growth factors, conditioned media, body fluids, as well as tumor promotors, lead to stimulation of hyaluronan synthesis in cultures of mesenchymal origin, *e.g*. fibroblasts (Heldin et al, 1989; Westergren-Thorsson G, 1990; Suzuki et al, 1995), normal human mesothelial cells (Honda et al, 1991; Heldin et al, 1992; Heldin and Pertoft, 1993), and hepatic stellate cells (Heldin et al, 1991; Gressner and Haarmann, 1988; Vrochides et al, 1996). Among these stimulators, platelet-derived growth factor (PDGF)-BB, transforming growth factor-ß (TGF-ß)

and the tumor promotor TPA exhibited the highest activity. PDGF-BB and TGF-ß mediated their stimulatory activity on hyaluronan synthesis at least in part through protein kinase C (PKC), since the PKC inhibitor calphostin C inhibited most of the hyaluronan synthesis induced by the growth factors. Direct activation of PKC by phorbol esters also stimulated hyaluronan production, and the combination of either PDGF or TGF-ß and TPA gave an increased effect. The stimulations by PDGF and TGF-ß were dependent on protein synthesis since cycloheximide inhibited their effects. In contrast, the effects mediated by TPA were not (Suzuki et al, 1995) (**Figure 3**). Thus, these results indicate that PKC is involved in the transduction of the effects of growth factors on hyaluronan biosynthesis in foreskin fibroblasts and normal human mesothelial cells, and that the effects involve direct or indirect activation of existing HAS molecules, as well as induction of new enzyme molecules or other factors involved in the transduction of growth factor signaling (Suzuki et al, 1995). The insulin-growth factor (IGF)-I and epidermal growth factor (EGF) also enhance the production of hyaluronan in mesothelial cell cultures derived from pericardium through a receptor tyrosine-kinase-involved transmembrane signaling process (Honda et al, 1991).

In inflammed tissues, hyaluronan accumulation is induced following infiltration by inflammatory cells which secrete growth factors and cytokines, many of which are capable of stimulating hyaluronan synthesis. *In vitro* studies on hyaluronan biosynthesis in human fibroblasts derived from various organs, revealed differential responses to cytokines, *e. g*. tumor necrosis factor (TNF)-α, interleukin (IL)-1α, interferon (IFN)-γ, and leukoregulin (Elias et al, 1988; Butler et al, 1988; Smith et al, 1995; Sampson et al, 1992). For example, treatment of human orbital fibroblasts (Smith et al, 1995) by leukoregulin, a cytokine released by activated T lymphocytes, resulted in a 3-fold higher stimulation of hyaluronan synthesis compared to leukoregulin stimulation of dermal fibroblasts (Mauviel et al, 1991). The leukoregulin-induced accumulation of hyaluronan in orbital fibroblasts was dependent on ongoing protein synthesis and was not mediated through the PKC pathway (Smith et al, 1995). However, prostaglandin E_2 stimulated hyaluronan synthesis in mesothelial cell cultures obtained from pericardium through a cAMP signaling pathway (Honda et al, 1993). Further *in vitro* studies revealed that combinations of IFN-γ and TNF-α augmented the synthesis of high molecular mass hyaluronan whereas combination of IL-1 and TNF-α induced the production of hyaluronan chains of low molecular mass (Sampson et al, 1992). These findings may be important for our understanding of the presence of abnormally low molecular weight hyaluronan chains (about 50 kDa) in several inflammatory disorders, such as in reumatoid arthritis where the hyaluronan found in joints has a low viscosity (Dahl and Husby, 1985). The differencies in the ability to synthesize hyaluronan may be due both to differences in intracellular signaling pathways (**Figure 3**) as well as to variations in the regulation of the expression of the three HAS isoforms (see below).

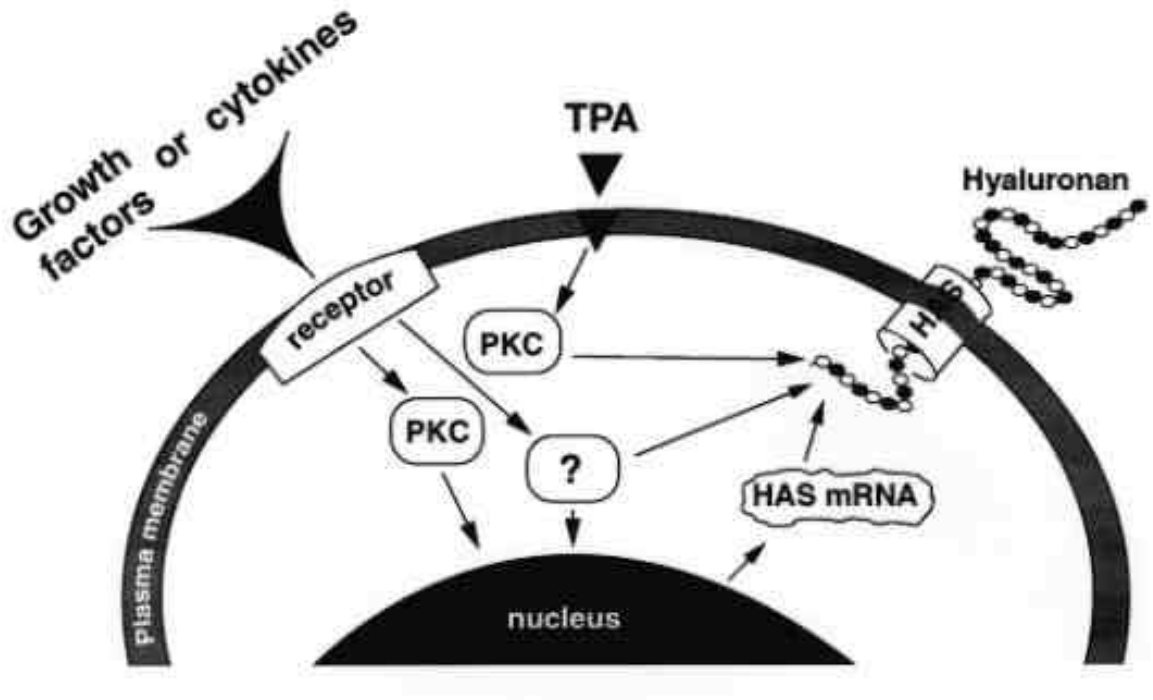


Figure 3. Schematic depiction of mechanisms that regulate hyaluronan synthesis.

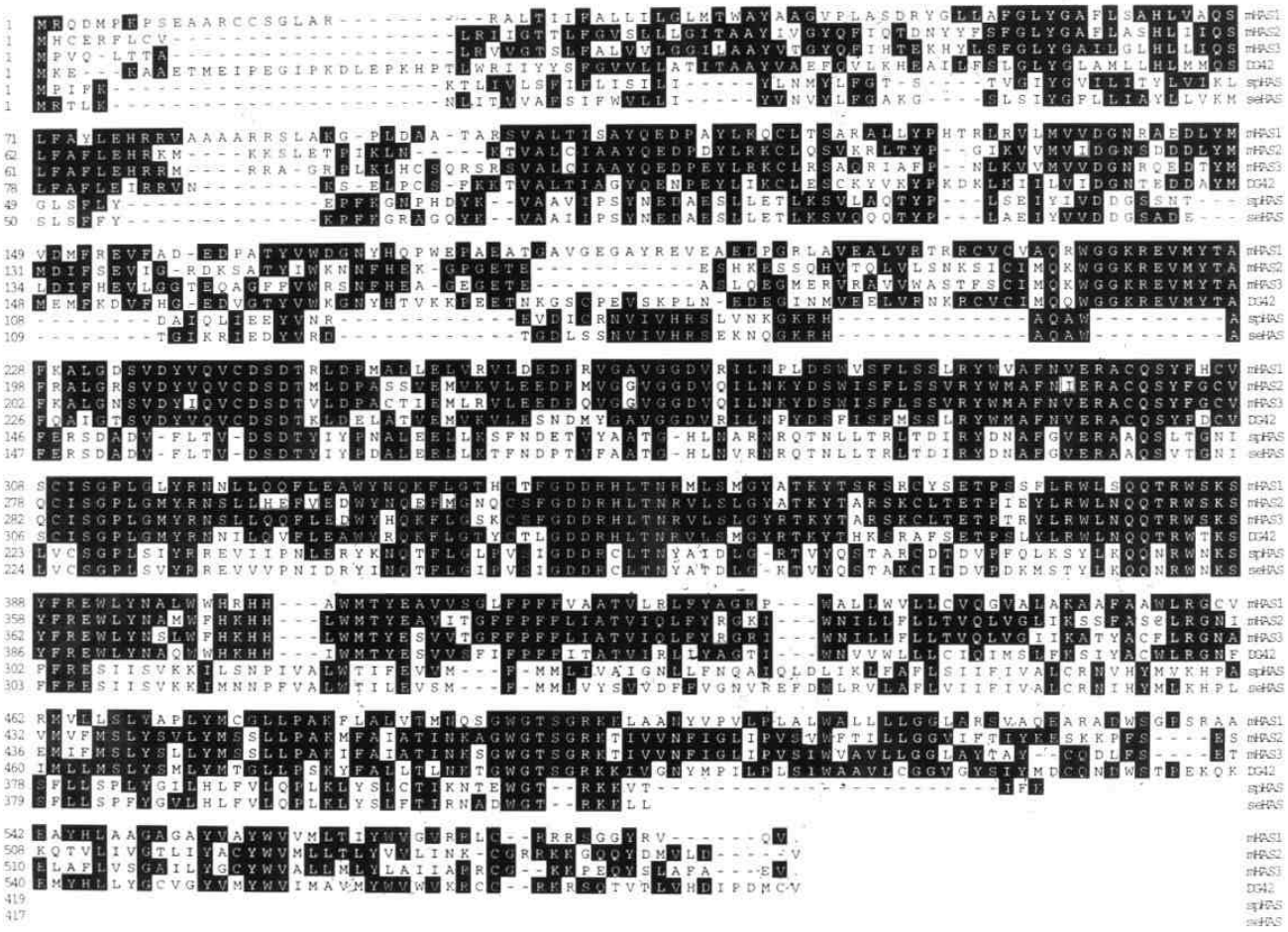


Figure 4. Comparison of amino acid sequencies between vertebrate HAS enzymes (mouse, mHAS1, mHAS2, mHAS3; frog, DG42) and bacteria enzymes (*Streptococcus pyogenes*, spHAS; *Streptococcus equisimilis*, seHAS). Conserved residues are boxed.

Non-steroidal anti-inflammatory drugs, such as indomethacin, have been shown to inhibit *in vitro* synthesis of hyaluronan by skin fibroblasts (August et al, 1994). Glucocorticoid and thyroid hormones also inhibit hyaluronan synthesis in skin fibroblast cultures (Smith et al, 1982; Smith, 1984). The glucocorticoid-dependent prevention of hyaluronan synthesis has physiological implications since it has been demonstrated that glucocorticoid therapy induces skin atrophy due to depletion of hyaluronan and proteoglycans which leads to closer packing of collagen fibrils (Lehman et al, 1983). In addition to these naturally occuring hormones, carboxylic acids, such as n-butyrate, have also been demonstrated to inhibit hyaluronan synthesis (Smith, 1987). The mechanisms involved in their inhibitory action on hyaluronan synthesis are not known.

B. Bacterial and vertebrate hyaluronan synthases

Although hyaluronan was isolated about 60 years ago, the genes involved in its biosynthesis were identified and characterized only recently. Their identification has been approached by isolating stable acapsular mutants of Streptoccocci via transposon mutagenesis and subsequent identification of the DNA flanking the inserted transposon (Dougherty and van de Rijn, 1994; DeAngelis et al, 1993a,b). These studies led to the identification of group A Streptococci genetic locus for hyaluronan synthesis termed *has* (hyaluronic acid synthesis). The *has* operon is composed of three genes, *has*A, *has*B, and *has*C. The *has*A gene codes for a hyaluronan synthase designated spHAS for *S. pyogenes* HAS (Weigel et al., 1997). The *has*B gene

encodes UDP-glucose dehydrogenase (converts UDP-glucose to UDP-GlcA) and *has*C gene exhibits homology to UDP-glucose pyrophosphorylase (converts glucose 1-phosphate and UTP to UDP-glucose). The mechanisms that regulate the expression of the *has* operon are yet not known. However, the *has* operon is adjacent to genes involved in DNA repair (DeAngelis and Weigel, 1995). Operons similar to the *has* operon in *Streptococcus pyogenes* are also found in other Gram-positive bacteria such as *Streptococcus pneumonia*; the Cap3B glycosyltransferase enzyme which produces a cellobiuronic acid is encoded in an operon flanked by UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase (Arrecubieta et al, 1996). The spHAS protein has a molecular weight of 42 kDa (DeAngelis and Weigel, 1994), possesses 4 transmembrane domains and one inner membrane-associated domain (Weigel et al, 1997; Tlapak-Simmons et al, 1998) with a large intracellular loop which contains the catalytic activity of the enzyme. However, its exact membrane topology has not yet been experimentally derived. The spHAS protein shows about 10% similarity to the rizobium *nodC* gene product (a fungal chitin synthase) (Atkinson and Long, 1992), and 30% similarity to the *Xenopus laevis* protein DG42 which was expressed transiently in frog embryos (Developmentally expressed during Gastrulation) (Rosa et al, 1988; DeAngelis et al, 1993a). Recently the hyaluronan synthase from the highly encapsulated strain of group C *Streptococcus equisimilis*, seHAS, was also cloned (Kumari and Weigel, 1997). The two bacterial synthases exhibit about 70% amino acid sequence similarity and are of similar size (**Figure 4**). There is, however, a difference in the rate of hyaluronan synthesis; seHAS polymerizes the growing hyaluronan chains 2-fold faster. More recently a new hyaluronan synthase from the bacterial pathogen type A *Pasteurella multocida*, PmHAS, was cloned (DeAngelis et al, 1998). PmHAS is twice the size of other bacterial HAS (972 amino acids versus 417 residues) and shows higher amino acid similarity to other bacterial glycosyltransferases, such as galactose transferase, than to the known HAS molecules from streptococci. Moreover, PmHAS is predicted to have only two transmembrane domains and possibly both termini are intracellular; this implies that a large part of the enzyme is located outside of the cell. It is a challenge to elucidate the mechanisms through which different bacterial HAS polymerize the same product.

The similarity of spHAS with DG42, whose function at that time was unknown, led to a series of experiments, *e.g* infection of mammalian cells with a DG42 vaccinia virus construct (Meyer and Kreil, 1996), as well as transfection of recombinant enzyme in yeast cells (DeAngelis and Achyuthan, 1996; Pummill et al, 1998) showing that the gene product exhibited hyaluronan synthesizing activity. Thus, DG42 was the first vertebrate enzyme found which had hyaluronan synthase activity. However, other studies have shown that DG42 synthesizes chitin even more efficiently (Semino et al, 1996; Varki, 1996). In 1996, almost simultaneously, several groups reported the molecular cloning of two mammalian HAS genes designated HAS1 and HAS2 (Watanabe and Yamaguchi, 1996; Itano and Kimata, 1996; Shyjan et al, 1996; Spicer et al, 1996). One year later a third HAS gene, HAS3, was identified (Spicer et al, 1997) which was found to exhibit 57%, 71%, 56% and 28% amino acid sequence similarities to HAS1, HAS2, DG42, and spHAS, respectively (**Figure 4**). The similarities between the same HAS gene from different mammalian species are larger than 95%. An important question raised is of course how the expression and enzymatic activity of HAS are regulated.

The mammalian HAS gene family (Spicer et al, 1997; Spicer and McDonald, 1998) has most likely arisen by sequencial gene duplication and divergence. Family members share characteristics such as similarity in flanking genes, location within the genome and location of exon-intron boundaries (Ruddle et al, 1994; Brown et al, 1995; Aruga et al, 1996). Mammalian HAS are located in different chromosomes, HAS1 on human chromosome 19q13.3-q13.4, HAS2 on 8q24.12, and HAS3 on 16q22.1. However, similarities in exon-intron boundaries as well as similarities in the expression pattern of HAS genes in developing mouse embryos (Spicer et al, 1997; Spicer and McDonald, 1998) support the notion that mammalian HAS isoforms belong to an ancient gene family and that the related genes appeared through sequential gene duplication events.

Surprisingly, a glycosyltransferase with the ability to synthesize hyaluronan has recently been described in Chlorella virus PBCV-1 that infects a chlorella-like green algae (DeAngelis et al, 1997). This is the first report where hyaluronan is found outside vertebrates and pathogens. It is important to point out here that the PBCV-1 genome possess genes that encode, in similarity with *A Streptococci*, UDP-Glc dehydrogenase and fructose-6-phosphate amidotransferase that is involved in the UDP-GlcNAc metabolic pathway. Therefore it is likely that the HAS operon in group A *Streptococci* evolved in context with other capsule polysacharide operons in procaryotic organisms as well as in some virus genomes. Further studies are needed in order to understand the evolution of HAS enzymes.

The expression pattern of mRNAs for the three HAS isoforms in response to growth factors differs, indicating different functional roles of the synthases (Sugiyama et al, 1998). Moreover, studies in our laboratory suggest that the three eukaryotic HAS isoforms are expressed differentially, and possess different intrinsic properties in their abilities to polymerize hyaluronan and in their interactions with other

proteins (unpublished observations). Recently, Yamada and his collegues (1998) have reported the structural organization of the mouse HAS1 gene; a CCAAT box is located in the promoter region of the HAS1 gene upstream of the transcription initiation site, as well as binding sites for AP-2 (activated in response to cAMP and phorbol ester), MyoD (regulatory gene for skeletal myogenesis), SPY and Sox-5 genes (expressed during embryogenesis), and IRF-1, IRF-2, and p53. Further studies by site-directed mutations in the HAS1 gene and expression in transgenic mice may elucidate the regulation of HAS1 expression. Yet, nothing is known about the structural organization of the HAS2 and HAS3 genes.

III. Future prospects

Hyaluronan has preserved its simple primary structure throughout the evolution in contrast to the diversification seen in other macromolecules of the extracellular matrix. In inflammatory conditions, such as lung fibrosis, myocardial infarctions, as well as in certain invasive tumors, increased levels of hyaluronan are observed in tissues and exudates. This accumulation of hyaluronan often leads to organ dysfunction and increased tumor invasion and, therefore, the excess hyaluronan has to be eliminated. Increased knowledge about the molecular mechanisms which regulate the activities of hyaluronan synthases will make it possible to design specific inhibitors for hyaluronan synthases which may be of clinical value.

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