MULTIPLE EXOSTOSES GENE, EXT1 AND HEPARAN SULFATE BIOSYNTHESIS

by

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Abstract

Hereditary multiple exostoses (HME), a dominantly inherited genetic disorder characterized by multiple cartilaginous tumors, is caused by mutations in members of the *EXT* gene family, *EXT1* and *EXT2*. The corresponding gene products, exostosin-1 (EXT1) and exostosin-2 (EXT2) are type II transmembrane glycoproteins which form a Golgi-localized hetero-oligomeric complex that catalyzes the polymerization of heparan sulfate (HS). Although the majority of the etiological mutations in EXT are splice-site, frameshift or nonsense mutations that result in premature termination, a significant number of missense mutations have also been identified.

To test the etiological missense mutant of the EXT1 proteins for their ability to synthesize HS *in vivo*, a functional assay that detects HS expression on the cell surface of an EXT1-deficient cell line was used. Of the twelve reported missense mutations, eight were defective in HS biosynthesis, but surprisingly, four were phenotypically indistinguishable from wild type EXT1. Three of these four phenotypically wild type EXT1 like mutations affected amino acids that were non-conserved among vertebrates and invertebrates, whereas all of the HS biosynthesis null mutations affected only conserved amino acids. Further, substitution or deletion of each of these four residues did not abrogate HS biosynthesis. Taken together, these results indicated that not all reported etiological missense mutations abrogated HS-Pol activity. These mutations may instead interfere with as yet undefined functions of EXT involved in HME pathogenesis.

The fact that HME is an autosomal dominant genetic disorder suggests that one mutant copy of the gene should be sufficient to cause disease. However, no dominant negative phenotype was observed, even when mutant and wild-type forms of EXT1 proteins were co-transfected in a ratio of 10:1. Furthermore, the two adjacent amino acid residues G339 and R340, which account for five out of twelve known etiological missense mutations of the EXT1 gene, were localized in a consensus cleavage site for furin, a proprotein convertase. Results from both

in vitro and *in vivo* experiments suggested that the putative cleavage sites were not used and EXT1 protein was expressed in its full-length form.

During the course of this study, nine HS deficient cell lines were isolated based on an HSV-1 resistant phenotype. Surprisingly, EXT1 alone corrected the HS deficiency of all nine mutant cell lines, although four other EXT genes (EXT2, EXTL1, EXTL2 and EXTL3) have now been identified and likely harbour glycosyltransferase activities that contribute to the synthesis of HS. These observations suggest that some glycosyltransferases which are involved in HS biosynthesis may be essential for cell survival and/or may exist in multiple copies. Because none of the nine HS deficient cell lines have detectable defects in EXT2, and only EXT1 corrects the HS deficiency, EXT1 could be the sole enzyme that polymerizes HS in mammalian cells.

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LIST OF ABBREVIATIONS

3-OST	3-O-sulfotransferase
ACV	acyclovir
BHK	baby hamster kidney
BHV	bovine herpes virus
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CS	chondroitin sulfate
DEAE	diethylaminoethyl-
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DS	dextran sulfate
dsDNA	double-stranded deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
EXT	exostosin
EXTL	exostosin-like
FBS	fetal bovine serum
Fc	fragment crystallizable (of immunoglobulins)
FGF-2	fibroblast growth factor-2
FITC	fluorescein isothiocyanate
FMDV	type O foot and mouth disease virus
GAG	glycosaminoglycan

Gal	galactose
GalNAc	N-acetylgalactosamine
GFP	green fluorescent protein
GlcA	glucuronic acid
GlcA-T	glucuronic acid transferase
GlcNAc	N-acetylglucosamine
GlcNAc-T	N-acetylglucosamine transferase
GPI	glycosylphosphatidylinositol
HA	hyaluronic acid
HBD	heparin binding domain
HCMV	human cytomegalovirus
Hh	hedgehog
HHV	human herpesvirus
HIV	human immunodeficiency virus
HME	hereditary multiple exostoses
HPLC	high performance liquid chromatography
HS	heparan sulfate
HS-Pol	heparan sulfate polymerase
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
Hve	herpesvirus entry mediator
IdoA	iduronic acid
IgG	immunoglobulin G
Ihh	Indian hedgehog
IP	immunoprecipitation
KS	keratan sulfate

KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Luria broth
LGS	Langer-Giedion syndrome
LOH	loss of heterozygosity
M6P	mannose-6-phosphate
M6PR	mannose-6-phosphate receptor
MAb	monoclonal antibody
mEXT	murine exostosin
MOI	multiplicity of infection
NDST	N-deacetylase-N-sulfotransferase
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electophoresis
PAPS	adenosine 3' phosphate 5' phosphosulfate
PBS	phosphate buffered saline
PFU	plaque forming units
PG	proteoglycan
Prr1	poliovirus receptor related protein-1
Prr2	poliovirus receptor related protein-2
PRV	pseudorabies virus
Ptc	patched
PTHrP	parathyroid hormone related protein
PVR	poliovirus receptor
RIPA	radioimmunoprecipitation assay
RPM	revolutions per minute
SDS	sodium dodecyl sulfate
ST	swine testes

TGF-β	transforming growth factor-β
TGN	*trans* Golgi network
TNF	tumour necrosis factor
Ttv	tout-velu
UDP	uridine diphosphate
UTR	untranslated region
VZV	varicella zoster virus
XLHED	X-linked hypohidrotic ectodermal dysplasia

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CHAPTER 1: Introduction

1.1 Overview

Viruses are obligate intracellular parasites that are unable to replicate on their own, and thus must enter a host cell and depend on the host cell biosynthetic machinery to replicate. The study of viruses, therefore, often leads to a greater understanding of host cell biology.

To gain entry into the host cell, viruses use host cell surface molecules that normally serve as receptors for intracellular signaling. Herpes simplex virus (HSV) uses heparan sulfate (HS) glycosaminoglycans (GAGs) as receptors for initial attachment to the host cell surface. I have used the HS binding ability of HSV to study the effect of a group of etiological mutations in a putative tumor suppressor gene, EXT1, on the biosynthesis of HS. Patients with inherited mutations in EXT1 develop multiple bone tumors, characteristic of a disease termed hereditary multiple exostosis (HME), and sometimes chondrosarcomas. In an attempt to study the enzymes that are involved in HS biosynthesis, I also took advantage of the fact that HSV uses cell surface HS as receptor to isolate a group of HS deficient cell lines.

The following introduction will address the structure and properties of GAGs, highlighting HS and its ability to facilitate viral entry. I will also review the process of HSV infection with an emphasis on viral entry. Finally, the involvement of EXT1 in HS biosynthesis, bone development, along with the genetics and pathology of HME will be discussed.

1.2 Glycosaminoglycans and Proteoglycans

Proteoglycans (PGs) are ubiquitous molecules present as integral membrane proteins of animal cells and as components of the extracellular matrix. The core proteins of PGs are modified by the addition of long sulfated polysaccharides, called glycosaminoglycans (GAGs). The GAG substitution depends on the ability of the protein to serve as an acceptor for xylosyltransferase, the enzyme that begins the synthesis of most types of GAGs. The versatility of PGs and their capacities for multiple interactions with other molecules gives them the ability to function as a multipurpose receptor in cellular interactions. They can bind together

extracellular matrix components, mediate the binding of cells to the matrix, and capture soluble molecules such as growth factors into the matrix and at the cell surface (Kjellen 1991; Lindahl et al. 1998).

1.2.1 Glycosaminoglycans

GAGs are unbranched heteropolysaccharides consisting of specific repeating disaccharide units (Lidholt and Lindahl 1992). Usually one monosaccharide of the disaccharide repeats is a hexuronic acid, either glucuronic acid (GlcA) or iduronic acid (IdoA), and the other sugar is a hexosamine, either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc). One or both of the sugars are variably N- and O-sulfated, thereby contributing to the diversity of these structures and their high negative charge density. The most common GAGs include heparin, HS, dermatan sulfate and chondroitin sulfate (CS) (Table 1.1). These can be linked through an O-glycosidic linkage to a serine residue in a protein core.

1.2.2 Glycosaminoglycans structure

GAGs are linear polysaccharides consisting of disaccharide repeats of hexuronic acid and hexosamine (Kjellen 1991). The structure of the most common members of the mammalian GAG family are illustrated in Figure 1.1 for heparin/HS and hyaluronan (HA), and in Figure 1.2 for chondroitin sulfate (CS), and dermatan sulfate (DS). HS and heparin are sulfated GAGs, which are composed of alternating units of β1,4-linked hexuronic acid and α1,4-linked glucosamine. The glucosamine is mostly N-acetylated or N-sulfated, but rarely unsubstituted and O-linked sulfate groups can be present at positions C-3 and/or C-6. The hexuronic acid unit is either GlcA or IdoA and where an O-sulfate group can be present at the C-2 position, it is usually found on IdoA rather than GlcA (Fig.1.1A). Heparin has the highest negative charge density of any known biological macromolecule, and is normally stored in cytoplasmic granules of mast cells. HS, on the other hand, has ubiquitous distribution on the cell surfaces and in the extracellular matrix. HS is generally less sulfated than heprain and has more varied structure (Salmivirta et al. 1996).

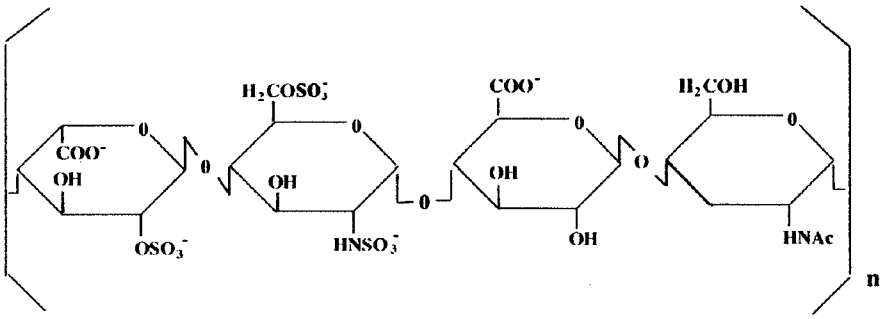
Table 1.1 Occurrence and properties of glycosaminoglycans

Polysaccharide	MW (kDa)	Repeating period monosaccharides	Sulfate per disaccharide unit	Examples of occurrence
Hyaluronic acid	4 – 8000	D-glucuronic acid D-glucosamine	0	connective tissue, skin, synovial fluid, umbilical cord cartilage, vitreous humour
Chondroitin 4- and 6-sulfates	5 – 50	D-glucuronic acid D-galactosamine	0.1 – 1.3	cartilage, cornea, bone, skin, arterial wall
Dermatan sulfate	15 – 40	L-iduronic acid D-galactosamine	1.0 – 3	skin, heart valve, tendon, arterial wall
Heparan sulfate	30[a]	D-glucuronic acid or L-iduronic acid D-glucosamine	0.4 – 2	lung, arterial wall, ubiquitous on most cell surfaces
Heparin	6 – 25	D-glucuronic acid or L-iduronic acid D-glucosamine	1.6 – 3	lung, liver, skin, intestine (mast cells)
Keratan sulfate	4 – 19	D-galactose D-glucosamine	0.9 – 1.8	Cartilage, cornea, invertebral disc

Table reproduced from Lindahl and Hook (Lindahl and Hook 1978)
[a]Average MW of HS chain (Sanderson et al. 1989).

(A) Heparin and Heparan Sulfate:

IdoA β1-4 $GlcNSO_3^-$ α1-4 GlcA β1-4 GlcNAc α1



(B) Hyaluronan:

4 GlcA β1-3 GlcNAc β1

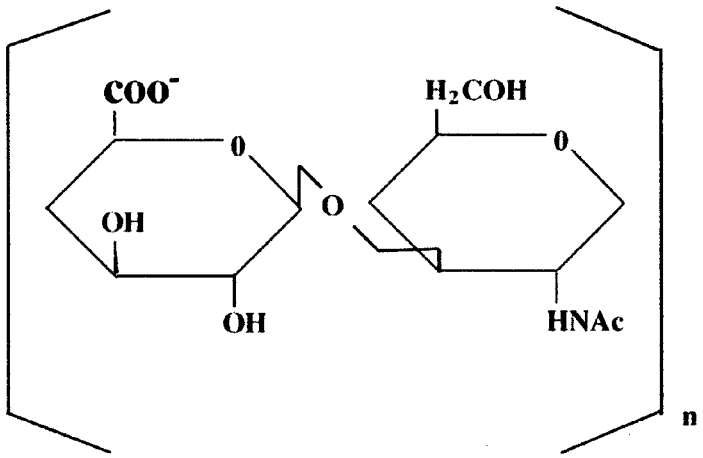
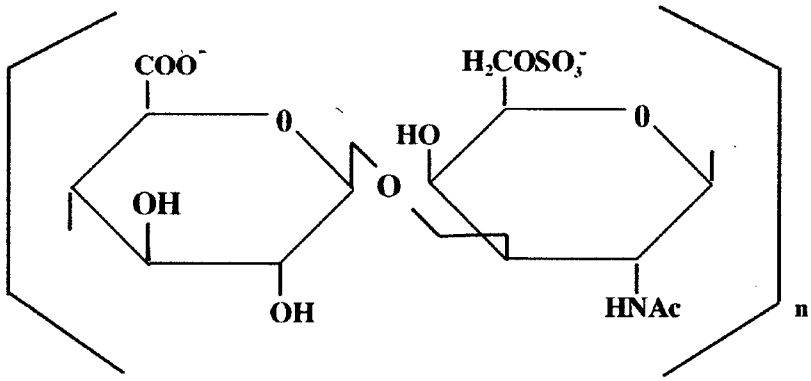


Figure 1.1 Common structure of (A) heparin and heparan sulfate and (B) hyaluronan.

(A) Chondroitin sulfate
4 GlcA β1-3 GalNAc(6S) β1



(B) Dermatan sulfate
4 IdoA β1-3 GalNAc(4S) β1

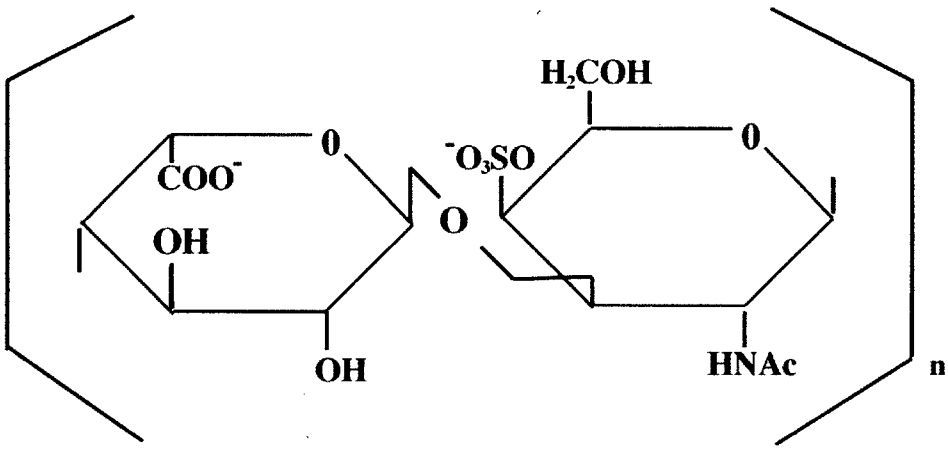


Figure 1.2 Common structure of (A) Chondroitin sulfsate and (B) Dermatan sulfate.

HA is a nonsulfated polymer composed of repeating units of (GlcA β1,3GlcNAc β1,4), the same sugar residues as in HS and heparin, but with different glycosyl linkages (Fig.1.1B) (Laurent and Fraser 1992).

CS and DS share a similar biosynthetic pathway with heparin and HS. CS and DS are also sulfated GAGs but with repeating units of β1,3-linked hexuronic acid and β1,4-linked N-acetyl-D-galactosamine (GalNAc) (Fig.1.2A and 1.2B). The GalNAc residues can be substituted with O-linked sulfate groups at the C-4 and/or C-6 positions whereas the hexuronic acid unit may be sulfated at position C-2 or, more rarely C-3 (Kjellen 1991). The polysaccharide is considered to be CS if all the hexuronic acids are GlcAs and DS if some of the hexuronic acid units are IdoAs.

1.2.3 Heparan sulfate domain structure

Heparin chains usually have an overall high sulfate content whereas HS chains generally have non sulfated as well as highly sulfated parts (Gallagher and Turnbull 1992). The distribution of N-sulfated glucosamine units, which are essential for generation of the fully modified product is not random, but seems organized into domains (Maccarana et al. 1996). These domains can be divided into three types; (1) regions with only N-acetylated glucosamine units, NA domains, (2) regions with only N-acetylated and N-sulfated glucosamine sequences, NA/NS domains, and (3) regions with only N-sulfated glucosamine units, NS domains (Fig.1.3) (Lindahl et al. 1998).

1.2.4 Biosynthesis of HS/CS common tetrasaccharide linker region

The two most abundant GAGs on plasma membrane PGs are HS and CS. Both HS and CS biosynthesis occurs by the concerted action of several membrane bound enzymes located in the endoplasmatic reticulum (ER) and Golgi compartments (Salmivirta et al. 1996; Silbert and Sugumaran 1995).

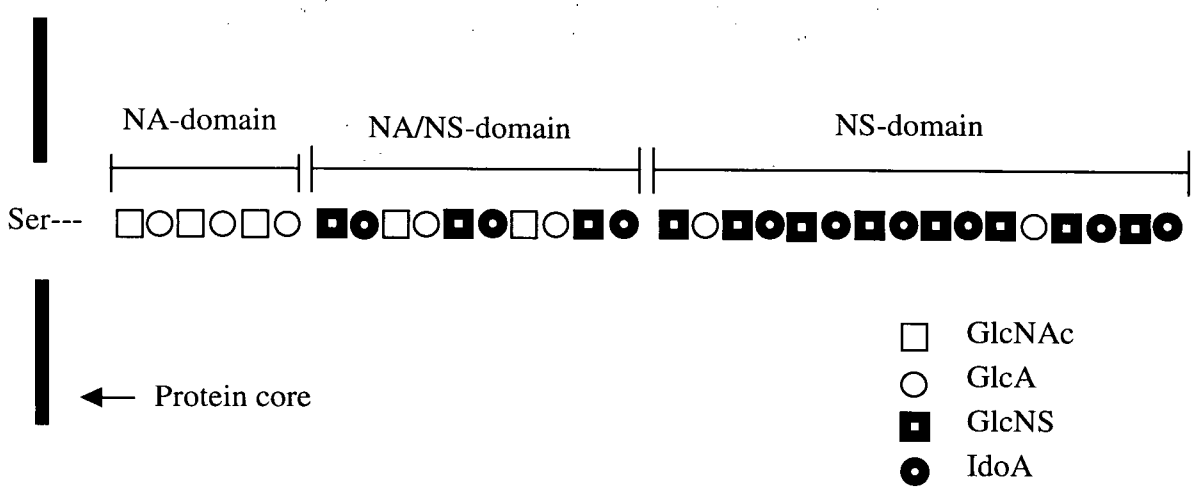


Figure 1.3 Domain structures of the HS chains.
Common O-linked sulfate groups within each domain are noted. The Xyl-Gal-Gal linkage region is represented by three short lines(---).

Figure 1.4 illustrates the sugars and their corresponding glycosyltransferases involved in the formation of the linkage region and the HS and CS chains, and represents a general overview of biosynthesis. The first enzyme involved in the formation of the linkage region is a xylosyl transferase (Xyl-T) which adds Xyl, from UDP-Xyl, to the serine residue in the HS/CS attachment motif in the protein core (Kearns et al. 1993). Xyl-T activity has been found in ER and in cis-Golgi compartments (Vertel et al. 1993) and it seems to initiate both HS and CS linkage regions (Esko 1985). The next enzyme acting in the formation of the linkage region, galactosyltransferase I (Gal-TI), was found to co-precipitate with antibodies raised against Xyl-T, and it also bound to immobilized Xyl-T (Schwartz and Dorfman 1975). These properties might reflect that Xyl-T follows the core protein from the ER to the early Golgi compartment where it directs Gal-TI to its substrate. Linkage regions of both HS and CS chains can have a phosphate group attached to the Xyl residue (Fransson et al. 1985).

Two consecutive Gal residues are added on the Xyl unit by the action of Gal-TI and Gal-TII, respectively, from UDP-Gal. Gal-TI adds the first Gal residue in the linkage region in the synthesis of both HS and CS (Esko 1987), and both Gal-TI and Gal-TII reside in the cis- and medial-Golgi fractions (Sugahara et al. 1988). Glucuronyl transferase I (GlcA-TI) completes the linkage region. This enzyme is different from the HS and CS GlcA-TIIs, which are involved in the GAG chain elongation step (Lidholt and Lindahl 1992). GlcA-TI has been co-localized with CS GlcA-TII to both the medial- Golgi and trans-Golgi network but is distributed differently compared to Gal-TI and Gal-TII (Sugumaran et al. 1998). GlcA-TI, also common for HS and CS linkage region synthesis, was the first glucosyltransferase in HS/CS biosynthesis to be cloned. This was accomplished by the use of polymerase chain reaction primers designed from sequence alignment of other glycosyltransferases (Kitagawa et al. 1998).

1.2.5 Heparan sulfate chain formation

HS will be formed if a GlcNAc sugar is added by GlcNAc transferase I (GlcNAc-TI) to the completed linkage region. The GlcNAc-TI is distinct from the HS chain elongation GlcNAc

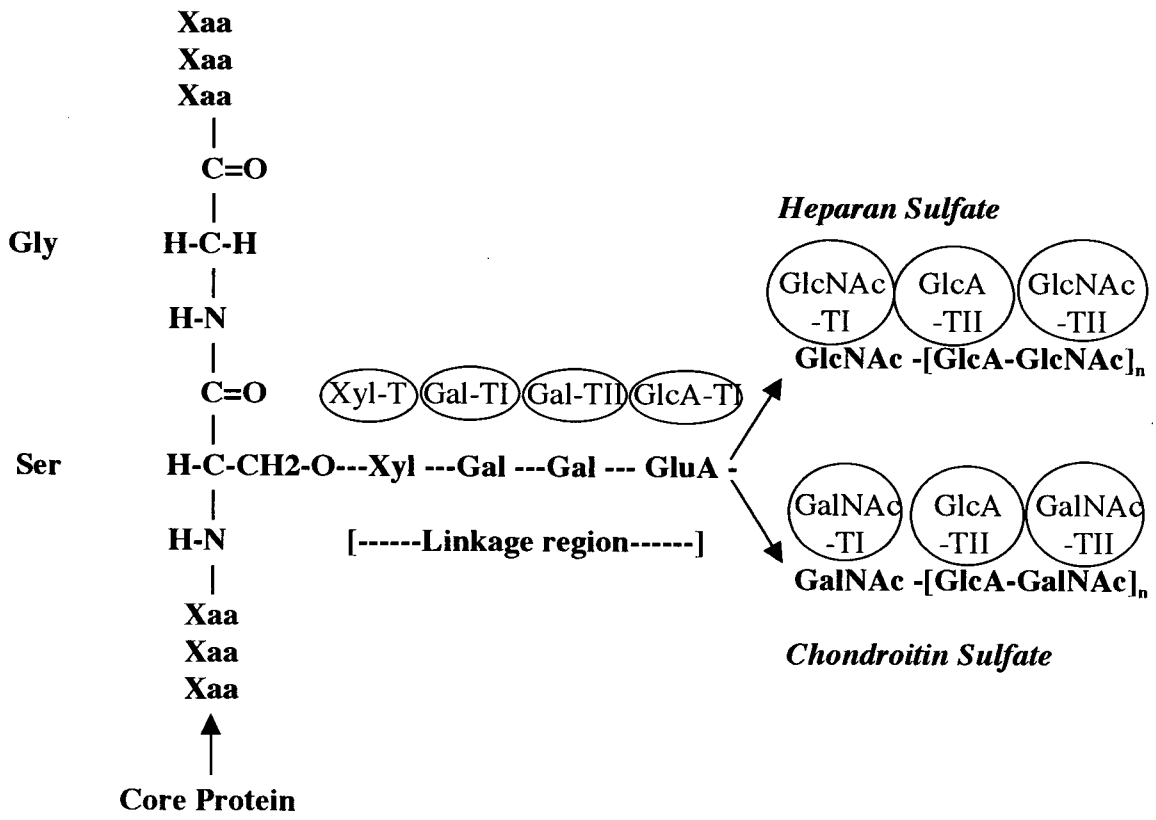


Figure 1.4 The sugar units and their corresponding glycosyltransferases involved in HS and CS biosynthesis.

Abbreviations are as follows; Gly (glycine), Ser (serine), Xaa (any amino acid), Xyl (xylose), Gal (galactose), GlcA (glucuronic acid), GlcNAc (N-acetylglucosamine), GalNAc (N-acetylgalactosamine).

transferase, GlcNAc-TII (Zhang et al. 1995). On the other hand, if a GalNAc sugar is added to the completed linkage region by the GalNAc-TI enzyme, it is committed to synthesis of a CS backbone.

After the first GlcNAc sugar was added to the common tetrasaccharide linker, subsequent steps involve the alternating transfer of GlcNAc and D-glucuronic acid (GlcA) monosaccharide units, from the corresponding UDP-sugar nucleotides to the growing chains (Lidholt and Lindahl 1992).The mechanism for termination of the chain is unknown. Following polymerization of the HS backbone, the polymer is modified through a series of consecutive reactions that are initiated by N-deacetylation and N-sulfation of GlcNAc, both of which are catalyzed by a single bi-functional enzyme in the Golgi (Aikawa and Esko 1999). This sulfotransferase reaction, and those reactions that follow, obtain sulfate from the sulfate transporter 5'-phosphosulfate 3'-phosphoadenosine (PAPS). Next, adjacent GlcA subunits are converted to IdoA by an epimerase (Crawford et al. 2001). The presence of IdoA residues in the GAG chain adds conformational flexibility to the polymer, and therefore allows the HS polymer to adopt a greater range of three dimensional structures, providing more potential binding sites for different proteins (Lindahl et al. 1998). The epimerization step is followed by extensive 2-O-sulfation of GlcA/IdoA (Kobayashi et al. 1996) and 6-O-sulfation of N-sulfoglucosamine (Habuchi et al. 1998). Some minor reactions include 3-O-sulfation of GlcNAc (Shworak et al. 1997; Shworak et al. 1999). Most of the modification reactions are incomplete, and only a fraction of the potential target units are actually attacked by the corresponding enzymes. These cooperative and incomplete enzymatic reactions lead to an extremely heterogeneous saccharide sequence with a broader range of biological properties (Lindahl et al. 1998).

1.3 Human pathogens that use HS as a gate of entry.

The structural complexity of GAGs, in addition to their abundance on the surface of almost all mammalian cells, has made them attractive targets for a number of important pathogens. Over time, many pathogenic microorganisms have evolved to exploit cell surface PGs

as receptors for attachment, a process, which ultimately facilitates invasion. The interaction of specific proteins on the surface of microorganisms (adhesins) with GAGs on the PGs (receptors) enables the microbes to take the first step towards establishing an infection.

These pathogens include bacteria, like *Chlamydia trachomatis* and *Neisseria gonorrhoeae* as well as protozoans like *Leishmania* (Rostand and Esko 1997). For viruses, herpes simplex virus (HSV) was the first virus shown to bind HS (WuDunn 1989). Since then, other herpesviruses have been found to use HS as an initial receptor, including human cytomegalovirus (HCMV) (Compton et al. 1993), human herpesvirus 7 (HHV-7) (Secchiero et al. 1997) (Table 1.2), pseudorabies virus (PrV) (Mettenleiter et al. 1990), and bovine herpesvirus type 1 (BHV-1) (Okazaki et al. 1991). Recent reports have also implicated HS as a receptor for human immunodeficiency virus (HIV) (Patel et al. 1993), adeno-associated virus (AAV) (Summerford and Samulski 1998) and type O foot and mouth disease virus (Jackson et al. 1996). Studies of the nature of the interactions between those microbes and the cell surface GAGs reveal that most of the microbe binding activity consists of reversible, low-affinity interactions. Thereby the microbe requires subsequent high-affinity interactions with specific cell surface receptors to complete the process of entry into the host cell.

1.4 The family of Herpesviruses

The herpesvirus family is large and contains more than 100 members that infect organisms from oysters to humans. Herpesvirus particles consist of (i) a large linear double-stranded DNA genome, ranging in size from about 100,000 to 250,000 base pairs; (ii) an icosahedral capsid, which packages the genome and has 162 morphological units; (iii) an amorphous tegument surrounding the capsid, consisting of a group of virus-encoded proteins that are critical for the establishment of infection and transcription of early genes, and (iv) a lipid-containing envelope derived from intracellular membranes (Fig 1.5).

Table 1.2 Heparin-binding proteins of the human herpesviruses and roles in viral entry.

Subfamily	Virus	Glycoprotein	Role in entry	Other comments
Alpha-	HSV-1 and HSV-2	gC	Binds HS & CS to mediate virus binding to cells	Binds C3b to inhibit complement activation
		gB	Binds HS & CS to mediate virus binding to cells; required for entry	Participates in membrane fusion for entry,
		gD	Binds 3-O-sulfated HS to mediate entry	Also binds protein receptors to mediate entry
	VZV	gB	Binds to HS; soluble gB can inhibit infection	
Beta-	CMV	gB	Binds to HS; soluble gB can inhibit infection	Binds to another receptor to induce interferon-responsive genes
		gM	Binds to HS; probably the principal ligand for HS in virus binding	Another viral glycoprotein forms part of the HS binding complex
	HHV7	gB	Binds to heparin and cell surface HS	
		gp65	Binds to heparin and HS	

Table reproduced from Shukla and Spear, 2001 (Shukla and Spear 2001).

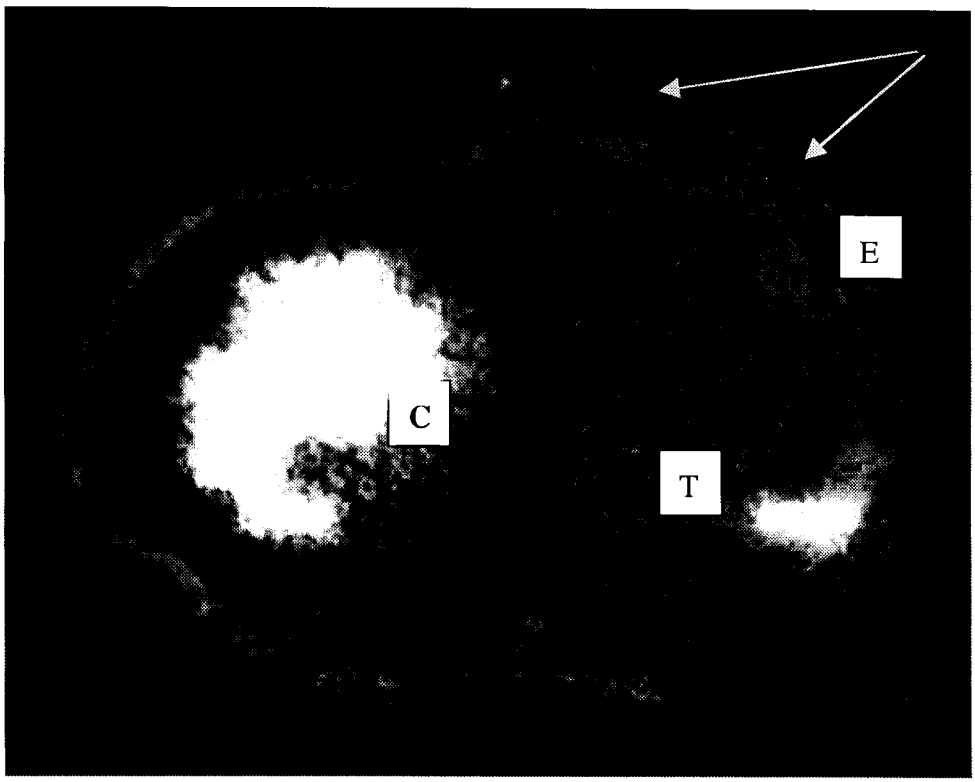


Figure 1.5 Electron micrograph of the HSV particle (x 100,000).

The HSV virion consists of a double-stranded (ds) linear DNA genome contained within an icosahedral capsid (C). Surrounding the capsid is the amorphous tegument (T). The virion envelope (E) contains glycoprotein spikes, which are indicated by the arrows. This image was obtained from the University of Cape Town website (**http://www.uct.ac.za/depts/mmi/stannard/emimages.html**), and was originally published by Stannard and colleagues in 1987 (Stannard et al. 1987).

Eight herpesviruses, differing markedly in their biology, are known to infect humans. These include herpes simplex types 1 and 2 (HSV-1, HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), HHV-7, and HHV-8, also known as Kaposi's sarcoma-associated herpesvirus (KSHV). Based on genomic analysis and other biological characteristics, the herpesviruses are classified into three subfamilies: alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae. HSV and VZV belong to the subfamily alphaherpesvirinae. Betaherpesvirinae consist of HCMV, human HHV-6 and HHV-7, and typically have longer replicative cycles. The gammaherpesvirinae are lymphotrophic, targeting B and T lymphocytes, and are represented by EBV and the recently identified HHV-8/KSHV.

1.4.1 Clinical pathology of HSV-1 and HSV-2

There are two serotypes of HSV, designated HSV-1 and HSV-2. HSV infections occur worldwide and are one of the most common afflictions affecting mankind. HSV-1 preferentially infects the oral mucosa and is associated with facial vesicular lesions, and it can also cause keratitis and encephalitis in certain individuals. HSV-2 is primarily associated with severe recurrent genital lesions, but is also associated with neonatal infections. Worldwide, 70% of people over the age of 40 have antibodies against HSV-1 (Roizman and Spear 1994). In the United States, the seroprevalence of HSV-2 in people 12 years of age or older was 21.9 percent between 1988 and 1994, although less than 10 percent of all those who were seropositive reported a history of genital herpes infection (Fleming et al. 1997). Improvements in the prevention and treatment of HSV infections are needed, particularly since genital ulcers may facilitate the transmission of the human immunodeficiency virus.

1.4.2 A brief overview of the HSV life cycle

The viral replicative cycle consists of two distinct phases; the lytic phase and the latent phase. The viral replicative cycle is relatively short for HSV, lasting approximately 18 hours, and

results in the production of infectious viral progeny and the destruction of the host cell (Ward and Roizman 1994). In cell culture, virally infected cells become round in appearance and lose their adhesive properties. Other cytopathic effects include degradation of cell nucleoli and chromatin. The lytic cycle of HSV can be divided into three stages: (a) viral entry, (b) viral replication, and (c) viral egress. For the latent phase, the HSV genome is able to remain in a quiescent episomal form for years, until an as-yet-unknown stimulus reactivates the virus and causes a lytic infection.

1.4.3 The HSV entry pathway into host cells

Enveloped viruses enter cells by inducing fusion between the viral envelope and a cell membrane. This membrane fusion can be triggered in at least two ways, resulting in different pathways of entry. For example, the binding of Influenza A virus to a cell induces endocytosis of the virus, followed by acidification of the endosome, which can trigger fusion between the viral envelope and the endosomal membrane (Schoen et al. 1996). Alternatively, the binding of a virus to a cell may result in multiple receptor-ligand interactions at the cell surface that can trigger fusion between the viral envelope and the plasma membrane. Most herpesviruses apparently enter cells via the latter pathway. They can fuse directly with the plasma membrane of the cell.

Entry of HSV-1 and HSV-2 into their host cells requires the binding of virus to receptors on the cell surface, which is followed by fusion of the virion envelope with the plasma membrane. This process is complex and likely involves multiple interactions between viral glycoproteins and cell surface components. Current evidence suggests that of the 11 viral envelope glycoproteins, only gB, gD, gH, and gL are essential for the process of viral entry in cell culture (Shukla and Spear 2001). Furthermore, while either gC or gB can mediate the binding of virus to cells, gB, gD, gH, and gL are all required for the membrane fusion that leads to viral entry (Fig. 1.6). An additional glycoprotein, gK, has been shown to be absent from the

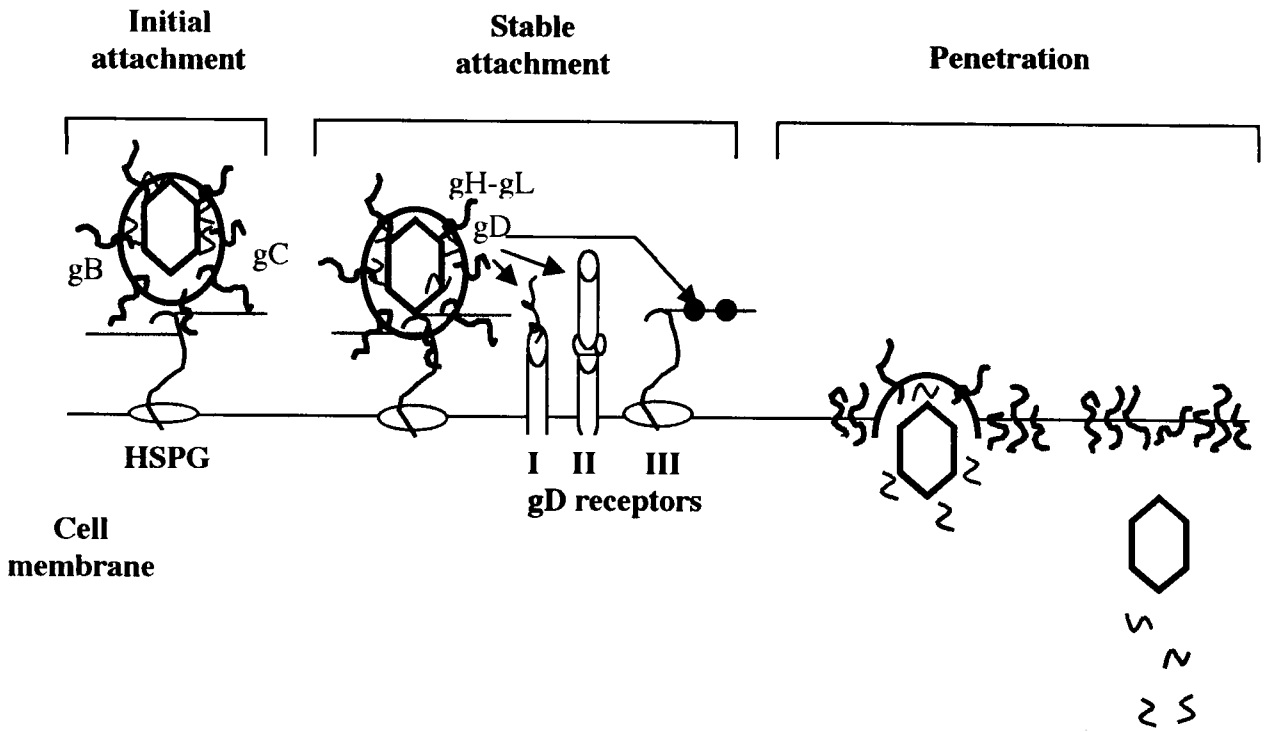


Figure 1.6 Entry of HSV-1 into cells.

The initial contact of virus with cell is usually the binding of virus to HS chains on cell surface proteoglycans (HSPG). Either of the viral glycoproteins gB or gC can mediate this binding. Then viral gD can bind to any one of several entry receptors, including HVEM (I), a member of the TNF-receptor family; nectin-1 or nectin-2 (II), two related members of the immunoglobulin superfamily; or sites generated in heparan sulfate by the action of specific 3-O-sulfotransferases (III). The binding of gD to one of its receptors activates viral membrane-fusing activity, which requires the action of gB and the gH-gL complex as well as gD and a gD receptor. Fusion between the viral envelope and cell membrane liberates the viral nucleocapsid and tegument into the cell cytoplasm. Adapted from Shukla and Spear, 2001 (Shukla and Spear 2001).

virus envelope; however, it is required for the production of infectious virions (Hutchinson et al. 1995).

1.4.4 Two-step process in virus attachment

1.4.4.1 Initial binding

The current model for HSV infection indicates that gB and gC are involved in the initial attachment phase through the interaction of positively charged glycoprotein structures with negatively charged HS (Lycke et al. 1991) and CS GAGs (Banfield et al. 1995) located on the cell surface PGs. Cells treated with heparinases or altered by mutations that prevent HS biosynthesis have reduced capacity to bind virus and are at least partially resistant to virus infection (Shieh et al. 1992; WuDunn 1989). In fact, HSV-1 or HSV-2 can be used to select for cells mutated in enzymes required for HS biosynthesis (see Chapter 3)(Banfield et al. 1995; Gruenheid et al. 1993) and to screen for cells in which the mutations have been complemented (McCormick et al. 1998). Both HSV-1 gB and gC extracted from virions and soluble forms of gB and gC bind to heparin or heparan sulfate or both (Herold et al. 1991; Trybala et al. 2000). Absence of gC from virions reduces the ability of virus to bind to cells and absence of both gB and gC virtually eliminates binding (Herold et al. 1994). Thus, it has been concluded that the initial interaction of HSV with cells is binding to cell surface HS and that gB, gC, or both can mediate this binding.

1.4.4.2 Stable attachment

Viral entry sometimes requires interactions of viral proteins with coreceptors or entry receptors that are distinct from those that mediate initial virus binding. Such entry receptors are probably required by all the herpesviruses. The search for these receptors can be greatly facilitated by identification of a cell line to which virus binds but fails to penetrate due to lack of entry receptors. For example, taking advantage of the fact that Chinese hamster ovary (CHO) cells are resistant to infection by HSV-1, Montgomery et al. introduced pools of human cDNAs

and screened for plasmids that could render the cells susceptible to viral entry (Montgomery et al. 1996). It was then possible to identify the cell surface molecules that served as entry receptors and the viral ligand for these receptors, which proved to be gD. Surprisingly, at least three classes of human gene products could each independently bind to gD to serve as entry receptors (Spear et al. 2000). These include a member of the TNF-receptor family named HVEM (HveA) (Montgomery et al. 1996), two members of the immunoglobulin family designated nectin-1 (HveC) (Geraghty et al. 1998) and nectin-2 (HveB) (Warner et al. 1998), and a specific modification in HS that generates sites for the binding of gD (Shukla et al. 1999) (Table 1.3). Any one of these gD receptors is sufficient to mediate viral entry. The interaction of gD with one of its receptors triggers fusion between the viral envelope and cell membrane, which requires the participation of gB and gH-gL as well as gD and its receptor (Fig 1.6).

1.4.5 HveA/HVEM/ TNFRSF-14

The search for stable attachment receptors for HSV, as with the search for initial attachment receptors, was made possible by employing HSV-resistant cell lines. By screening a human cDNA expression library for genes that could mediate entry into HSV-resistant CHO-K1 cells, a single cDNA was isolated which rendered the cells 100 to 1000 fold more susceptible to HSV-1 upon transfection (Montgomery et al. 1996). Sequencing of the cDNA revealed that it encoded a 283 amino acid protein, with three complete and one partial cysteine-rich repeats characteristic of members of the tumour necrosis factor (TNF)/nerve growth factor receptor family. The protein was designated Herpesvirus entry mediator (HVEM), later renamed Herpesvirus entry mediator A (HveA), and officially named tumour necrosis factor receptor superfamily 14 (TNFRSF-14) to indicate its place in an established family of proteins. HveA-transfected CHO-K1 cells also became susceptible to HSV-2 (Table 1.3), but not to the related porcine pseudorabies virus PRV nor bovine herpesvirus 1, indicating that HveA is not a general

Table 1.3 Human Proteins that Serve as gD Receptors for HSV-1 and HSV-2 Viral entry

Entry mediator		Mediates entry of	
Protein family	Names	HSV-1	HSV-2
TNF receptor	HveA (HVEM;ATAR; TNFRSF-14)	Yes[a]	Yes
Related subfamily of the immunoglobulin superfamily	HveB (Nectin-2; Prr2)	No	Yes
	HveC (Nectin-1; Prr1)	Yes	Yes
Sulfotranferase	3-OST-3_A & 3-OST-3_B	Yes	No

[a] "Yes" and "No" indicates that the protein has been tested for entry activity in HSV-resistant cell lines and is able or not to facilitate entry for each virus.
Adapted from Shukla and Spear (Shukla and Spear 2001).

mediator of alphaherpesvirus entry. HveA is mainly expressed in lymphoid cells (Montgomery et al. 1996). Deletion of the cytoplasmic tail from HveA has no effect on the ability of HveA to mediate HSV entry (Montgomery et al. 1996). Thus, signal transduction seems not to be required for viral entry.

1.4.6 HveB/Prr2/Nectin-2

In addition to human lymphoid cells, HSV can also infect cells of neuronal and epithelial origin. Therefore other receptors must exist for HSV entry. The finding that certain mutant strains of HSV-1 are unable to use HveA as a stable attachment receptor spurred yet another search for an HSV receptor. By repeating the screening of a human cDNA library for HSV-entry mediating cDNAs, another single cDNA was isolated that could confer susceptibility to infection (Warner et al. 1998). This cDNA could mediate infection of several mutant strains of HSV-1, and wild type HSV-2 into CHO cells. However, it failed to support entry of wild type HSV-1. Sequencing of this cDNA revealed that it was identical to a previously described member of the immunoglobulin superfamily, poliovirus receptor related protein-2 (Prr2) (Eberle et al. 1995). This protein was designated Herpesvirus entry mediator B (HveB), indicating its new-found function. Recently, HveB was renamed as nectin-2 and further was found to function as cell-cell adhesion molecules (Takahashi et al. 1999). HveB is expressed in human neuronal cell lines, fibroblastic cells, keratinocytes, and like HveA, primary activated T lymphocytes. However, antibodies to HveB did not block infection of many of these cells, suggests other receptor(s) may exist in the surface of these cells. The observed differences in the ability of HSV-1 and HSV-2 strains to use HveB may account, in part, for differences in viral tissue tropisms and pathogenicity (Spear et al. 2000). It will be important to learn whether HveB, with its specificity for one serotype, contributes to differences in the biology of HSV-1 and HSV-2.

1.4.7 HveC/Prr1/Nectin-1

Neither HveA nor HveB function as a co-receptor for both serotypes into epithelial cells,

the mucosal epithelia being the site of initial replication upon HSV infection of the host. For this reason, Geraghty and colleagues set out to isolate a common co-receptor for HSV-1 and HSV-2 (Geraghty et al. 1998). Considering the ability of HveB/Prr2 to act as a stable attachment receptor for HSV, the next logical step was to test the Prr2 homologs, poliovirus related receptor-1 (Prr1) (Eberle et al. 1995). In their study, Geraghty et al. found that Prr1 protein, designated as herpesvirus entry protein C (HveC), was able to mediate entry of both HSV-1 and HSV-2 viruses into resistant CHO cells. Prr1 was later renamed as nectin-1. Despite their abilities to act as herpesvirus entry mediators, HveA and HveB do not display the tissue distribution necessary to mediate infection of the epithelial and neural tissues that HSV normally infects. Remarkably, HveC is expressed in high levels in human cells of both epithelial and neural origin (Geraghty et al. 1998). Thus, HveC is the prime candidate for the secondary receptor that allows both HSV-1 and 2 to infect epithelial cells on mucosal surfaces and spread to cells of the nervous system.

1.4.8 3-O-sulfated heparan sulfate

The previously identified gD-binding receptors for HSV, HveA, HveB, and HveC are all cell surface proteins, encoded by cDNAs isolated from a human cDNA library. Surprisingly, yet another round of screening cDNAs, this time from a mouse cDNA library, for the ability to mediate entry into CHO-K1 cells resulted in the isolation of a cDNA closely related to the human gene 3-O-sulfotransferase 3 (3-OST-3) (Shukla et al. 1999), which modifies HS late in biosynthesis (Lindahl et al. 1998). The sites in HS that can serve as gD receptors for entry only function for HSV-1 and are generated by the action of specific 3-O-sulfotransferases (3-OSTs) (Shukla et al. 1999). At least five isoforms of 3-OSTs (3-OST-1, 3-OST-2, 3-OST-3_A, 3-OST-3_B, and 3-OST-4) are found in humans (Shworak et al. 1999). Whereas 3-OST-1 has been shown to generate binding sites for antithrombin (Shworak et al. 1997), 3-OST-3_A and 3-OST-3_B generate binding sites for HSV-1 gD (Liu et al. 1999; Shukla et al. 1999). Accordingly, CHO cells expressing 3-OST-1 remain resistant to HSV-1 entry whereas CHO cells expressing 3-OST-3_A or 3-OST-3_B are susceptible to HSV-1 entry. While the precise structure of the gD-binding site

generated in HS by 3-OST-3_A or 3-OST-3_B is not yet known, part of the structure can be deduced from disaccharide analysis of HS modified by these enzymes, which appear to be identical in substrate specificity. Figure 1.7 shows the structure generated by either 3-OST-3_A or 3-OST-3_B (3-OST-3s) in comparison with that generated by 3-OST-1. Unlike the HveA, B and C stable attachment receptors, 3-OST-3s are widely expressed in human tissues, suggesting that in tissues that do not express any of the Hve-receptors, 3-OST-3s modifications of HS may compensate and permit virus entry.

1.4.9 Viral penetration

Enveloped viruses, such as HSV, can either enter cells by fusion with the plasma membrane or by endocytosis. Agents that block endocytosis do not inhibit HSV entry, which suggests that HSV fuses with the plasma membrane (Fuller et al. 1989). Furthermore, it has been shown that HSV entry by endocytosis results in degradation of the virus and non-productive infection (Campadelli-Fiume et al. 1988).

The HSVglycoproteins gB, gD gH and gL are required for viral penetration and are essential for productive HSV infection (Cai et al. 1988; Hutchinson et al. 1992). HSV mutants deleted for any of these four glycoproteins are able to attach to cells but unable to penetrate. Furthermore, anti-gB, gD and gH neutralizing antibodies permit attachment to cells but prevent penetration (Fuller et al. 1989). The activity of gH is dependent on gL (Hutchinson et al. 1992). gH and gL from a hetero-oligomer complex which is incorporated into virions. Co-expression of gH and gL is required for normal post-translational modifications, folding and intracellular transport of both glycoproteins (Roop et al. 1993).

Much of the current understanding of the functions of gB, gC, gD, gH, and gL comes from studies of deletion mutants lacking these proteins (Roizman 1999). The conclusions drawn from these studies are predicated on the assumption that the absence of one protein does not affect virion composition with respect to the others. Domains critical for viral penetration have

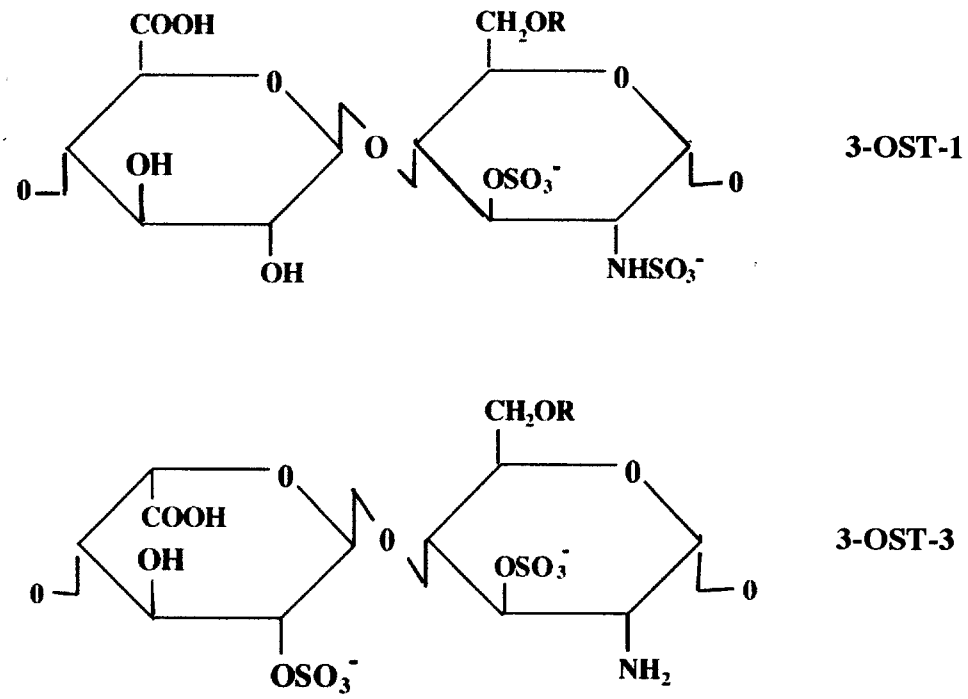


Figure 1.7 Structural features of disaccharides in HS within the sites modified by 3-OST-1 and 3-OST-3_A or 3-OST-3_B.

Substrate recognition by each enzyme probably involves binding to more than two residues in the polymer. The sulfate group added by each enzyme is shown in red. The disaccharide found in 3-OST-1 modified HS is part of the pentasaccharide to which antithrombin binds. The disaccharide found in 3-OST-3 modified HS is part of the binding site for gD, the size and overall structure of which is not yet known. (Adapted from Spear et al. 2000).

been mapped for HSV-1 gB. Anti-gB monoclonal antibodies that inhibit viral penetration map to amino acid residues 241 to 441 in the gB molecule, a region that is centrally located within the ectodomain (Highlander et al. 1988). With respect to gD, all three classes of host cell surface receptors for HSV entry interact with gD and serve as gD Receptors (Fig. 1.6 and Table 1.2). It is of interest to point out that HveB is a coreceptor for HSV-2 entry but not for wild type HSV-1 strain unless they have amino acid substitutions at position 25 or 27 of HSV-2 gD (Lopez et al. 2000).

Despite these findings, the molecular mechanisms underlying HSV penetration remain unclear. It is evident, however, that HSV-1 gB, gD, gH, gL and also gC can associate with one another in the virion envelope (Handler et al. 1996). Chemical cross-linking studies have shown that these glycoproteins can from homodimers and hetero-oligomers of gD linked to gB, gC linked to gB and gD, and gH-gL linked to gD and gC. The gH-gL complex does not appear to associate with gB (Handler et al. 1996a). However, complexes of gB and gD which were present during attachment disappeared as penetration proceeded. It is possible that during penetration, the glycoproteins undergo a conformational change or that the virion envelope undergoes a physical change (such as during the formation of a fusion pore). Together, these findings support the concept that the HSV viral glycoproteins form a complex during entry (Handler et al. 1996a; Handler et al. 1996b).

1.5 Characterization of GAG-deficient cell lines

As part of a broad study to identify host cell factors that facilitate HSV infection, a selection procedure was developed to isolate HSV-resistant mouse cells (gro mutants) (Tufaro et al. 1987). In this procedure, mouse L cells were infected with HSV-1 at an MOI of 1-3 PFU/cell, and after three days, any surviving cells were clonally expanded. It has been shown previously that HSV infection of a gro mutant, gro2C, which synthesizes CS but not HS, is 90% resistant to infection relative to parental control cells (Gruenheid et al. 1993) (Table 1.3). The residual infection remained sensitive to inhibition by soluble HS, however, suggesting that gro2C cells

possess a GAG-dependent pathway of infection. Considering the levels of residual infection of gro2C cells, it was proposed that it should be possible to select for a variant of gro2C cells even less susceptible to infection that was defective in additional components of the virus entry pathway.

When gro2C cells were challenged by 'herpes selection', a cell line was isolated that was nearly uninfectible by HSV-1. This cell line, termed sog9, harbors additional defects in the GAG synthesis pathway such that no HS or CS GAGs are expressed on the cell surface (Table 1.4).

HSV-1 infection of these cells is independent of GAGs, and can be restored to the level of gro2C cell infection by incubating the cells in a low concentration of a heparin/HS analog, dextran sulfate, which presumably coats the virus allowing it to bind to the cell surface by way of a dextran sulfate scavenger receptor (Dyer et al. 1997). This study provides compelling genetic evidence that HSV possesses a 'second' pathway for infection, and that CS as well as HS moieties are replaceable elements in the initial attachment of the virus to the host cell. Besides being used by HSV and other pathogens as a gateway to enter the host cells, HS expression is also likely involved in the bone development as suggested by the fact that mutation in either EXT1 or EXT2, which encode HS polymerase, is linked to the development of bone tumours.

1.6 Hereditary bone tumours – an overview

Bone development is a highly regulated process sensitive to a wide variety of hormones, inflammatory mediators and growth factors. Bone at the tissue level undergoes remodeling: it is continuously being resorbed and rebuilt (or formed). A negative balance between bone resorption and formation is the basis of many bone diseases (Stickens and Evans 1998).

1.6.1 Bone development

Bone is a complex tissue composed of cells, collagenous matrix and inorganic elements. The growth, development and maintenance of bone are influenced by a wide variety of cytokines, growth factors and hormones. Long bones grow by extension at either end in growth

Table 1.4 Relative infectivities of HSV on control and mutant cell lines

Virus	Relative infectivity(%)[a]		
	L(control)	gro2C	sog9
HSV-1 (KOS)	100	10	0.3
HSV-1(F)	100	20	0.8
HSV-2(G)	100	3.3	1

[a]Titers of serial 10-fold dilutions of virus stocks grown in Vero cells were determined on cell monolayers. Results from at least three determinations were averaged and expressed relative to control L cell infection. This data first appeared in (Banfield et al. 1995).

zones situated between the marrow-filled diaphysis (which is bone) and two growth plates, which separate bone from the bulbous, cartilagenous ends (called epiphysis) (Fig 1.8). These bones grow via a strictly coordinated event, involving chondrocytes and osteocytes in the growth plate.

Chondrocytes, cells that synthesize cartilage matrix in the epiphyses, proliferate, become larger and eventually apoptose. This leaves cavities that are subsequently invaded by osteocytes, present in the diaphyses, which replace cartilage with bone. The hypertrophy and apoptosis of the chondrocytes in the growth plate is thought to be caused, at least in part, by the presence of the perichondrial ring that surrounds the growth plate and prevents diffusion of nutrients and oxygen into the cartilage matrix. This ring contains bone produced by cells of the periosteum (a thin layer of connective tissue that lines the outside of bone); its outward advance precedes bone formation in the growth plate. As the bone-cartilage boundaries move outwards towards the bone ends, osteoclasts follow, reabsorbing bone and leaving a porous infrastructure in which marrow resides (Rodan 1992).

Osteoblasts are bone-forming cells, originating from cells resident in bone committed to the osteoblastic lineage. They synthesize and secrete most of the proteins of the bone matrix, including type I collagen and noncollagenous proteins. They possess high levels of alkaline phosphatase, which participates in mineralization. Proteins, produced by osteoblasts, spill over into the blood and are used as indicators of bone formation. In addition to the matrix-forming ability, cells of the osteoblastic family (osteocytes, lining cells, and maybe other cells) participate in the regulation of bone turnover. They respond to parathyroid hormone, glucocorticoids, vitamin D, sex steroids, insulin, prostaglandins, growth factors, and so on. Considering this degree of complexity and strict coordination of events, it should not come as a surprise that disruptions in chondrocyte and osteocyte cell surface architecture often lead to developmental abnormalities.

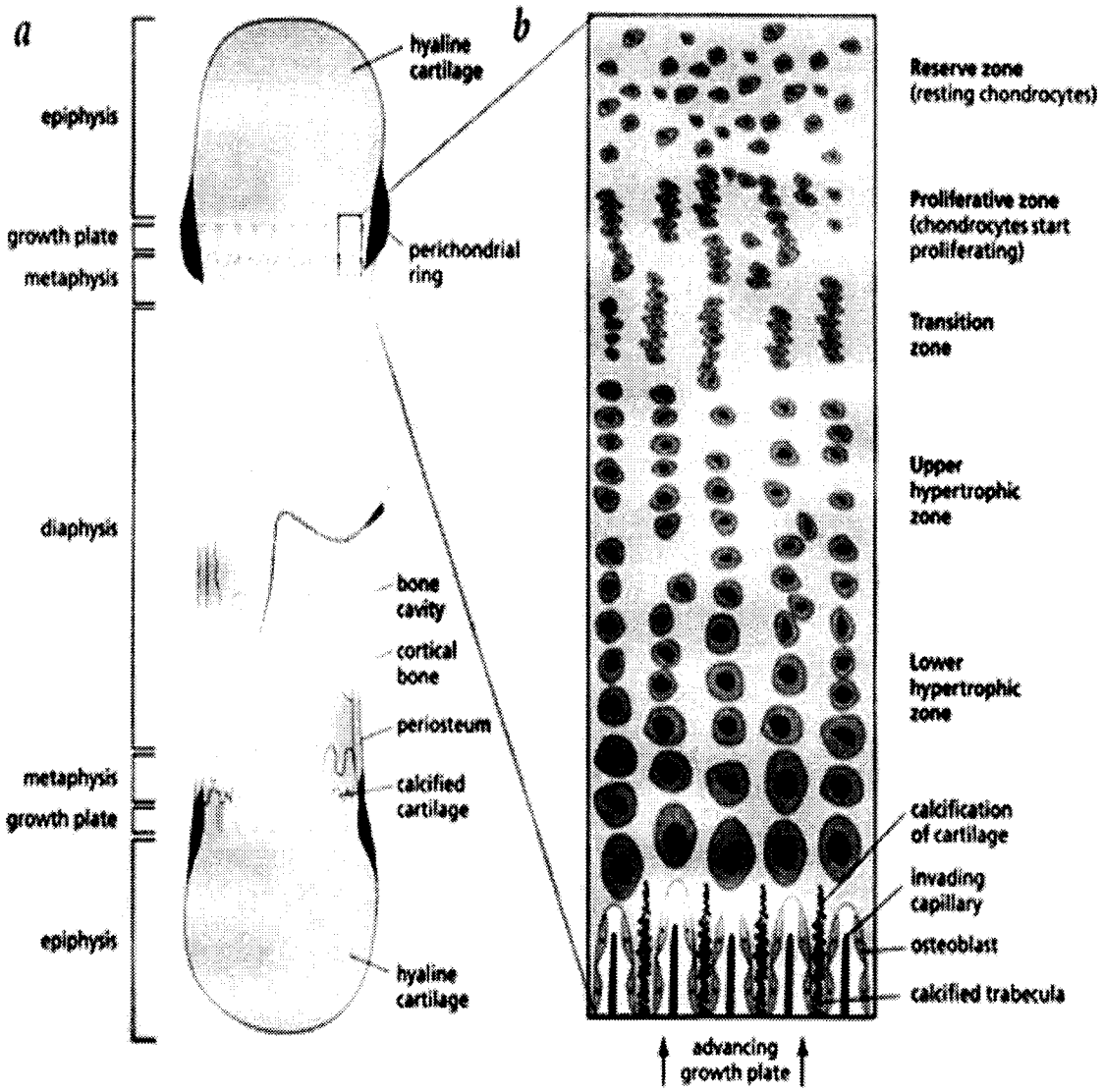


Figure 1.8 Schematic model of bone development.
From (Stickens and Evans 1998).

1.6.2 Hereditary multiple exostoses

Hereditary multiple exostoses (HME) is an autosomal dominant condition characterised by the development of multiple osteochondromas (exostoses) from the metaepiphyseal areas of the long bones. The exostoses are either sessile (broad based) or pedunculated and they vary widely in size and number (Fig. 1.9) (Solomon 1963; Stickens and Evans 1998). They can be present at birth and continue to appear and grow throughout childhood and into puberty. Exostoses are usually located in the metaephyseal region of the proximal humerus, distal radius and ulna, proximal tibia, and distal femur, but can also be found on the pelvis and scapula (Leone et al. 1987; Schmale et al. 1994). HME not only affects humans, but also is found to be naturally occuring in horses. A comparative study of five generations of selectively bred horses with HME, and five generations of HME families, conducted over 15 years, provided invaluable information regarding HME in both humans and horses. Leone et al. (Leone et al. 1987) concluded that the pattern of HME in the horse is similar to that in humans, making it an ideal model for the study of both the physical as well as the hereditary aspects of the disease.

HME can cause multiple and severe complications including pain, restricted range of joint movement, deformities and shortening of the long bones, and nerve or blood vessel compression. The most severe complication is malignant transformation of an exostosis into a chondrosarcoma, which occurs in 0.5-2% of cases (Schmale et al. 1994; Wicklund et al. 1995).

1.6.3 EXT family of proteins

HME is genetically heterogenerous as three disease-linked loci, EXT1-3 have been identified so far. EXT1 has been mapped to chromosome 8q23-24 (Cook et al. 1993), EXT2 to chromosome 11p11-p12 (Wu et al. 1994), and EXT3 to chromosome 19p (Le Merrer et al. 1994). Linkage studies have identified EXT1 and EXT2 as the two major disease loci in HME families, while EXT3 appears to be a minor locus (Legeai-Mallet et al. 1997). The EXT family

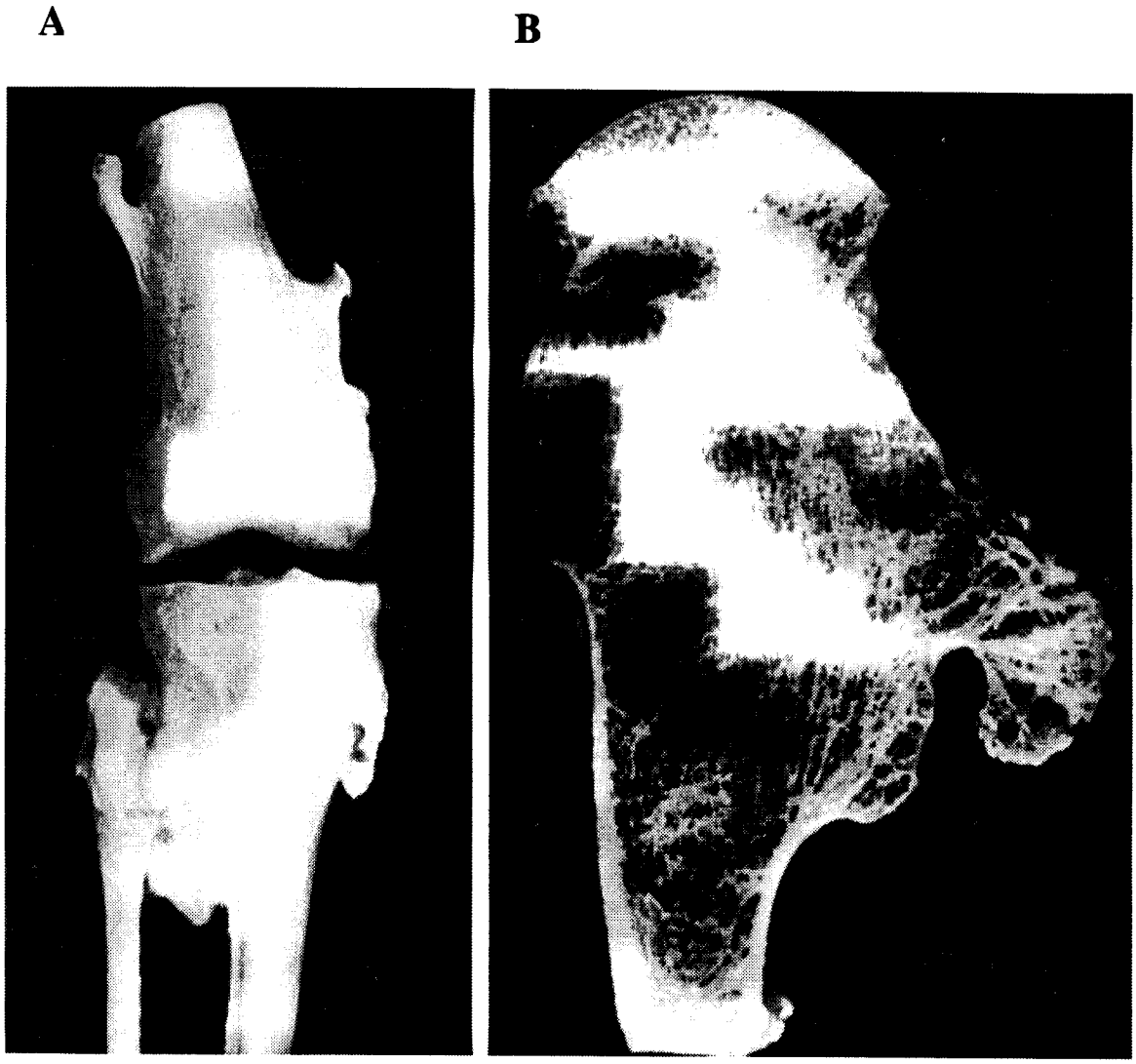


Figure 1.9 The multiple exostoses phenotype.

A dissected right knee joint of a 67-year-old man displaying exostoses projections on the femur, tibia and fibula. (b) A section through the proximal end on the right femur displays a HME lesion, as well as width and trabecular pattern changes. Adapted from Leone and Phillips (Leone et al. 1987).

of genes also have orthologs in rodents (Clines et al. 1997; Lin and Wells 1997; Lohmann et al. 1997; Stickens and Evans 1997; Wei et al. 2000), *Drosophila melanogaster* (Bellaiche et al. 1998) and *Caenorhabditis elegans* (Clines et al. 1997; Lohmann et al. 1997). In humans, the corresponding proteins, exostosin-1 (EXT1) and exostosin-2 (EXT2) are ubiquitously expressed glycoproteins of 746 and 718 amino acids, respectively (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996). EXT1 and EXT2 have a predicted type II transmembrane glycoprotein structure, and both proteins localize predominantly to the endoplasmic reticulum when over-expressed in cells (Fig. 1.10) (McCormick et al. 2000; McCormick et al. 1998).

Three additional loci designated EXTL1, EXTL2, and EXTL3 have been identified and mapped to chromosomes 1 (1p36, 1p11-12p) and 8 (8p12) respectively. However, no HME family has yet been linked to these loci (Van Hul et al. 1998; Wise et al. 1997; Wuyts et al. 1997). Loss of heterozygosity (LOH) at the EXT1, EXT2, and EXT3 loci has been observed among patients with EXT related and unrelated chondrosarcomas suggesting that the EXT genes are tumour suppressors (Bovee et al. 1999; Hecht et al. 1995; Hecht et al. 1997; Raskind et al. 1995).

So far, only two EXT genes have been cloned, namely EXT1 and EXT2 (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996). Despite extensive genetic characterization, the function of the EXT proteins remained unknown until 1998 when the study of a HS-deficient cell line, sog9, revealed that EXT1 is involved in HS biosynthesis (McCormick et al. 1998). Biochemical studies have since confirmed that EXT1 and EXT2 both possess the GlcNAc-T and GlcA-T activities representative of an HS-Polymerase (HS-Pol) (Lind et al. 1998). However, the HSV functional assay has shown that EXT2 cannot substitute for EXT1 *in vivo* (McCormick et al. 2000).

1.6.4 EXT-like proteins

A recent report indicates that all three EXT-like proteins harbor α1,4GlcNAc-T activity. EXTL1 is able to transfer α1,4-GlcNAc onto a GlcA acceptor sugar (Kim et al. 2001). The

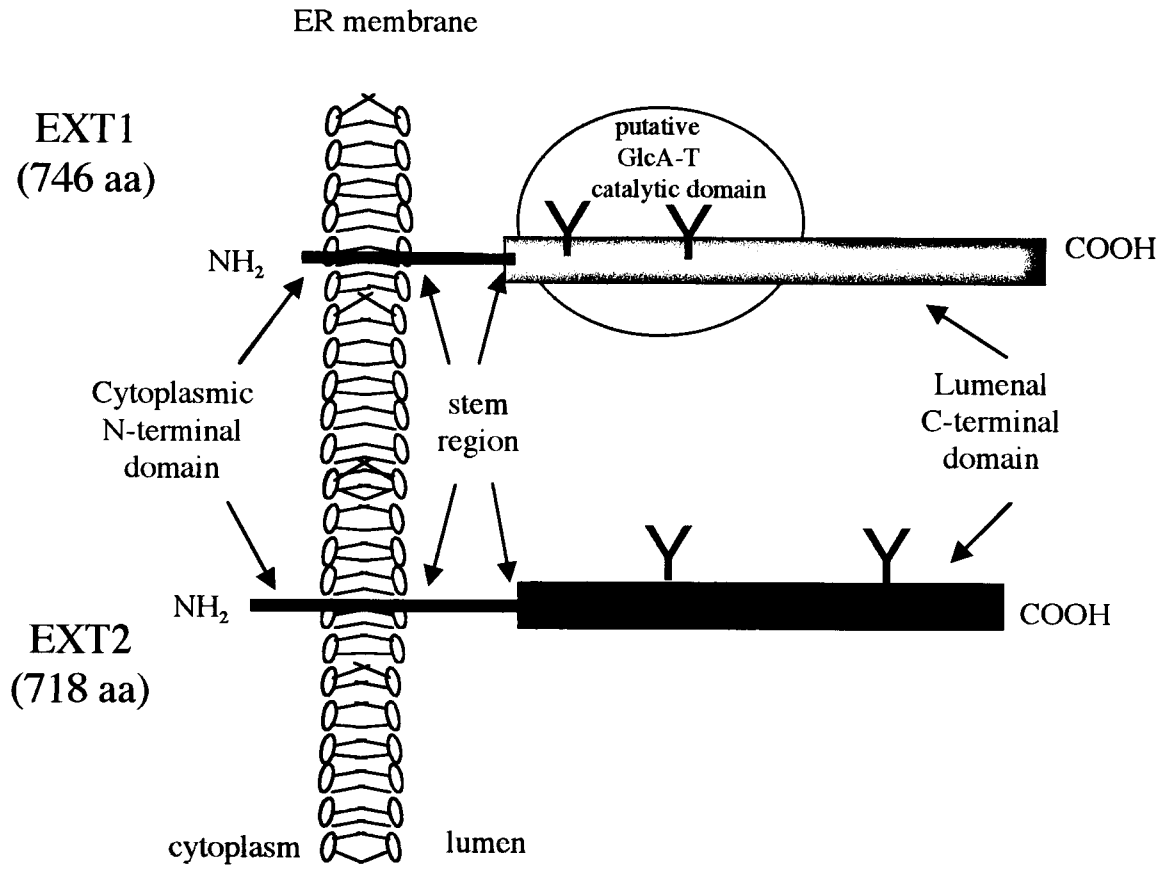


Figure 1.10 Topology of EXT1 and EXT2 in the ER membrane.

EXT1 and EXT2 are both type II transmembrane proteins with a short N-terminal cytoplasmic domain, a single hydrophobic transmembrane domain followed by a large C-terminal domain containing the stem region and catalytic domain(s). Locations of proposed N-glycosylation sites are indicated with a **Y**.

EXTL2 protein is an N-acetylhexoaminyltransferase that could transfer either GlcNAc or GalNAc to the common linkage-region core tetrasaccharide (Kitagawa et al. 1999). The EXTL3 protein shows both GlcNAcT-I and GlcNAcT-II activities, which could be involved in the biosynthetic initiation and elongation, respectively, of HS precursor chains (Kim et al. 2001). A recent study of a *C. elegans* EXTL3 ortholog suggests that a single protein, Rib-2, is able to carry out both the HS chain initiation and polymerization steps. This report indicates that the biosynthetic mechanism of HS in *C. elegans* is similar in that a protein, Rib-2, harbors both initiation and elongation activities (Kitagawa et al. 2001). However, there are important differences, unlike Rib-2, EXTL-3 only harbors one of the two glycosyltransferases that is involved in HS chain elongation (Kim et al. 2001). Further discussions in EXT-like proteins are stated in Chapter 3 (Fig. 3.9).

1.6.5 Genotype-phenotype correlation in HME

Extensive genetic analysis of HME patients' genomic DNA over the past few years has led to the identification of a number of mutations in both *EXT1* and *EXT2* that appear to be disease-related. The majority result in premature termination, although a small number of missense mutations have also been identified. Interestingly, significant differences in clinical severity of HME disease have been observed, but until recently, the cause of this clinical variability has remained unclear. Indeed, a recent study involving an extensive mutational analysis of the EXT1 and EXT2 genes in a large series of HME families has established an interesting genotype-phenotype correlation (Francannet et al. 2001). Francannet and collegues show that EXT1 mutations caused the most severe forms of the disease and were exclusively responsible for the degeneration of exostosis into chondrosarcoma. On the contrary, EXT2 mutations were frequently associated with more moderate phenotypes.

1.7 Hypotheses and Objectives

The research presented in this thesis involves the investigation of the correlation between

HS biosynthesis and HME. At the time of this study was initiated, the mechanism of HSV-1 attachment to the host cell surface was not well understood. Our laboratory was therefore focused on the study of HSV-resistant cell lines with defects in GAG biosynthesis to better understand HSV entry (Banfield et al. 1995; Banfield et al. 1995; Gruenheid et al. 1993). Although it was determined that one of the isolated cell lines, termed sog9, was unable to synthesize any cell surface GAGs, and thus was 500 fold more resistant to HSV infection than its parental cell line, the cause of the GAG defect was not known for the next few years. To address this problem, Dr. Craig McCormick screened a human cDNA library for genes able to restore the defect in GAG biosynthesis in sog9 cells and thereby identified the EXT1 protein as an HS polymerase (McCormick et al. 1998). In fact, EXT1 gene had already been identified earlier as a putative tumor suppressor gene involved in HME (Cook et al. 1993) before its biochemical activity was revealed. Taken together, these results raised several important questions regarding HSV entry, HS biosynthesis and HME.

Since GAG biosynthesis is important for viral infection and EXT1 expression rescues HS biosynthesis in sog9 cells, I hypothesized that by stably expressing extra copies of the human EXT1 gene in a naturally EXT1 expressing cell line, followed by challenging with HSV-1, a new set of resistant cell lines may be selected. These new resistant cell lines may be deficient in other components of the HS GAG synthesis pathway besides EXT1. Alternatively, these cell lines could be deficient in other cellular factors involved in HSV-1 attachment or fusion during viral entry.

Following the isolation of the EXT1 gene, the focus of our laboratory switched to address the role of the EXT1 protein in HS polymerization, and the correlation between HS biosynthesis and HME. I used the HSV infectivity assay to test the putative etiological missense mutant EXT1 proteins for their ability to catalyze HS biosynthesis in sog9 cells. This assay is based on the ability of HSV to infect cells by attaching to cell surface HS. Moreover, this assay is particularly useful because it measures cellular HS biosynthesis and transduction to the cell surface. I

hypothesized that if HS polymerization is related to the disease pathogenesis of HME, then the HS polymerase activity of EXT1 constructs bearing disease-causing mutations should be altered or impaired. Furthermore, these specific mutants may be useful for pinpointing the different functional domains of the EXT1 protein, thus leading to a better understanding of the roles of these mutations in the pathogenesis of HME.

In the present study, I evaluated the effect on cellular HS-Pol activity of HME etiological mutations identified in the EXT1 locus of affected individuals. The results obtained raise the possibilities that some of these published mutants may not relate to disease pathogenesis, or on the other hand, that EXT1 possesses additional functions not yet identified. The results presented in this thesis provide insight into the link between HS biosynthesis and HME disease, and should prove extremely useful for further studies of the role of HS in normal bone development and the molecular pathogenesis of HME.

CHAPTER 2 Materials and Methods

2.1 Materials

A complete list of chemical reagents and suppliers appears in Appendix II.

2.2 Cells, Viruses, and plasmids

The parental murine L cell used was the clone 1D line of Lmtk$^-$ mouse fibroblasts. The procedures for the isolation of the mutant gro2C and sog9 cell lines were described previously (Banfield et al. 1995; Gruenheid et al. 1993) as was their specific defect in the *EXT1* gene (McCormick et al. 2000). Vero cells were a gift from Dr. S. McKnight. BHK cells were obtained from the ATCC. BHK, Vero, L, gro2C, sog9 and L-EXT1 mutant cells were cultured at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Inc.) with 10% fetal bovine serum (Life Technologies Inc.). Stable cell lines were generated by transfection using LipofectAMINE PLUS reagent (Life Technologies Inc.), followed by selection in media supplemented with 700 μg/ml Geneticin (G-418 Sulfate) (Life Technologies Inc.). The three Chinese hamster ovary (CHO) cell lines were generously provided by Dr. Stephen H. Leppla (Gordon et al. 1995). The wild-type and furin-deficient (FD11) CHO cells were maintained in DMEM/F12 (1:1) (Life Technologies Inc.) supplemented with 5% fetal bovine serum. The stably transfected FD11+furin#5-1 CHO cells, were grown in the same media supplemented with 400 ug/ml Geneticin. A mutant HSV-2 strain, L1BR1, which has a modified β-galactosidase gene inserted into the US3 protein kinase gene under the control of the HSV-1 beta 8 promoter was obtained as a generous gift from Y. Nishiyama (Nishiyama et al. 1992). R8102, a mutant HSV-1 F strain which has the β-galactosidase gene inserted between UL3 and UL4, under the ICP27 promoter, was a gift from B. Roizman. R8102 displays LD50 values similar to those observed for the wild type HSV-1 strain F (B. Roizman, personal communication). All viruses were propagated and titered on Vero cells.

Two commercially available mammalian expression vectors were used for all EXT constructs: pcDNA3.1myc-his (Invitrogen) and pEGFP-N1 (Clontech). All DNA was amplified in *E. coli* DH5α (Life Technologies Inc.) and prepared for transfection using standard plasmid purification systems (Qiagen and Life Technologies Inc.). All stable EXT1-expressing cell lines were generated by lipofectAMINE-mediated transfection with pEXT1, followed by selection in media supplemented with 700 μg/ml Geneticin (G418). Clonal versions of these cell lines were derived from individual transfected colonies.

2.3 Construction of EXT fusion proteins and etiological mutant proteins

All EXT mutants were constructed by site-directed PCR mutagenesis using the previously described wild-type EXT1 constructs, pEXT1myc-his and pEXT1gfp (McCormick et al. 2000; McCormick et al. 1998). All oligonucleotide primers (Table 2.1), Taq DNA polymerase and restriction enzymes were obtained from Life Technologies Inc., unless otherwise stated.

pEXT1 was isolated from a HeLa cell cDNA library in pcDNA3.1 (Invitrogen) (McCormick et al. 2000). pEXT1myc was constructed by PCR amplification of the EXT1 coding region using primers complementary to the translation start site (5'-CGG GAT CCC GCA GGA CAC ATG CAG GCC AAA AAA CGC TAT TTC ATC C-3') and the region preceding the translation stop site (5'-TTT TCC TTT TGC GGC CGC TTT TTT CCT TAA GTC GCT CAA TGT CTC GGT A-3'), which contained BamHI and NotI restriction enzyme sites, respectively. Following digestion with BamHI and NotI, the PCR products were ligated into pcDNA3.1/Myc-His B (Invitrogen). pEXT1gfp was constructed by excision of EXT1 from pEXT1myc with *Bam*HI and *Sst*II, followed by ligation into the *Bgl*II and *Sst*II sites in the pEGFP-N1 expression vector (Clontech).

The Q27K, D164H, N316S, A486V, and P496L etiological mutants, as well as the corresponding conserved/severe change and deletion mutants were all constructed following Stratagene's QuikChange site-directed mutagenesis protocol. Briefly, complementary oligonucleotides bearing the desired mutation were used to amplify by PCR the entire pEXT1gfp

Table 2.1 Oligonucleotide primers used for PCR mutagenesis of EXT1[a]

Primer	DNA sequence (5'--3')
Q27K-Fwd	TTTTATTTCGGAGGCCTGAAGTTTAGGGCATCG
Q27K-Rev	CGATGCCCTAAACTTCAGGCCTCCGAAATAAAAC
Q27A-Fwd	TTTTATTTCGGAGGCCTGGCGTTTAGGGCATCG
Q27A-Rev	CGATGCCCTAAACGCCAGGCCTCCGAAATAAAAC
Q27del-Fwd	TTTTATTTCGGAGGCCTGTTTAGGGCATCG
Q27del-Rev	CGATGCCCTAAACAGGCCTCCGAAATAAAAC
D147E-Fwd	CATCGAGGGCTCCAGGTTCTACACCTCGGAGCCCAGCCAGGCGTG
D147E-Rev	CTCAGGACAAAGAGGCACGCCTGGCTGGGCTCCGAGGTGTAGAAC
D164H-Fwd	CTGGATACTTTAGACAGACACCAGCTGTCACCTCAGTATGTG
D164H-Rev	CACATACTGAGGTGACAGCTGGTGTCTGTCTAAAGTATCCAG
D164E-Fwd	CTGGATACTTTAGACAGAGAACAGCTGTCACCTCAGTATGTG
D164E-Rev	CACATACTGAGGTGACAGCTGTTCTCTGTCTAAAGTATCCAG
D164del-Fwd	CTGGATACTTTAGACAGACAGCTGTCACCTCAGTATGTG
D164del-Rev	CACATACTGAGGTGACAGCTGTCTGTCTAAAGTATCCAG
D202E-Fwd	TTAATTTATATTCCGGCACTTGGCCTGAGTACACCGAGGACGTGG
D202E-REV	CCGATGTCAAACCCCACGTCCTCGGTGTACTCAGGCCAAGTGCCG
R280G-Fwd	GGAAGAGGTACCTGACAGGGATAGGATCAGACACCGGGAATGCCT TATATC
R280S-Fwd	GGAAGAGGTACCTGACAGGGATAGGATCAGACACCAGCAATGCCT TATATC
R280-Rev	GAGTTATCCCAGAAGTGGCTGCGCGCGGGGTACCCACAATCCTCTC AGG
N316S-Fwd	CACAAGGATTCTCGCTGCGATCGAGACAGCACCGAGTATGAGAAG
N316S-Rev	CTTCTCATACTCGGTGCTGTCTCGATCGCAGCGAGAATCCTTGTG
N316A-Fwd	CACAAGGATTCTCGCTGCGATCGAGACGCCACCGAGTATGAGAAG
N316A-Rev	CTTCTCATACTCGGTGGCGTCTCGATCGCAGCGAGAATCCTTGTG
N316del-Fwd	CACAAGGATTCTCGCTGCGATCGAGACACCGAGTATGAGAAG
N316del-Rev	CTTCTCATACTCGGTGTCTCGATCGCAGCGAGAATCCTTGTG
R340-Fwd	CGGGATCCCGCAGGACACATGCAGGCCAAAAAACGCTATTTCATCC
R340S-Rev	CAAAGCCTCCAGGAATCTGAAGGACCCAAGCCTGCTACCACGAGGAA CCAG
R340H-Rev	CAAAGCCTCCAGGAATCTGAAGGACCCAAGCCTGTGACCACGAGGAA CCAG

R340L-Rev	CAAAGCCTCCAGGAATCTGAAGGACCCAAGCCTGAGACCACGAGGAA CCAG
H340R-Rev	CAAAGCCTCCAGGAATCTGAAGGACCCAAGCCTGCGACCACGAGGAA CCAG
R340WT-Rew	CAAAGCCTCCAGGAATCTGAAGGACCCAAGCCTGCGACCACGAGGAA CCAG
A486V-Fwd	GCAGTCATCCATGTGGTGACGCCCCTGGTCTCTCAGTCC
A486V-Rev	GGACTGAGAGACCAGGGGCGTCACCACATGGATGACTGC
A486H-Fwd	GCAGTCATCCATCATGTGACGCCCCTGGTCTCTCAGTCC
A486H-Rev	GGACTGAGAGACCAGGGGCGTCACATGATGGATGACTGC
A486del-Fwd	GCAGTCATCCATGTGACGCCCCTGGTCTCTCAGTCC
A486del-Rev	GGACTGAGAGACCAGGGGCGTCACATGGATGACTGC
P496L-Fwd	CAGTCCCAGCTAGTGTTGAAGCTCCTCGTGGCTGCAGCC
P496L-Rev	GGCTGCAGACACGAGGAGCTTCAACACTAGCTGGGACTG
P496H-Fwd	CAGTCCCAGCACGTGTTGAAGCTCCTCGTGGCTGCAGCC
P496H-Rev	GGCTGCAGACACGAGGAGCTTCAACACGTGCTGGGACTG
P496del-Fwd	CAGTCCCAGGTGTTGAAGCTCCTCGTGGCTGCAGCC
P496del-Rev	GGCTGCAGACACGAGGAGCTTCAACACCTGGGACTG
TKF	CCCAAGCTTGGGGTGGCGTTGAAC
TKR	CTAGTCTAGACTAGTTCGGGCTTCCGTGTTTG
TK7G	CGGGGCTTGCGGGCCCACAGCCTCCCCCCCGATATGAGGAGC
HEXT1-SC	GTTAAGCTTGGTACCGAGCTCGGATCCCGCAGGACACATGCAGGC
JS1	CTGATCAGCGTCAGAGCGTT
JS8	CGCTTATGGACACACCACAC

[a] Mutated nucleotides are shaded in gray.

plasmid, using PfuTurbo DNA polymerase (Stratagene). The parental DNA template was then digested with Dpn1 and the nicked vector DNA bearing the desired mutation was transformed directly into *E. Coli* competent cells.

The R280G, R280S, R340S, R340H, R340L and the repaired H340R mutants were all constructed by PCR amplification using mutagenic primers, and then subsequent cloning into pEXT1myc-his using appropriate restriction enzymes. The G339D and R340C mutants were constructed as described previously (McCormick et al. 1998). The D147E and D202E mutants were introduced by way of a two-step PCR mutagenesis strategy, using an overlapping and complementary primer set bearing the desired mutation and two external primers. First, fragments were made with an approximately 40 bp over lap in the region which included the desired mutation, then the two over lapping PCR fragments were denatured, annealed together, extended for 15 minutes with Taq polymerase, and amplified by PCR using the outer-most primers to make a fragment which was cloned into the pEXT1myc-his vector.

To convert from myc epitope tags to green fluorescent protein tags, all EXT1 mutants were excised from the pEXT1myc-his vector with BamH I and Sac II, and re-ligated into the Bgl II and Sac II sites of the pEGFP-N1 vector such that they were in frame for an EXT1-gfp fusion protein.

2.4 Mutation repair of the viral *TK* from a clinical isolate

DNA from HSV-2 clinical isolate bearing an extra G at the 7 G stretch of the viral *TK* gene was used in a PCR with primers JS1 and JS8 to generate a 1.6-kb fragment representing the *TK* gene. This was then used as a template in a PCR with primers TKF and TKR to generate a 1,200-bp fragment representing the TK coding region and a short noncoding region at each end with a unique *Hin*dIII site and an *Xba*I. This was then ligated into pRc/CMV after digestion with *Hin*dIII and *Xba*I, yielding plasmid pTK-8G. To repair the mutation in the homopolymer sequence of this TK gene (bases 433 to 439 of the open reading frame), primers TK7G and TKSC were used to generate a fragment containing 7 G residues in place of the 8 G residues of

the mutant gene. The resulting fragment was then recombined into plasmid pTK-8G by digestion of the fragment and the plasmid with *Apa*I and pflM1, followed by ligation. Ten recombinants were selected, and the plasmid DNA was transfected into TK-deficient mouse L cells by using lipofectamine (Gibco BRL, Burlington, Ontario, Canada). After 2 days, cells were incubated with medium containing HAT supplement (Gibco BRL) to select for cells expressing TK. After 7 days of growth, the medium was removed and the monolayer was incubated with 0.1% methylene blue in 70% ethanol for 10 min to stain the cell colonies.

2.5 Viral stock production

Subconfluent monolayers of Vero cells in 150 mm dishes were incubated with virus at a multiplicity of infection (MOI) of 0.05 plaque forming units per cell in DMEM. After 1 hour, cells were washed once with phosphate buffered saline (PBS) and overlaid with DMEM/10% fetal bovine serum (FBS) for 3 days. Cells and media were collected into a 50 ml conical vial, and centrifuged at 600Xg for 10 min. Supernatant was collected, except for 1 ml which was used to resuspend the cell pellet. The resuspended cell pellet was then subjected to 3 freeze-thaw cycles in a dry ice/ethanol bath. The cell lysates were centrifuged at 600Xg for 10 min, and the virus-containing supernatants were pooled. The combined supernatants were distributed into aliquots and stored at –80°C.

2.6 Determination of virus titer

To determine the titer of virus stocks in various cell lines (i.e. Vero, L, sog9, etc.), 1 x 10^6 cells were plated in 6-well dishes and infected 18 hours later with serial 10-fold dilutions of virus stock in DMEM. After a 1 hour adsorption period, the inoculum was removed and cell monolayers were washed three times with PBS to remove unbound virus. Cell monolayers were then overlaid with DMEM containing 4% FBS and 0.1% pooled human IgG. As the infection progressed, the IgG neutralized any extracellular virus, while virus could still be transmitted by direct cell-to-cell contact, allowing a plaque to form. At 4 days post-infection, cell monolayers

were washed with PBS and plaques were visualized by staining with 70% methanol/5% methylene blue for 5 minutes.

2.7 HSV infectivity assay for EXT1 function

Sog9 cells were transiently transfected with wild-type EXT1 construct or EXT1 constructs bearing missense mutations for 30 hours, to allow for cDNA expression. Cell monolayers were than infected with either HSV-1 (R8102) or HSV-2 (L1BR1). Following 1 hour incubation, the inoculum was replaced with media containing pooled human IgG to allow either plaque formation for 4 days in the case of R8102 as described in 2.5 above or blue cells staining with X-gal after 10 hours, in case of L1BR1.

2.8 β-Galactosidase activity assay in cell extracts

Similar to the HSV infectivity assay, sog9 cells were transiently transfected for 30 hours and infected with the L1BR1 strain of HSV-2 for 10 hours. Cells were than washed with PBS and scraped into a microfuge tube. After recovery by centrifugation, cells were then lysed by 3 cycles of fast freezing and thawing. The supernatant of the cell lysates were incubated with o-nitrophenyl-β-D-galactopyranoside (ONPG) at 37^0C for 30 minutes or until a faint yellow color developed. Reactions were stopped by adding Na_2CO_3 and the optical density was read at a wavelength of 420nm.

2.9 Heparin-inhibition assay

Cell monolayers were rinsed twice with PBS and inoculated with HSV-1 diluted in DMEM previously mixed with various concentrations of heparin. After a 60 minutes. adsorption period at 37^0C, the inoculum was removed and the cells were washed three times with PBS to remove unbound virus. Cells were then overlaid with DMEM containing 4% FBS and 0.1% IgG. At 4 days post-infection, cell monolayers were washed with PBS and plaques were visualized by staining with 70% methanol/5% methylene blue for 5 minutes.

2.10 Immunoprecipitation of radiolabeled EXT proteins

Cell lines were transfected with GFP or myc-his tagged EXT constructs. After 18 hours, cells were radiolabeled with 100 μCi/ml ^{35}S-methionine (ICN) in methionine and cysteine-free DMEM (ICN) for 12 hours at 37°C. Cells were washed with PBS and lysed in Triton lysis buffer (2% Triton X-100, 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1.U./ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin) at 4°C for 15 minutes. The lysates were centrifuged at 12,000 xg for 15 minutes, and pre-cleared for 30 minutes with 25 μl of protein G-Sepharose (Pharmacia) at 4°C. The lysates were then incubated with 0.5 μg of mouse anti-myc monoclonal antibody (Invitrogen) or 0.5 μg of mouse anti-GFP monoclonal antibody (Clontech) for 2 hours, followed by incubation with 25 μl of Protein G-Sepharose for 1 hour. The lysates were centrifuged at 12,000 x g for 10 seconds, and washed two times with 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, two times with 10 mM Tris-HCl pH 7.4, 500 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, and two times with 10 mM Tris-HCl pH 7.4. The pellet was suspended in 30 μl of SDS-PAGE sample buffer and boiled for 15 minutes, prior to SDS-PAGE. Gels were dried and exposed to BioMAX MR film (Kodak).

2.11 Anion exchange chromatography of glycosaminoglycans

Biochemical labeling of GAGs was performed by a modification of procedures described previously by Bame and Esko (Bame 1989). Briefly, GAGs were radiolabeled by incubation of cells for 24h with 60 μCi of [^{35}S]sulfate and/or 60 μCi [^{3}H] glucosamine hydrochloride per ml in DMEM-FBS modified to contain 10 μM sulfate. The cells were washed three times with cold PBS and solubilized with 1.5 ml of 0.1 M NaOH at 25°C for 15 minutes. Extracts were adjusted to pH 5.5 by the addition of concentrated acetic acid and treated with 2 mg of protease (Sigma) per ml in 0.32 M NaCl-40 mM sodium acetate, pH 5.5, containing shark cartilage chondroitin sulfate (2 mg/ml) (Sigma) as carrier, at 37°C for 12 hours. For some experiments, portions of the radioactive material were treated for 12 hours at 37°C with 10 mU of chondroitin ABC lyase (Sigma) or 0.5 U of heparitinase (Sigma). The radioactive products were quantified by

chromatography on DEAE-Sephacel (Pharmacia) by binding in 50 mM NaCl followed by elution with 1 M NaCl. For HPLC analysis, the GAG samples were desalted by precipitation with ethanol. Following centrifugation, the ethanol precipitates were suspended in 20 mM Tris, pH 7.4, and resolved by anion exchange HPLC with a TSK DEAE-3SW column (15 by 75 mm; Beckman Instruments). Proteoglycans were eluted from the column with a linear 50 to 700 mM NaCl gradient formed in 10 mM KH_2PO4 (pH 6.0). All buffers contained 0.2% Zwittergent 3-12 (Calbiochem) to extend the life of the column. The HPLC samples were normalised by the amount of cellular proteins using Bio-Rad Protein assay, which was based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein.

CHAPTER 3: Selection of Mutant cell lines defective in Heparan sulfate biosynthesis

3.1 Introduction:

The entry of herpes simplex virus (HSV) into the host cell can be divided into three phases: initial binding, stable attachment and penetration. Many of the advances in the understanding of HSV entry into host cells have come from the study of HSV resistant cell lines, in particular, the characterization of their defects. In our laboratory, we generated HSV resistant cell lines using murine fibroblast L (tk-) cells with HSV-1. Clonal expansion of HSV-1 resistant colonies resulted in the isolation of the Gro2C cell line, which is 90% resistant to HSV-1 infection relative to parental L cells. It was determined that the resistance resulted from the abrogation of synthesis of heparan sulfate (HS), a ubiquitous cell surface glycosaminoglycan (GAG) (Gruenheid et al. 1993). A second cycle of selection using the Gro2C cells as the parental cell line resulted in the isolation of Sog9 cell line. Sog9 does not express HS or chondroitin sulfate (CS) on the cell surface and thus exhibits 500-fold resistant to HSV infection in comparison to parental L cells (Banfield et al. 1995). In order to characterize the specific defect in this mutant cell line, Sog9 cells were transiently transfected with pools of a human cDNA expression library, followed by HSV-1 infection. Several cycles of screening and subdividing cDNAs resulted in the isolation of the EXT1 gene (McCormick et al. 1998).

The expression of EXT1 in Gro2C cells or Sog9 cells restored the susceptibility to HSV infection by restoring cell surface HS expression. This observation suggested that both mutant cell lines were deficient in EXT1 related activity. Biochemical evidence indicates that EXT1 protein harbors both glucuronic acid-transferase (GlcA-T) and N-acetylglucosamine-transferase (GlcNAc-T) activities, which are required for the biosynthesis of the HS GAG (Lind et al. 1998; McCormick et al. 2000). Since GAG synthesis is important for viral infection and EXT1 expression rescues HS synthesis in Gro2C cells and Sog9 cells, we hypothesized that by stably

expressing extra copies of the human EXT1 gene in L cells, followed by challenging with HSV-1, a new set of resistant cell lines may be selected. These new resistant cell lines may be deficient in other components of the HS GAG synthesis pathway besides EXT1. Alternatively, these cell lines could be deficient in other cellular factors involved in HSV-1 attachment or fusion during viral entry.

3.2 Results:

3.2.1 Isolation of nine HSV-resistant cell lines.

As part of a study to identify host cell molecules that are involved in HS GAG biosynthesis, genetic selection was used to isolate human EXT1 expressing murine L cell lines capable of surviving exposure to HSV-1. To do this, a stable cell line over expressing EXT1 (L-EXT1) was first generated, and cell monolayers were infected with HSV-1 and then incubated to allow for viral destruction of the monolayers. After 14 days, colonies of cells arose at the rate of approximately 5 for 10^7 cell seeded. Stable clonal cell lines were established from independent clones and have been propagated for more than 3 months. Cell variants were identified on the basis of a distinctive herpesvirus resistance phenotype, which was characterized by the fact that the monolayers of mutant cells were destroyed more slowly than control cells after exposure to HSV-1. A collection of nine HSV-1 resistant colonies, termed TL mutants, were isolated in this manner.

3.2.2 Analysis of the susceptibility to HSV-1 infection in mutant cells monolayers.

To determine the susceptibility of the mutant cell lines to HSV-1 infection, monolayers of mutant and control cells were exposed to HSV-1 expressing β-galactosidase. Following incubation, the inoculum was replaced with media containing human IgG to allow plaque formation for 4 days. In this assay, the number of plaques on the cells monolayers were reduced by 70%-90% compared with the parental L-EXT1 cells (Fig 3.1), consistent with a partial herpesvirus resistance phenotype.

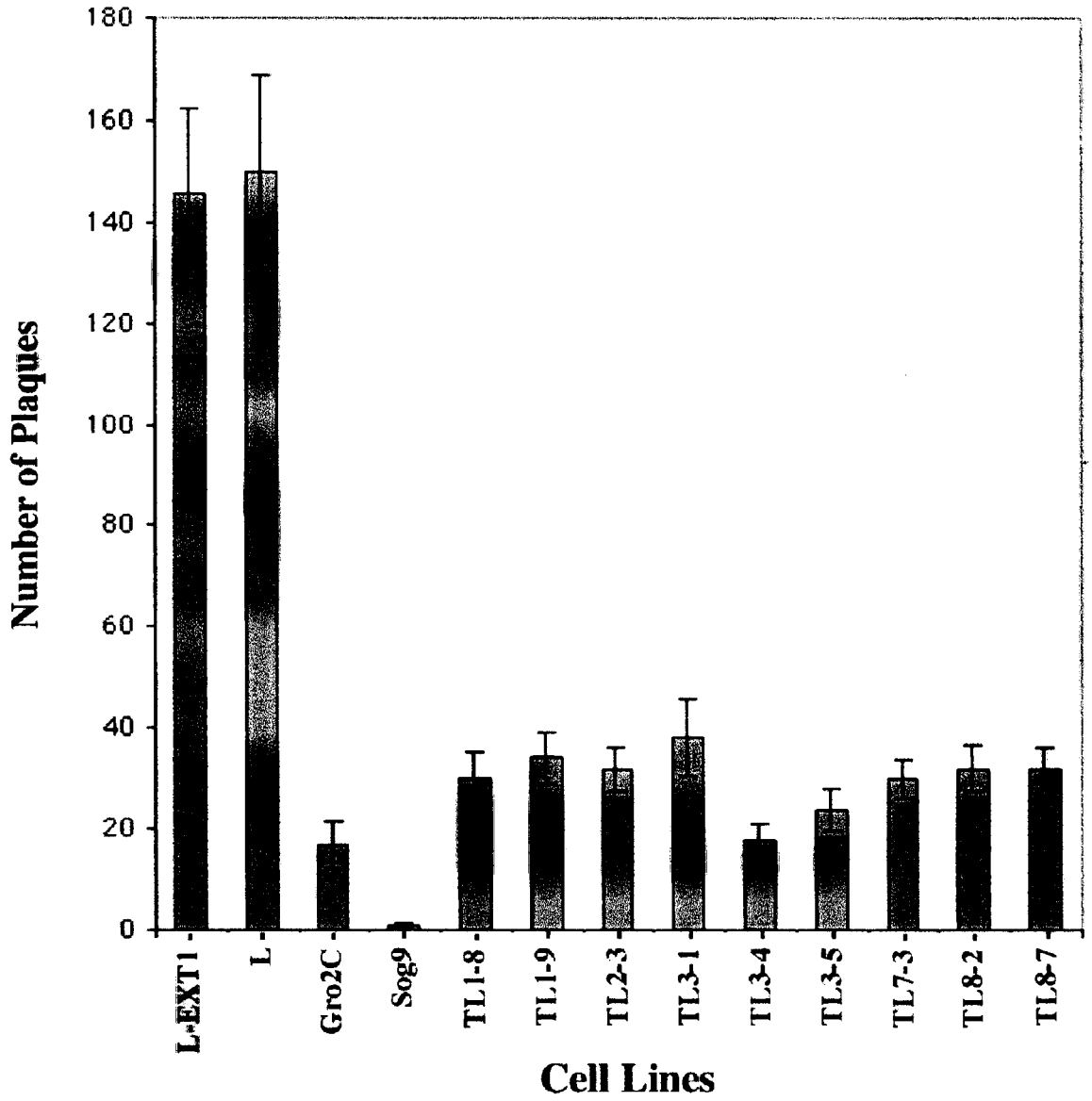


Figure 3.1 The different susceptibilities of TL mutant cell lines to HSV-1 infection measured by plaque formation.

Monolayers of different cell lines were inoculated with equivalent amounts of HSV-1. After 1 h of adsorption, the inoculum was removed and medium containing 0.1% pooled human IgG was added to the monolayers. Plaques were visualized after 4 days by methylene blue staining. TL mutant cell lines were generated by challenging stably human EXT1-transfected L cells (L-EXT1) with HSV-1. Two independent experiments were performed with similar results. Data shown are the average of three infected wells from a single experiment.

3.2.3 HSV-1 infection of L-EXT1 cell derived mutants is inhibited by heparin.

I next determined whether the partial herpesvirus resistance phenotype was due to the alteration of cell surface GAG expression on the mutant cells by using soluble heparin as a competitor. Heparin is a large, highly negatively charged molecule which when bound to the cell surface, may be acting as a HS GAG analog (WuDunn 1989). Gro2C, a murine L cell mutant that is 90% resistant to HSV-1 infection, failed to express HS GAG on the cell surface, and therefore was hypersensitive to the inhibition by heparin or heparan sulfate in comparison with its parental L cell line (Banfield et al. 1995; McCormick et al. 1998). In the present study, HSV-1 infection in control L-EXT1 cells was effectively inhibited by heparin in a dose-dependent manner (Fig 3.2). Moreover, HSV-1 infection of TL mutant cells was hypersensitive at any concentration of heparin tested. Taken together, these results indicate that TL mutant cells resemble Gro2C cells with regard to HSV-1 infection, and probably have altered GAG expression on the cell surface.

3.2.4 Characterization of GAG synthesis.

To test whether mutant cells acquired defects in the GAG synthesis pathway compared with the parental L-EXT1 cells, cell monolayers were radiolabeled to high specific activity with [^{35}S] sulfate and washed extensively to eliminate remaining traces of growth medium. GAGs were prepared from the intact cells and analyzed by anion-exchange high-pressure liquid chromatography (HPLC). Fractions eluted from the HPLC column were collected and counted by liquid scintillation spectroscopy. Consistent with previous results (Banfield et al. 1995; McCormick et al. 1998), control L-EXT1 cells and L cells synthesized two major sulfated peaks representing HS (fraction 40-50 Fig. 3.3) and chondroitin sulfate (CS; fractions 60-75, Fig. 3.3). In Gro2C cells, the profile was essentially the same except for the absence of the HS peak, while EXT1 expressing Sog9 cells synthesized HS only. By contrast, Sog9 cells had lost the ability to synthesize any of the major GAG, and therefore, no sulfate peak was observed (Fig. 3.3).

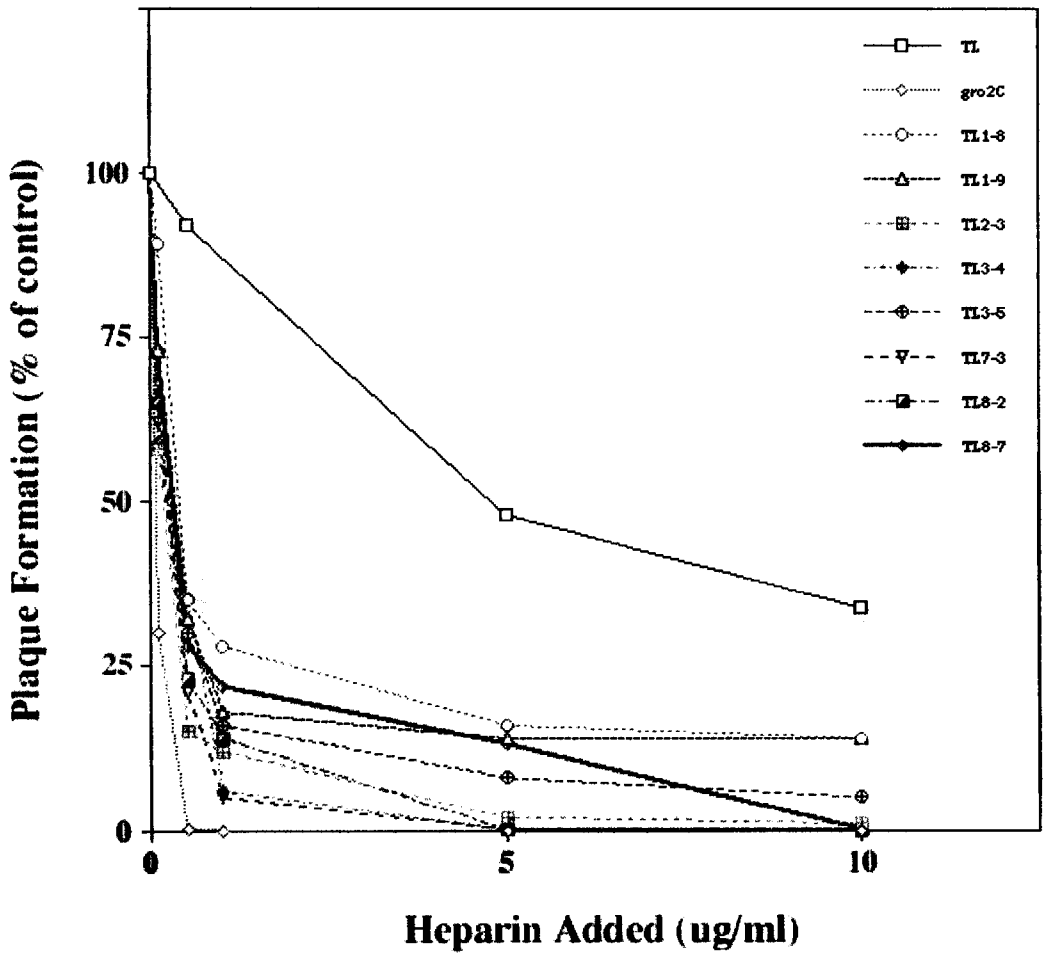


Figure 3.2 HSV-1 infection of TL mutant cell lines was hypersensitive to inhibition by heparin compared with their parental L-EXT1 cell line.
Cell monolayers were inoculated with HSV-1, which had been preincubated with various concentrations of soluble heparin from 0-10μg/ml. The inoculum was incubated with the cells for 60 min at 37^{0}C to allow for absorption of the virus. Plaques were visualized after 4 days by methylene-blue staining. Results are averages of two determinations, which did not vary more than 15% at any point.

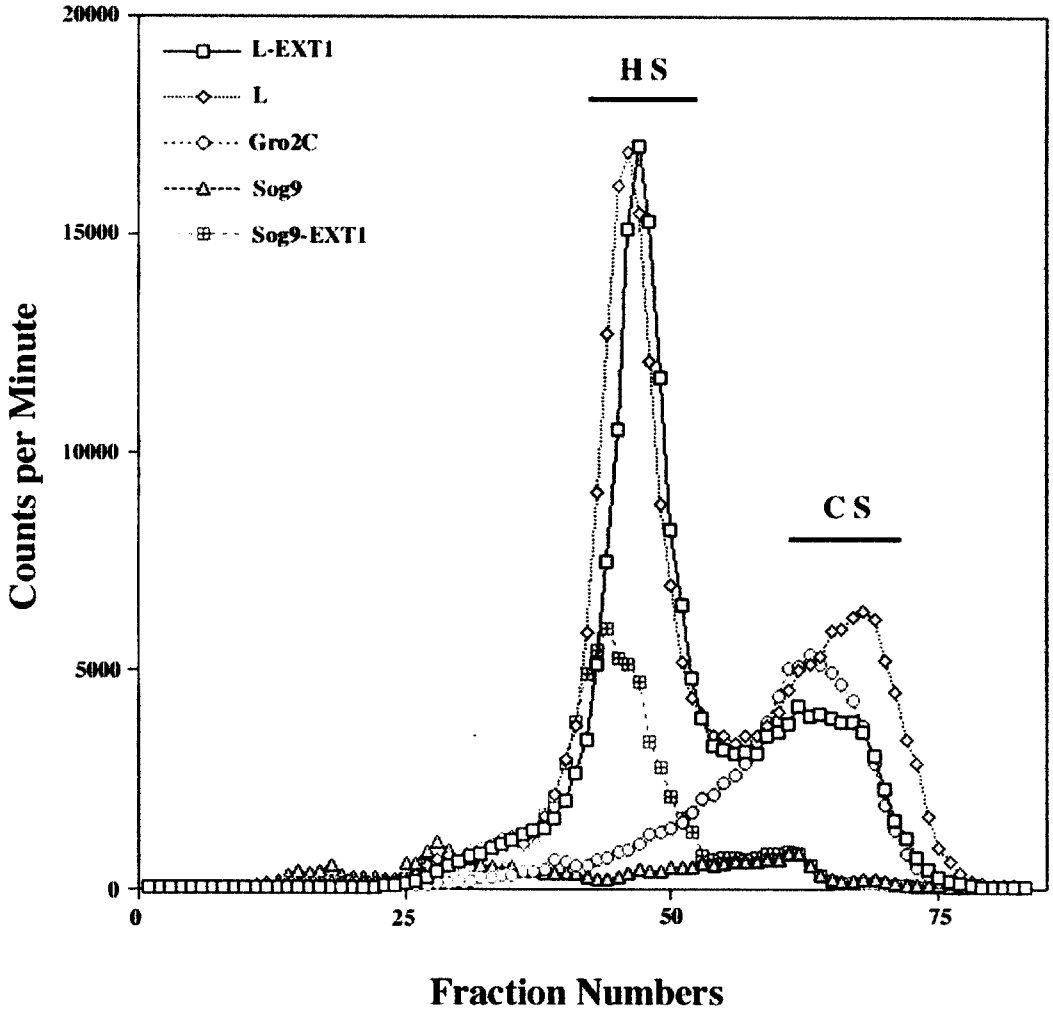


Figure 3.3 Anion exchange HPLC of cell-associated GAGs for control cell lines labeled with [^{35}S]-sulfate.
Cell monolayers were grown for 24 hours in the present of [^{35}S]-sulfate. GAGs were isolated and fractionated by HPLC. Samples were normalised by the amount of proteins. HS, elution position of heparan sulfate; CS, elution position of chondroitin sulfate.

Analysis of [^{35}S]GAGs from five of nine L-EXT1 derived mutant cell lines (TL) by anion-exchange HPLC showed that these GAGs consisted of material that coeluted with CS (Fig. 3.4). One possibility to account for the failure to detect sulfated heparin-like chains in L-EXT1 mutants was that those mutants are incapable of synthesizing the alternating disaccharide units characteristic of this GAG. To test this possibility, control cell and TL mutant cell monolayers were incubated with [^{3}H] glucosamine to label the carbohydrate chains. After 24 hours of labeling, GAG fractions from cell extracts and extracellular medium were pooled and analyzed by anion-exchange HPLC. As can be seen in figure 3.5, the control cells' GAGs form peaks that resemble those for the sulfate moieties observed in figure 3.3. When all five TL mutants were subjected to HPLC analysis, there was no detectable HS peak, despite the presence of normal or slightly elevated amounts of CS (Fig. 3.6).

3.2.5 Only EXT1 corrects the resistant mutants deficiency in HS biosynthesis.

EXT1 is a member of a family of proteins, which also includes EXT2, EXTL1, EXTL2 and EXTL3. Lind et al. (Lind et al. 1998) have reported that both EXT1 and EXT2 contain enzyme activities corresponding to HS GlcNAc-T and GlcA-T, although only low level (5%-10%) of enzyme activity has been seen for EXT2 compared with EXT1, in mammalian cell systems (Kitagawa et al. 2001; McCormick et al. 2000). However, it has been shown that EXT2 is unable to complement the specific defect in EXT1 in Sog9 cells or in CHO cells (McCormick et al. 2000; Wei et al. 2000).

To gain further insight into the specific defects in the mutant TL cell lines, EXT1 and EXT2 were introduced into the mutant cells and assayed for the expression of HS by HSV infection. As shown in Figure 3.7, EXT1 rescued the susceptibility of all nine TL mutant cell lines from HSV infection, which implied that EXT1 corrected the HS deficiency in those mutant cell lines. By contrast, the related protein EXT2 displayed no rescued activity in this assay

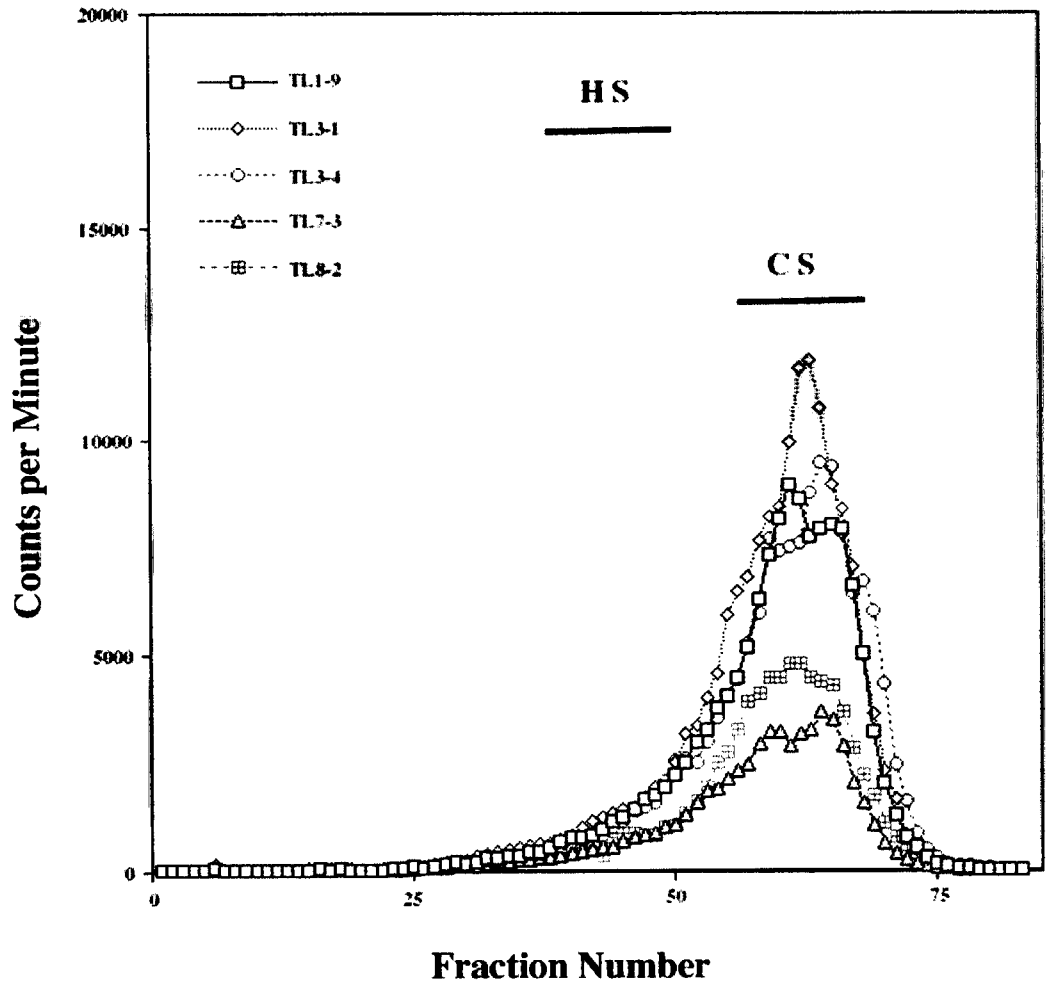


Figure 3.4 Anion exchange HPLC of cell-associated GAGs for five TL mutant cell lines labeled with [^{35}S]-sulfate.
Cell monolayers were grown for 24 hours in the present of [^{35}S]-sulfate. GAGs were isolated and fractionated by HPLC. Samples were normalised by the amount of proteins. HS, elution position of heparan sulfate; CS, elution position of chondroitin sulfate.

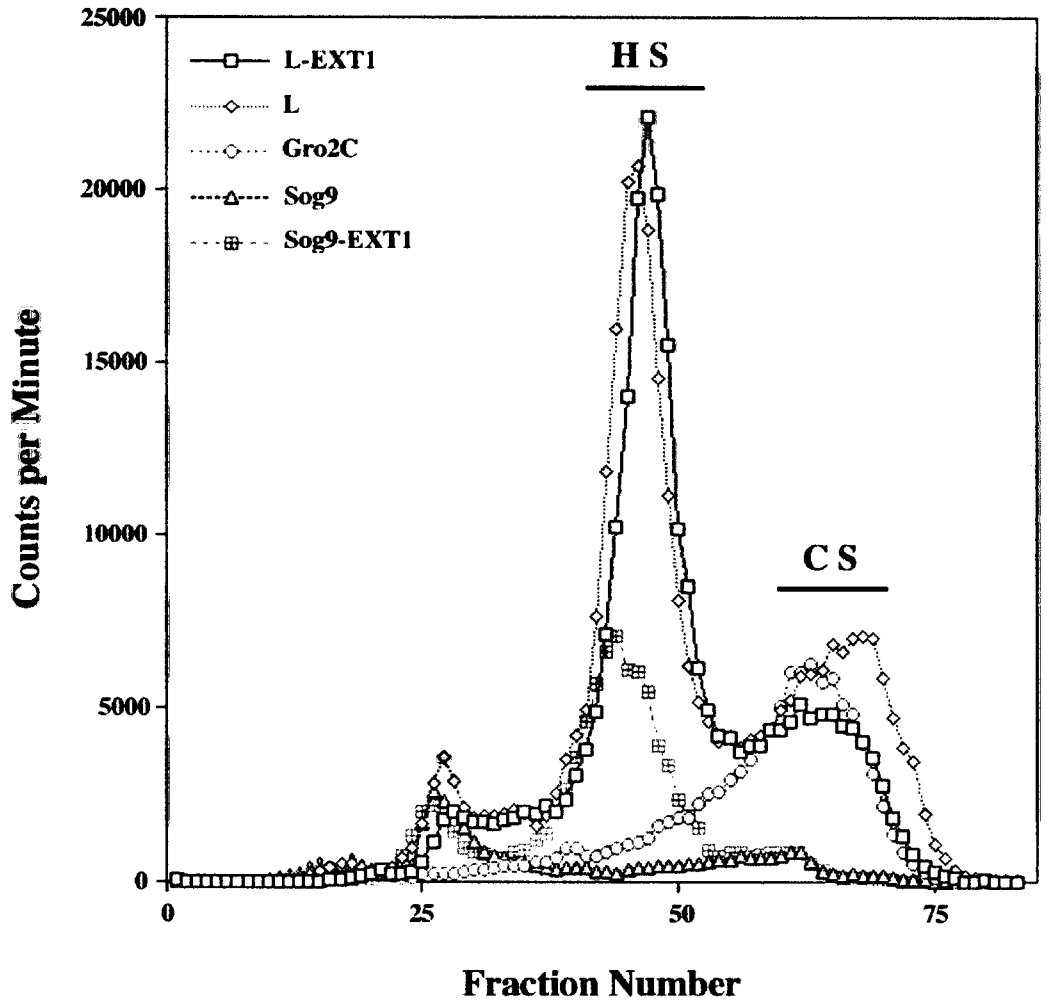


Figure 3.5 Anion exchange HPLC of cell-associated GAGs derived for control cell lines labeled with [^{3}H] glucosamine.
Cell monolayers were grown for 24 hours in the present of [^{3}H] glucosamine. GAGs were isolated and fractionated by HPLC. Samples were normalised by the amount of proteins. HS, elution position of heparan sulfate; CS, elution position of chondroitin sulfate.

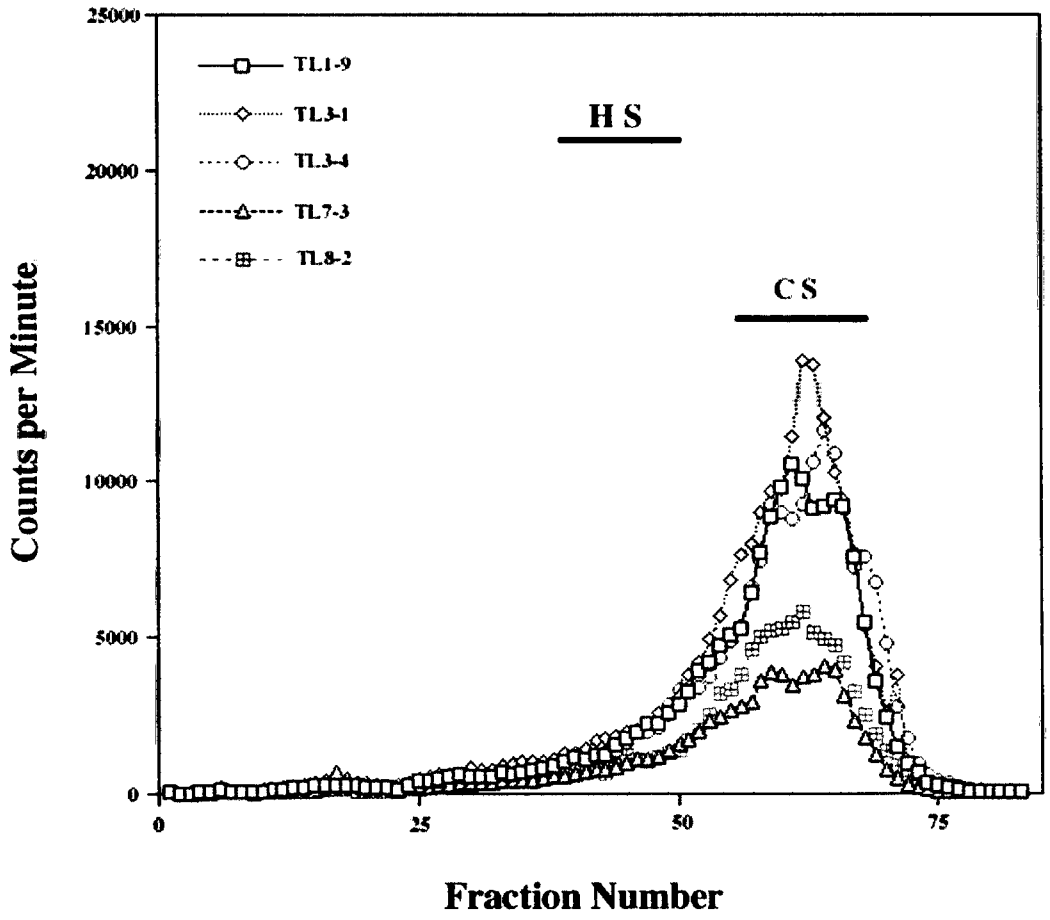


Figure 3.6 Anion exchange HPLC of cell-associated GAGs from TL derived cell lines labeled with [^{3}H] glucosamine.
Cell monolayers were grown for 24 hours in the present of [^{3}H] glucosamine. GAGs were isolated and fractionated by HPLC. Samples were normalised by the amount of proteins. HS, elution position of heparan sulfate; CS, elution position of chondroitin sulfate.

(Fig.3.7), implying that EXT2 alone is not able to catalyze the polymerization of HS in those TL mutant cell lines. Additional experiments also established that the EXT homologs EXTL2 (Kitagawa et al. 1999), and EXTL3 (Van Hul et al. 1998), and the HS-modifying enzyme N-deacetylase/N-sulfotransferase-2 (NDST2) (Eriksson et al. 1994) were inactive in this assay (data not shown), implying that the defect in HS expression observed was not due to a defect in any of these genes.

3.3 Discussion

The study presented in this chapter describes the isolation of a group of nine mammalian mutant cell lines with an HSV-1 resistant phenotype. Five of the mutant cell lines were characterised by using HPLC analysis. Any mutation that is essential for one of the numerous stages of the viral infection process, while being unnecessary for cellular survival, could produce such mutant cell lines.

Surprisingly, when five L-EXT1 derived mutant cell lines were subjected to anion exchange HPLC analysis, results suggest that they were all unable to produce or effectively transport any detectable amount of HS to their cell surface and as secreted HS in the media, despite the presence of normal or elevated amounts of CS chains. More strikingly, EXT1 alone corrected the HS deficiency of all nine L-EXT1 derived mutant cell lines (Fig. 3.7), although four other EXT genes (EXT2, EXTL1, EXTL2 and EXTL3) have now been identified and are likely to harbor glycosyltransferase activities that contribute to the synthesis of HS or heparin (Kim et al. 2001; Kitagawa et al. 1999; Lind et al. 1998).

Similar results have been published by Esko and his colleagues regarding the characterization of a collection of Chinese hamster ovary (CHO) cell mutants (pgsD) that fail to make HS. In Esko's studies, 16 HS deficient CHO mutants were corrected by the transfection of EXT1, whereas none of the CHO mutant cell lines transfected with EXT2, or EXTL1-3 corrected

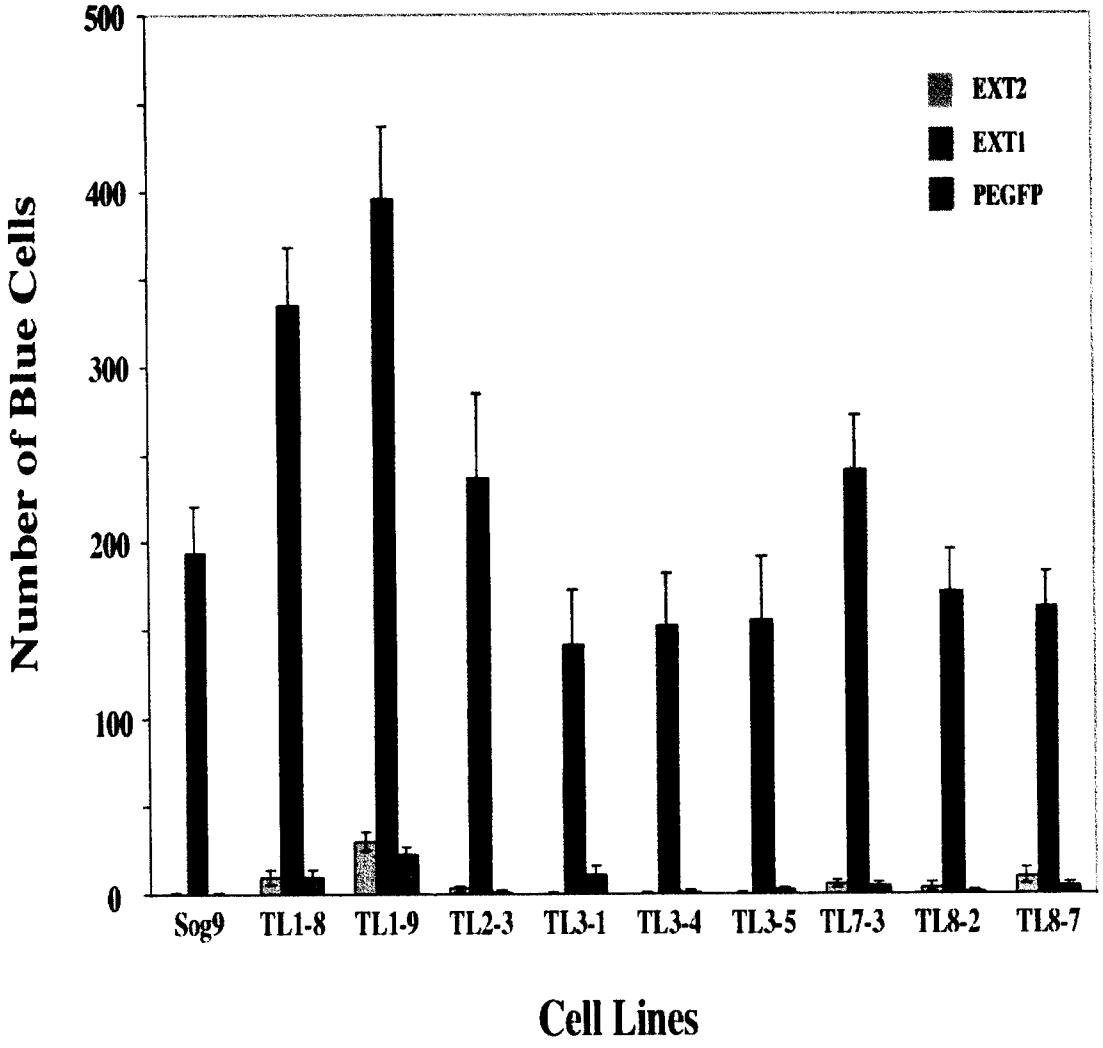


Figure 3.7 Effect of EXT1 and EXT2 expression on HSV-1 infection of TL mutant cell lines.

Subconfluent Sog9 cells and TL mutant cells were transfected with either control plasmid (PEGFP) or EXT1 or EXT2 and incubated for 30 h before infection with HSV-1 expressing β-galactosidase. At 10 h post infection, cells were stained with x-gal. Two independent experiments were performed with similar results. Data shown are the average of five fields counted from an infected well of a single experiment.

the defects (Wei et al. 2000). It is hard to understand why EXT1 is the most vulnerable gene for mutation when the HS deficiency phenotype was selected in both murine fibroblast cells and CHO cells where numerous genes are involved in the process of HS biosynthesis. A likely explanation may be that some glycosyltransferases which are involved in HS biosynthesis, are essential for cell survival and/or exist in multiple copies.

Originally, we hypothesized that by stably expressing extragenous copies of human EXT1 in a murine L cell line, termed TL, multiple copies of functional EXT1 would be intergrated and expressed. The fact that all nine TL mutants were corrected by once again transfecting in the *EXT1* gene (fig 3.7) may suggust that TL cells may in fact only express one functional copy of EXT1. Indeed, since stable cell lines are generally generated by homologus recombination, and human EXT1 is 94% homologous with murine EXT1 (Lin and Wells 1997), the human EXT1 construct may insert directly into the murine EXT1 locus of the host cell genome, thereby inactivating it and functionally replacing it by a single copy of human EXT1.

The EXT2 protein had been suggested to harbor both the GlcNAc and GlcA transferase activities that involved in HS polymerization, as for EXT1. However, recent data suggests that the low level of activity seen for EXT2 in mammalian cell systems could be due to contamination by EXT1, given that the enzymes form hetero-oligomeric complexes (Kitagawa et al. 2001). It has been shown that EXT1 is predominantly localized to the ER when over expressed in cells. However, when co-expressed with EXT2, both EXT1 and EXT2 proteins redistribute to the Golgi apparatus (Kobayashi et al. 2000; McCormick et al. 2000). EXT2 may therefore act as a chaperone protein and facilitate the EXT1/EXT2 complex for intracellular trafficking. Because none of the L-EXT1 cell mutants or CHO cell mutants deficient in HS expression have detectable defects in EXT2, and only EXT1 corrects the HS deficiency, EXT1 could be the sole enzyme that polymerizes HS in mammalian cells.

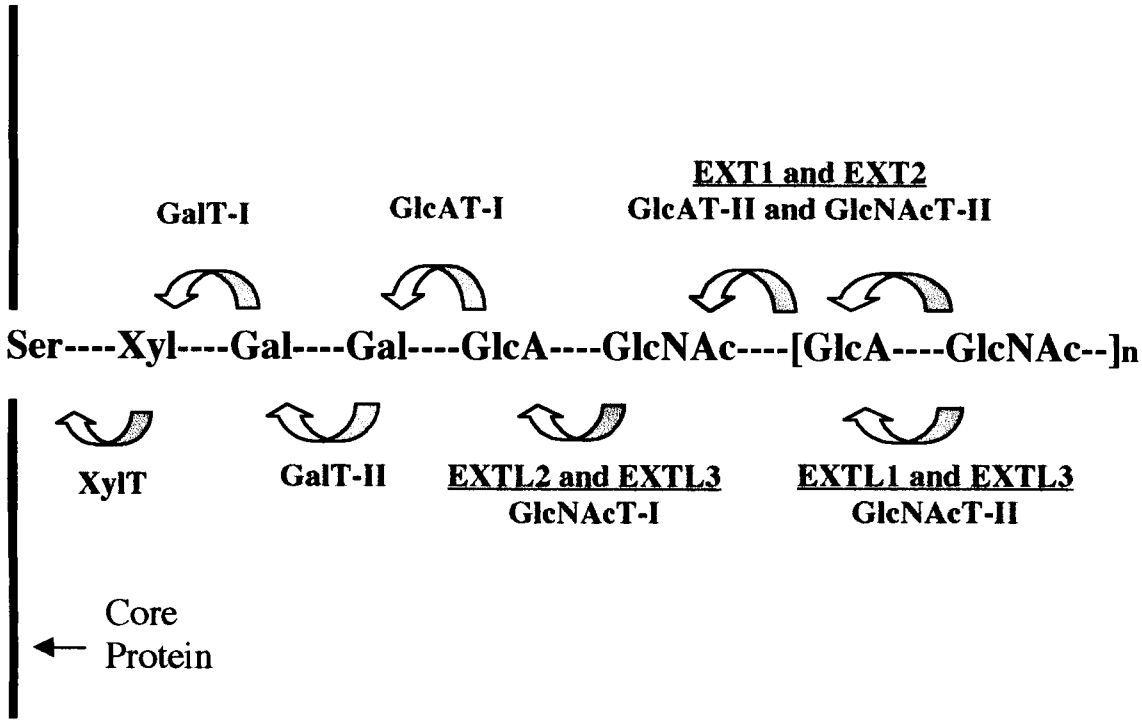


Figure 3.8 The sugar units and their corresponding glycosyltransferases involved in HS biosynthesis and the EXT proteins.
Abbreviations are as follows; Ser (serine), Xyl (xylose), Gal (galactose), GlcA (glucuronic acid), GlcNAc (N-acetylglucosamine).

Chapter 4: Analysis of two conserved aspartic acid residues located in a EXT1 putative catalytic domain

4.1 Introdction:

Glycosyltransferases catalyze the transfer of sugar residues from an activated donor substrate to an acceptor molecule. There are two types of glycosyltransferases and they can be categorized into processive enzymes and nonprocessive enzymes. Processive enzymes, such as hyaluronan synthase (Jing and DeAngelis 2000), and KfiC protein from Escherichia (E) coli K5 capsule gene (Griffiths et al. 1998), transfer multiple sugar residues to the acceptor. Nonprocessive enzymes, such as those involved in the synthesis of the repeat unit of succinoglycan (Lellouch and Geremia 1999), on the other hand, catalyze the transfer of a single sugar residue to the acceptor. Glycosyltransfer reactions have been studied in a wide variety of systems, and the enzymes responsible for these reactions have been characterized. In a number of cases, genes coding for glycosyltransferases have been isolated, making possible the identification of other glycosyltransferases based on sequence analysis.

Hydrophobic cluster analysis (HCA) has been used to detect three-dimensional similarities in proteins showing very limited sequence relatedness. For instance, HCA has allowed the accurate grouping of cellulases with only 11% to 15% identical amino acids into families of structurally related proteins (Henrissat et al. 1989). HCA of processive β-glycosyltransferases identified a conserved domain consisting of four alternating β-strands separated by three α-helices. Two conserved aspartic acid residues were identified in the C-terminal loops of the β 2- and β 4-strands within this domain, and it has been suggested that these might be the catalytic amino acids for the nucleophilic substitution reaction involved in β-bond formation between two sugar units (Saxena et al. 1995).

4.2 Result:

4.2.1 Sequence comparison of EXT1 and two other β-glycosyltransferases by HCA plot

Using the HCA-Plot program (Doriane Informatique, Le Chesnay, France), the predicted HCA plot pattern based on the EXT1 amino acid sequence was shown in Fig.4.1. The HCA program writes protein sequences on a duplicated net and circles clusters of hydrophobic amino acids (Ala,Val, Leu, Ile, Met, Phe, Gln). The prediction of β-strands and α-helices is based on the observed association of specific hydrophobic cluster shapes with secondary structures (Lemesle-Varloot et al. 1990). In general, wide bands of hydrophobic clusters are associated with α–helix structures, while narrow bands of hydrophobic clusters are associated with β-strands. The plots are then visually compared for similarity in the hydrophobic cluster patterns, limiting analysis to the predicted globular portions of the proteins. Plots were aligned using the results of amino acid sequence alignments as a starting point. Hydrophobic clusters with obvious similarities were used as anchors for the structural alignment. Alignment of the HCA plot of EXT1 with plots of other known β-glycosyltransferases revealed a conserved domain structure of repeating α–helix and β-strand motifs between amino acids 110-210, and vertical lines were drawn to indicate structurally conserved features (Fig. 4.2). Within this domain, two aspartic acid residues at 147 and 202 in EXT1 were identified as the highly conserved aspartic acid residues in the C-terminal loops of the β2 and β4 strands present in all of the β -glycosyltransferases (Fig. 4.2, Table 4.1). For reasons of clarity, only HCA alignments with KfiC protein from E. Coli K5 capsule gene cluster and hyaluronan synthase (HasA) from *Streptococcus pyogenes* (processive β-glycosyltransferases that utilize alternating UDP-GlcA and UDP-GlcNAc) are shown in Figure 4.2, however, most β-glycosyltransferases may be aligned in this way.

4.2.2 Effect of mutation of the two highly conserved aspartic acid residues

Based upon the identification of the conserved domain and the presence of the two highly conserved aspartic acid residues, site-directed mutations were made. The aspartic acid (D) residues at 147 and 202 within the domain were replaced with glutamic acid (E) residues. The

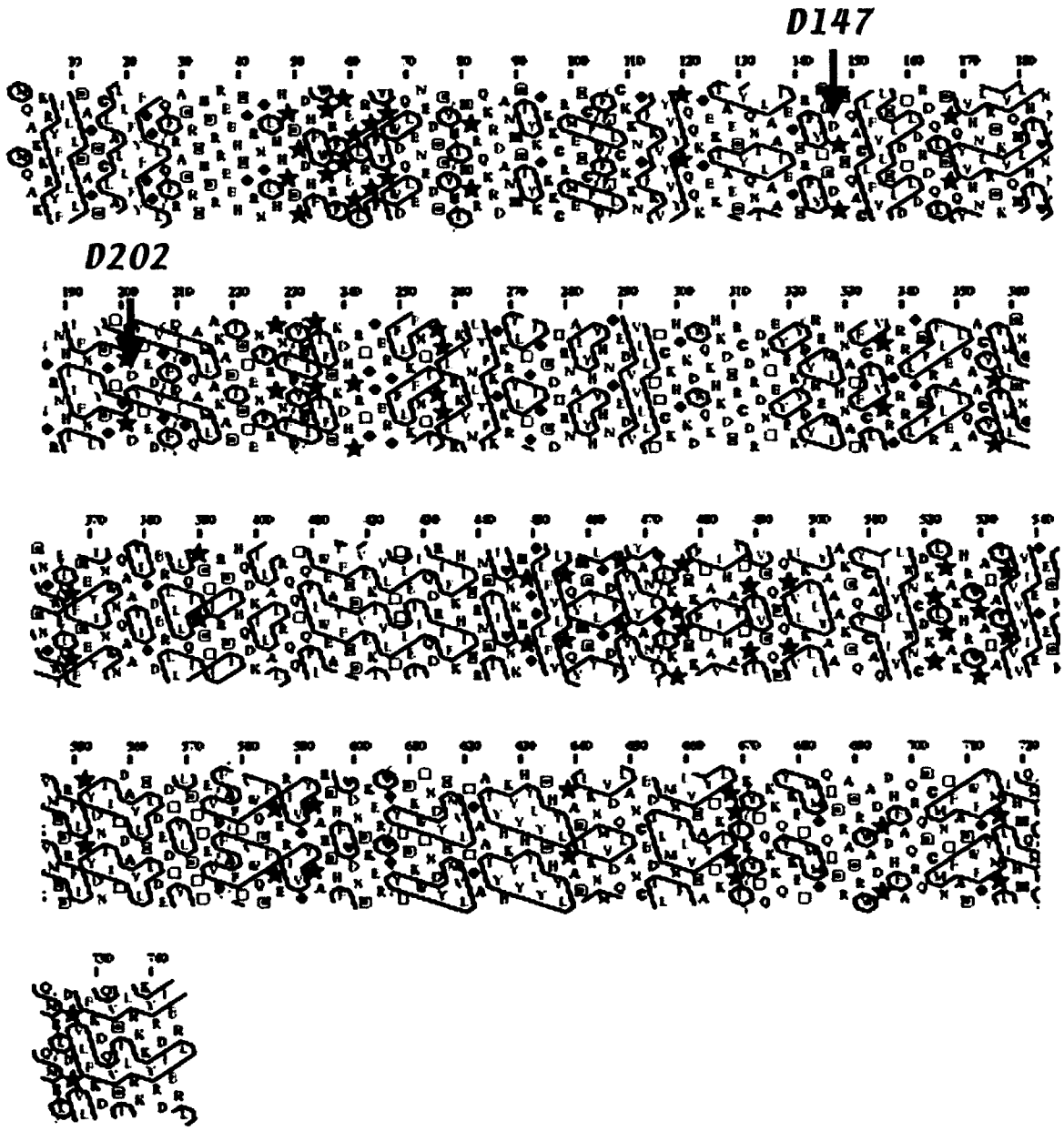


Figure 4.1 HCA plot of EXT1.
The plot was made with the HCA-Plot program (Doriane Informatique, Le Chesnay, France). In this plot, the protein sequence is written on a duplicated α-helical net and the contour of clusters of hydrophobic residues are automatically drawn. Two conserved aspartic acid residues, D147 and D202 are pointed out by red arrows. The standard one letter code for amino acids is used except for proline, glycine, serine, and threonine, which are represented by ★, ◆, ⊡, and □, respectively.

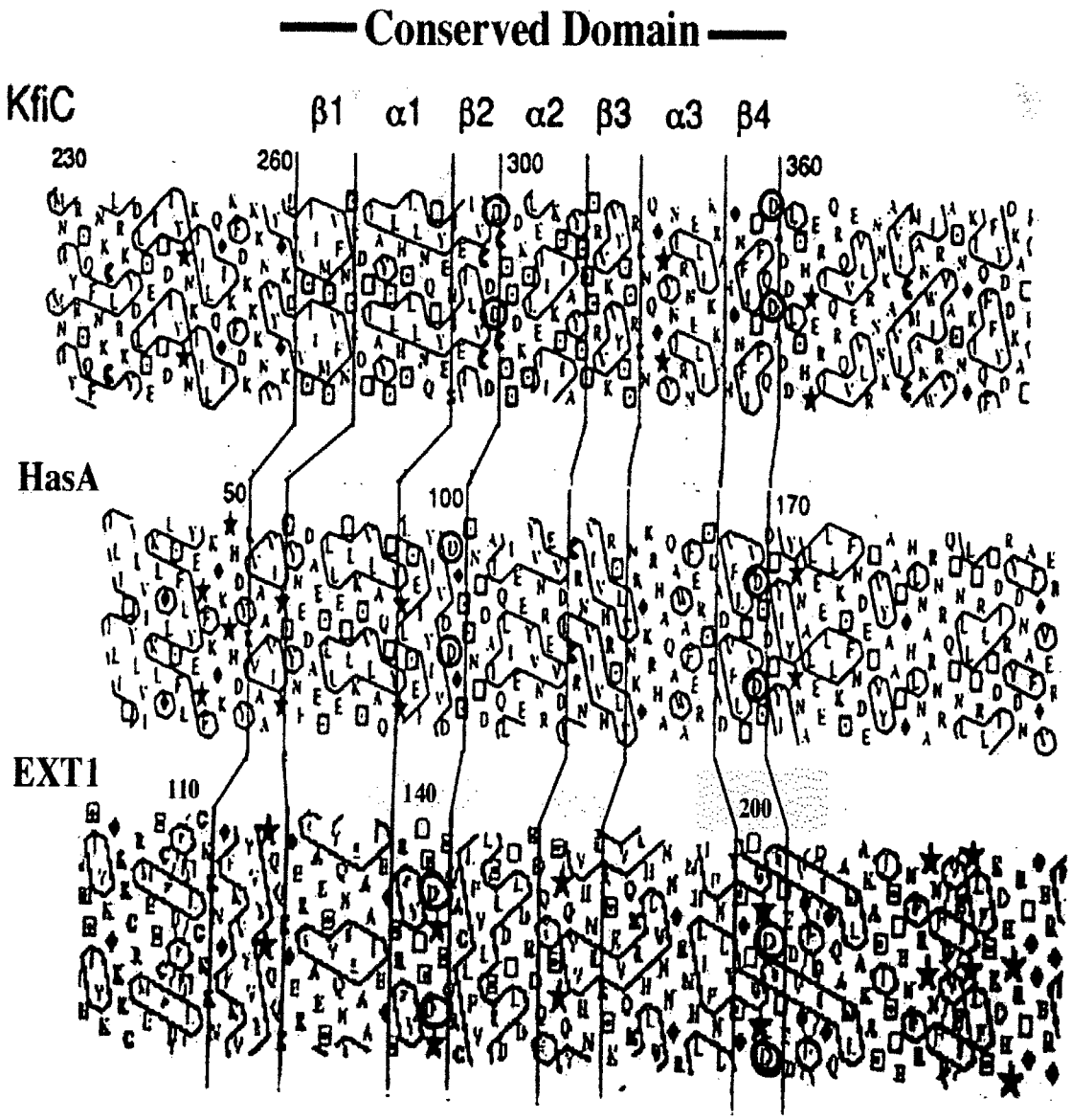


Figure 4.2 HCA alignment of the EXT1 protein with two other known β-glycosyltransferases.
The EXT1 protein is aligned with the KfiC protein from the E. Coli K5 capsule gene cluster (KfiC) and the hyaluronan synthase (HasA) from *Streptococcus pyogenes*. Vertical lines indicate the proposed correspondences between the sequences. The two strictly conserved aspartic acid residues are circled.

Table 4.1 Primary amino acid sequence of the HCA predicted conserved domain A in β-glycosyltransferases.

Gene	Organism	Sequence	
		D147 ***D202***	
EXT1	Human	144).YTS**D**PSQACLFV...YSGTWP**D**YTEDV	207
KfiC	E. coli	298) VCD**D**CSSDKSLEI...NFITFQ**D**ADDLS	358
HasA	Strep. Pyogenes	61) VD**D**GSSNTDAIQ...DVFLTV**D**SDTYIY	126

Base on the HCA alignment from Fig. 4.2, two conserved aspartic acid residues were identified in the C-terminal loops of the β2- and β4–strands within the conserved domain of β-glycosyltransferases. These two aspartic acid residues are shown in bold. The aspartic acid residues in EXT1 selected for mutagenesis studies are underlined. As suggested in grouping family of other β-glycosyltransferases (Henrissat et al. 1989), EXT1, Hfic, and HasA proteins are showing very limited primary amino acid sequence relatedness.

effect of the site-directed mutations in the EXT1 gene on heparan sulfate (HS) polymerase activity in Sog9 cells was determined by assaying the ability of these mutant EXT1 proteins to rescue HSV infectivity. This assay takes advantage of two biological reagents: herpes simplex virus (HSV), which utilizes cell surface HS as a primary receptor for infection, and a HS-deficient murine cell line, sog9. Sog9 cells harbor a specific defect in the EXT1 gene abrogating HS biosynthesis (McCormick et al. 2000), thus rendering these cells highly resistant to HSV infection (Banfield et al. 1995). When a functional EXT1 gene is transfected into sog9 cells, the defect is complemented, HS is synthesized and the cells become susceptible to HSV (McCormick et al. 1998). Surprisingly, both mutations were able to rescue the HSV infectivity in sog9 cells (Fig 4.3), although the same amino acid substitution of the two conserved aspartic acids in the Kfic protein abolished any detectable catalytic activity for the β addition of UDP-GlcA (uridine diphosphoglucuronic acid) to the oligosaccharide acceptors. Results suggested that these conserved aspartic acid residues, identified by using HCA plot, are catalytically important for β-glycosyltransferase activity in the Kfic protein (Griffiths et al. 1998).

4.3 Discussion

Exostosin-1, the corresponding gene product of EXT1, known to harbour HS polymerase activity, was analyzed using the protein sequence analysis tool HCA, that is based on the detection and comparison of structural segments in the hydrophobic core of globular protein domains. Exostosin-1, like E. coli KfiC protein, is thought to be a processive glycosyltransferase with one α– and one β-glycosyltransferase activity. Using HCA, a conserved secondary structure motif characteristic of β-glycosyltransferases was identified along with two highly conserved aspartic acid residues at positions 147 and 202. These units were changed by site-directed mutagenesis to glutamic acid and tested for the ability of the mutant EXT1 proteins to synthesize HS using our HSV infectivity assay. Surprisingly, mutation of either of the two aspartic acid residues did not abolish the enzyme activity as has been reported for the KfiC protein (Griffiths et al. 1998). These results suggest that these two aspartic acids in the EXT1 protein are not

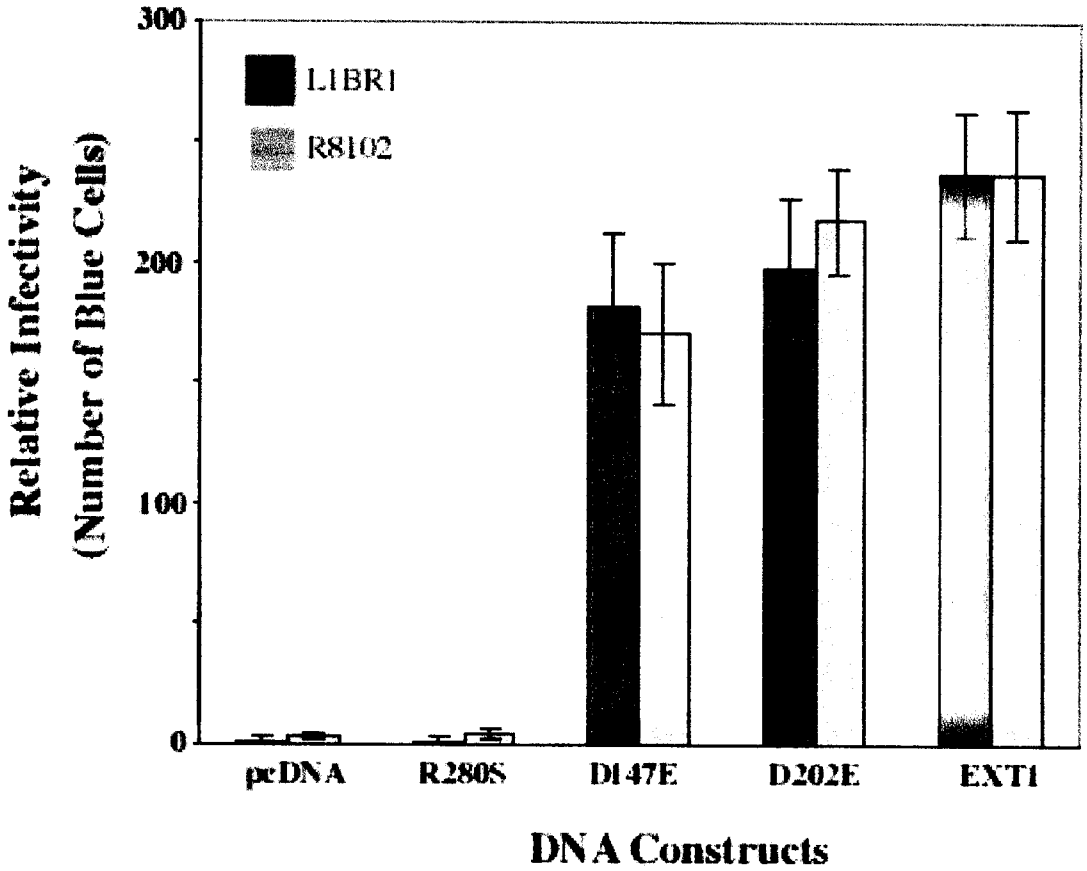


Figure 4.3 The effects of mutation on HS biosynthesis in cell culture.
Two highly conserved aspartic acid residues within the putative catalytic domain of EXT1 were mutated and their effect on HS biosynthesis in cell culture was measured by the HSV infectivity assay. This assay is based on the ability of HSV to infect cells by attaching to cell surface HS. At 10h post infection, cells were stained with X-gal. Two independent experiments were performed with similar results. Data shown are the average of five fields counted from an infected well of a single experiment. R280S EXT1 mutant construct, which has been described as null mutant in Chapter 5, is being used as negative control.
R8102 - HSV-1 (strain F) expressing β-galactosidase. L1BR1 – HSV-2 (strain 186) expressing β-galactosidase.

essential for the enzyme activity and, therefore, are not likely to be directly involved in the catalytic activity. A recent report has identified an EXT1 etiological missense mutation affecting aspartate residue at position 164 (Bovee et al. 1999), which is located between the two conserved aspartate residues identified in this chapter. When this aspartic acid residue was changed to glutamic acid residue (Table 5.2), the enzyme activity was completely abolished as reported for the KfiC protein (Griffiths et al. 1998). Therefore, aspartic acid residue at position 164 in the EXT1 protein may involved in the catalytic activity. Further study of aspartic acid 164 will be addressed in Chapter 5.

CHAPTER 5: Analysis of etiological missense mutations in EXT1

5.1 Introduction

Hereditary Multiple Exostoses (HME) is an autosomal dominant disorder characterized by the presence of cartilage-capped exostoses, which develop from the growth plate of all long bones. Exostoses are generally not apparent at birth, but develop during childhood and grow until puberty when linear bone growth ceases (Hennekam 1991; Schmale et al. 1994; Solomon 1963).

Genetic linkage has identified three different loci for phenotypically identical hereditary multiple exostoses: EXT1 is located on 8q24.1(Cook et al. 1993), EXT2 on 11p11-p12 (Wu et al. 1994) and EXT3 on 19p (Le Merrer et al. 1994). The majority of multiple exostoses cases are attributable to mutations within the EXT1 and EXT2 genes, with EXT3 being a minor locus (Le Merrer et al. 1994). The EXT1 gene was reported to show linkage in 44% to 66% of the HME families (Legeai-Mallet et al. 1997; Raskind et al. 1998), whereas EXT2 appears to be involved in only 27% of familial cases (Legeai-Mallet et al. 1997). The EXT1 and EXT2 proteins have a predicted type II transmembrane glycoprotein structure, and both proteins localize predominantly to the endoplasmic reticulum when over-expressed individually in cells (Lin and Wells 1997; McCormick et al. 2000; McCormick et al. 1998).

Despite extensive genetic characterization, the function of the EXT proteins remained unknown until 1998 when the study of a heparan sulfate (HS) deficient cell line, sog9, revealed that EXT1 is involved in HS biosynthesis (McCormick et al. 1998). Biochemical studies have since confirmed that EXT1 and possibly EXT2 possess the *N*-acetylglucosamine (GlcNAc) and D-glucuronic acid (GlcA) transferase (T) activities representative of an HS-Polymerase (HS-Pol) (Lin et al. 1998; McCormick et al. 2000). Moreover, the link between EXT and HME has been further affirmed by a study showing that EXT protein expression was significantly reduced and in some cases, absent, in HME-derived chondrocytes (Bernard et al. 2001). Extensive genetic analysis of HME patients' genomic DNA over the past few years has led to the identification of a number of mutations in both EXT1 and EXT2 that appear to be disease-related, the majority of

which result in premature termination. However, a small number of mutations have been identified, often repeatedly, affecting single amino acids mainly in the N-terminal half of the EXT1 and EXT2 proteins. Here in this chapter, I used HSV infectivity assay to test the putative etiological mutant EXT1 proteins for their ability to catalyze HS biosynthesis in Sog9 cells, as described in chapter 4. This assay is specific for EXT1 activity and does not function with other EXT members, including EXT2, which are not able to complement the defect in HS biosynthesis (McCormick et al. 2000). Moreover, this assay is particularly useful because it measures cellular HS biosynthesis and transduction to the cell surface.

I hypothesized that if HS polymerization is related to the disease pathogenesis of HME, then the HS polymerase activity of EXT1 constructs bearing disease-causing mutations should be altered or impaired. Furthermore, these specific mutants may be useful for pinpointing the different functional domains of the EXT1 protein, thus leading to a better understanding of the roles of these mutations in the pathogenesis of HME.

5.2 Results

5.2.1 Two Groups of Etiological Missense Mutant

To date, there are forty-nine different EXT1 mutations have been reported from patients with HME (Wuyts and Van Hul 2000). Although the majority of the etiological mutations in EXT1 are splice-site, frameshift, or nonsense mutations that result in premature termination, twelve missense mutations have also been identified (Wuyts and Van Hul 2000). These twelve missense mutations are found exclusively in the luminal portion of the protein in three of the eleven exons (Table 5.1).

5.2.2 Analysis of Mutant EXT1 Function in Cell Culture

To determine whether the HS polymerase activity of EXT1 is relevant to HME, cDNA containing all twelve putative etiological missense mutants that affecting seven different residues

Table 5.1 Etiological missense mutations identified in the EXT1 gene

HME etiological mutant proteins	Exon	cDNA change[a]	Reference
Q27K	1	C79A	Wuyts et al., 2000
D164H	1	G490C	Bovee et al., 1999
R280G	1	A838G	Wuyts et al., 1998 Raskind et al., 1998
R280S	1	G840C	Raskind et al., 1998
N316S	1	A947G	Bovee et al., 1999
G339D	2	G1016A	Philippe et al., 1997
R340C	2	C1018T	Philippe et al., 1997
R340S	2	C1018A	Wuyts et al., 1998
R340H	2	G1019A	Raskind et al., 1998
R340L	2	G1019T	Hecht et al., 1997
A486V	6	C1475T	Xu et al., 1999
P496L	6	C1487T	Xu et al., 1999

[a] All mutations were uniformly renumbered with the adenosine of the start codon = nucleotide position +1.

(Q27, D164, R280, N316, R340, A486, and P496), were constructed. These cDNA constructs were tested for over expression of the mutant protein could restore HSV susceptibility to Sog9 cells as measured by infection with a β-galactosidase-expressing HSV strain. Representative results for each of the seven residues is shown in Figure 5.1.

Based on their ability to rescue the defect in sog9 cells, the missense mutants fell into two groups: 'active ' mutants, which rescue HSV infectivity, and null mutants, which are inactive in the HSV infectivity assay, as summarized in Figure 5.2. Eight out of the twelve missense mutants belong to the group of null mutants. Five of these null mutants cluster together, affecting amino acids G339 and R340, while two affecting R280. The last mutant in this group is found in codon 164, which is located in a DXD motif, a sequence purported to be a key component of glycosyltransferase active sites (Busch et al. 1998; Wiggins and Munro 1998). Active mutants are spread out in exon 1 and exon 6 are not clustered (Fig.5.2).

Comparable results were observed for each of the missense constructs when the transfected Sog9 cell extracts were assay for β-galactosidase activity using ONPG (o-nitrophenyl-β-D-galactopyranoside) (Fig. 5.3).

5.2.3 Complementation of HS polymerase activity in EXT1 missense mutants

To determine whether EXT1 proteins bearing missense mutations are able to complement each other in trans, as has been observed for a bacterial β-glycosyltransferase, hyaluronan synthase (Jing and DeAngelis 2000), combinations of the null mutants were co-transfected into sog9 cells and assayed for rescue of HSV infectivity. Surprisingly, no tested combinations were able to complement each other. Moreover, when sog9 cells were co-transfected with null mutant and wild-type forms of EXT1, no measurable decrease in activity was observed, even when the amount of null mutant and wild-type forms were coexpressed in a ratio of 10:1 (Fig.5.4). These results indicate that wild-type EXT1 is able to polymerize HS in the presence of excess amounts of mutant forms of the EXT1 protein.

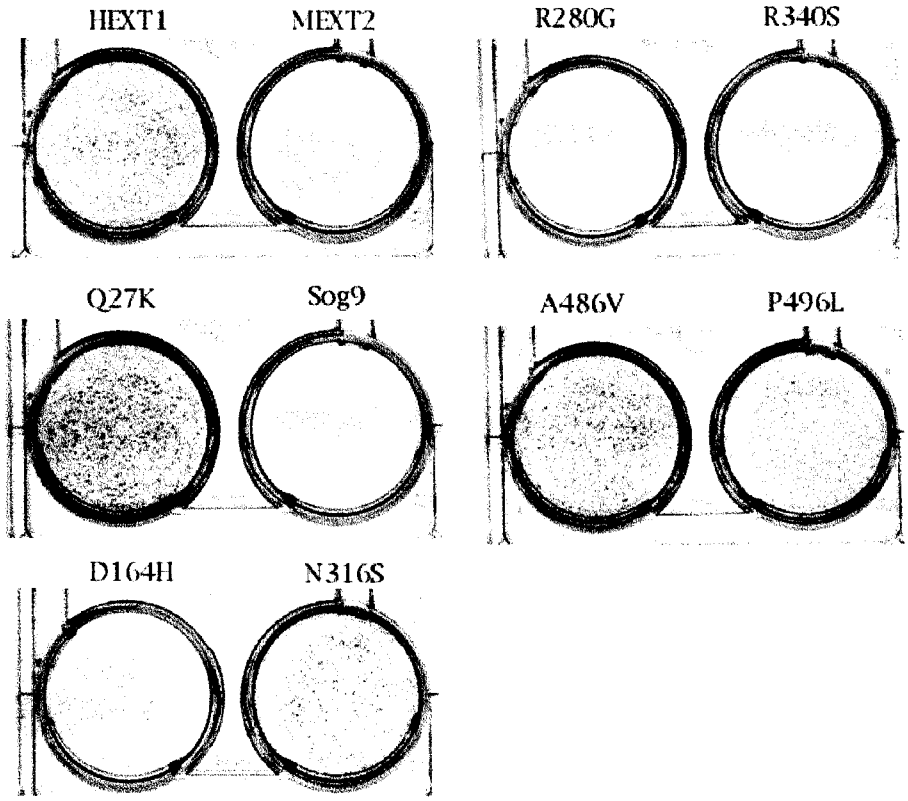


Figure 5.1 Cell-culture assay for EXT1 function.
HSV-resistant, HS-deficient mouse fibroblast sog9 cells were either mock-transfected (sog9) or stably transfected with cDNAs corresponding to wild-type EXT1, EXT2 or mutant EXT1 constructs. Cells were then infected with a β-galactosidase expressing HSV and were stained blue with X-gal. For the two residues affected by multiple etiological missense mutations (R280 and R340) only one mutation form is shown.

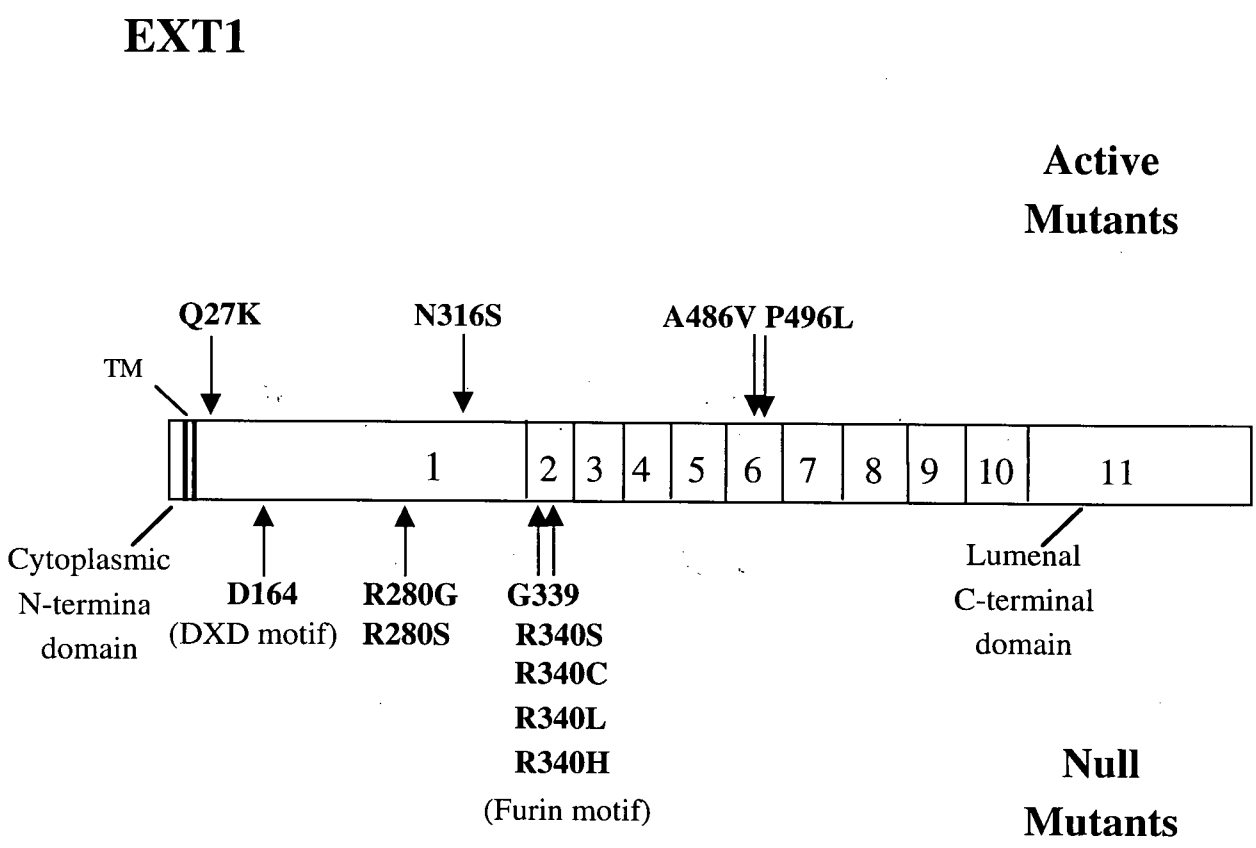


Figure 5.2 Two phenotypes for etiological EXT1 mutants.
Schematic representation of the EXT1 coding region, illustrating the 11 exons of the human EXT1 gene with all 12 of the currently reported missense mutations from HME patients. Mutant EXT1 forms can be divided into two categories: "active" mutants (top arrows), which were observed to synthesize HS, and null mutants (bottom arrows), which were observed to abrogate HS biosynthesis. TM-Transmembrane domain.

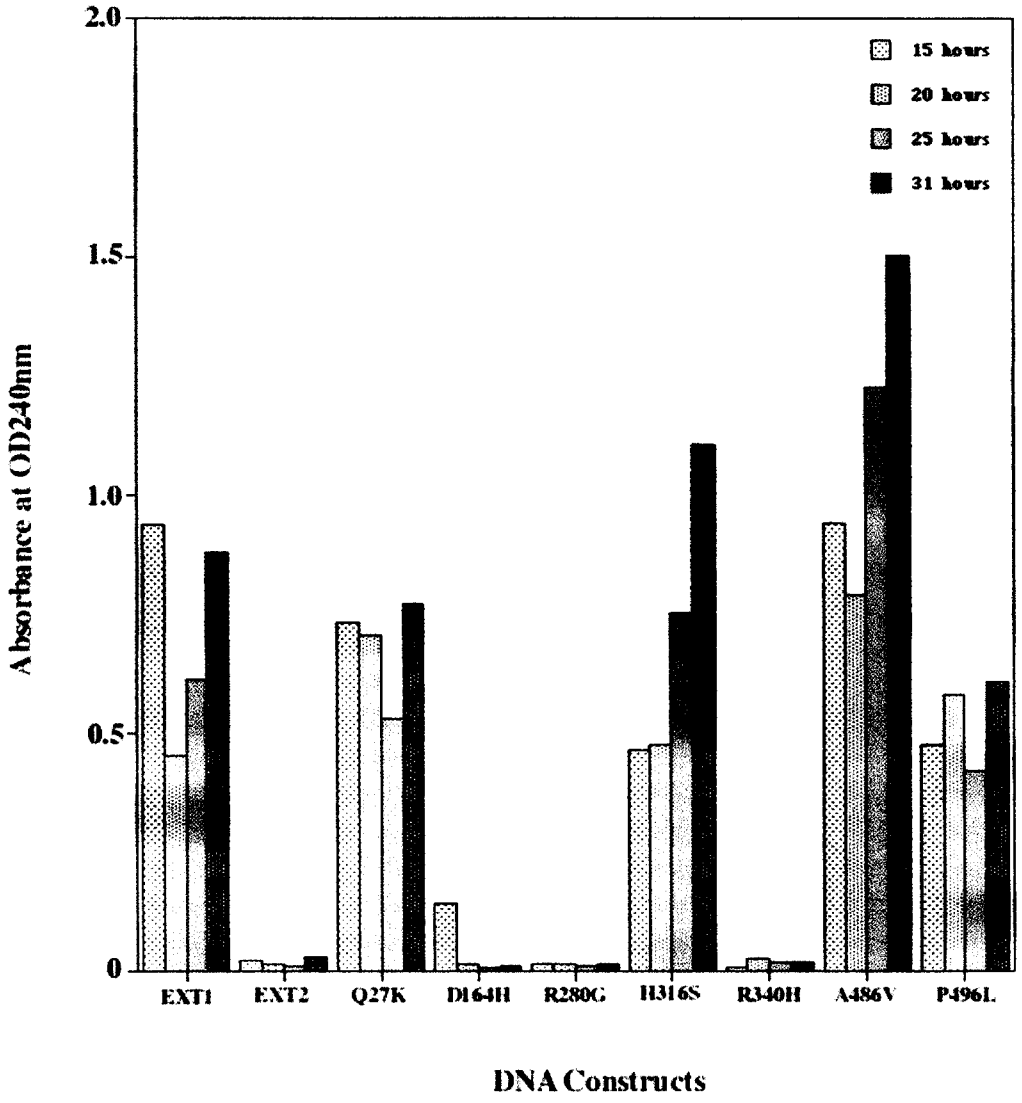


Figure 5.3 Enzymatic activity of EXT1 missense mutants measured by HSV infectivity assay.
Sog9 cells were transfected with missense mutants and incubated for 15h, 20h, 25h, and 31h before exposure to a β-galactosidase-expressing HSV. β-galactosidase activity was measured using ONPG. Results are shown for one single experiment.

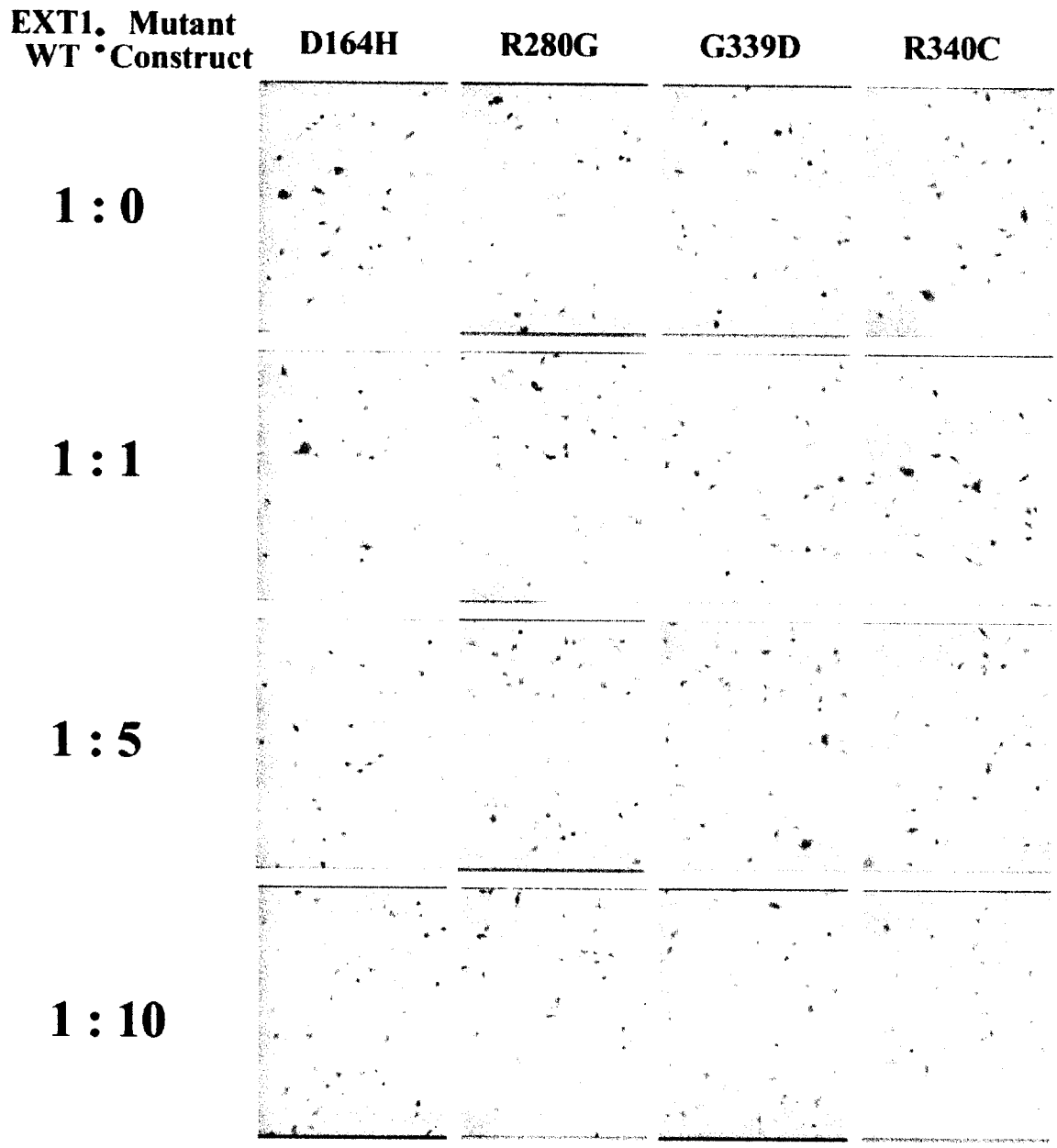


Figure 5.4 Mutant forms of EXT1 do not exert any detectable dominant negative effect on HS biosynthesis of wild-type EXT1.
EXT1 wild-type construct was transfected alone or cotransfected with null missense mutant constructs followed by HSV infection. Infected cells were stained with X-gal.

5.2.4 The intracellular trafficking of EXT1 missense mutants

It has been shown that EXT1 and EXT2 are predominantly localized to the ER when expressed alone in the cell. However, when co-expressed, both proteins redistribute to the Golgi apparatus (Kobayashi et al. 2000; McCormick et al. 2000). In agreement with these published results, the subcellular localization studies shown in Fig 5.5, reveal the same difference in subcellular localization between cells expressing wide-type EXT1 alone and those co-expressing EXT1/EXT2. Interestingly however, it appears that when EXT1 constructs containing HME-linked missense mutations R340H, R340L, and R340S were transfected alone, the subcellular localization patterns for these mutant EXT1 proteins are indistinguishable from that observed when wild-type EXT1 and EXT2 are co-expressed in the same cell (Fig. 5.5). A similar observation has been reported previously for another missense mutant affecting the same amino acid residue, R340C (McCormick et al. 2000). By contrast, missense mutations affecting positions other than R340 appeared to exhibit the same subcellular localization pattern as the EXT1 wild-type construct (Fig. 5.5).

5.2.5 Anion exchange chromatography of GAGs from etiological mutant EXT1 expressing cells.

To investigate GAG synthesis directly in Sog9 cells stably expressing mutant EXT1 proteins, anion-exchange chromatography of radiolabeled cell surface GAGs was performed. The results, illustrated in Figure 5.6, show a single major peak of GAGs eluted at a similar position as in the wild-type EXT1 for each of the "active" mutants. The data show that these mutants express HS to levels comparable to those of wild-type EXT1. In agreement with their null phenotype in the HSV infectivity assay (Fig. 5.1), none of the null mutant forms could catalyse the polymerization of HS.

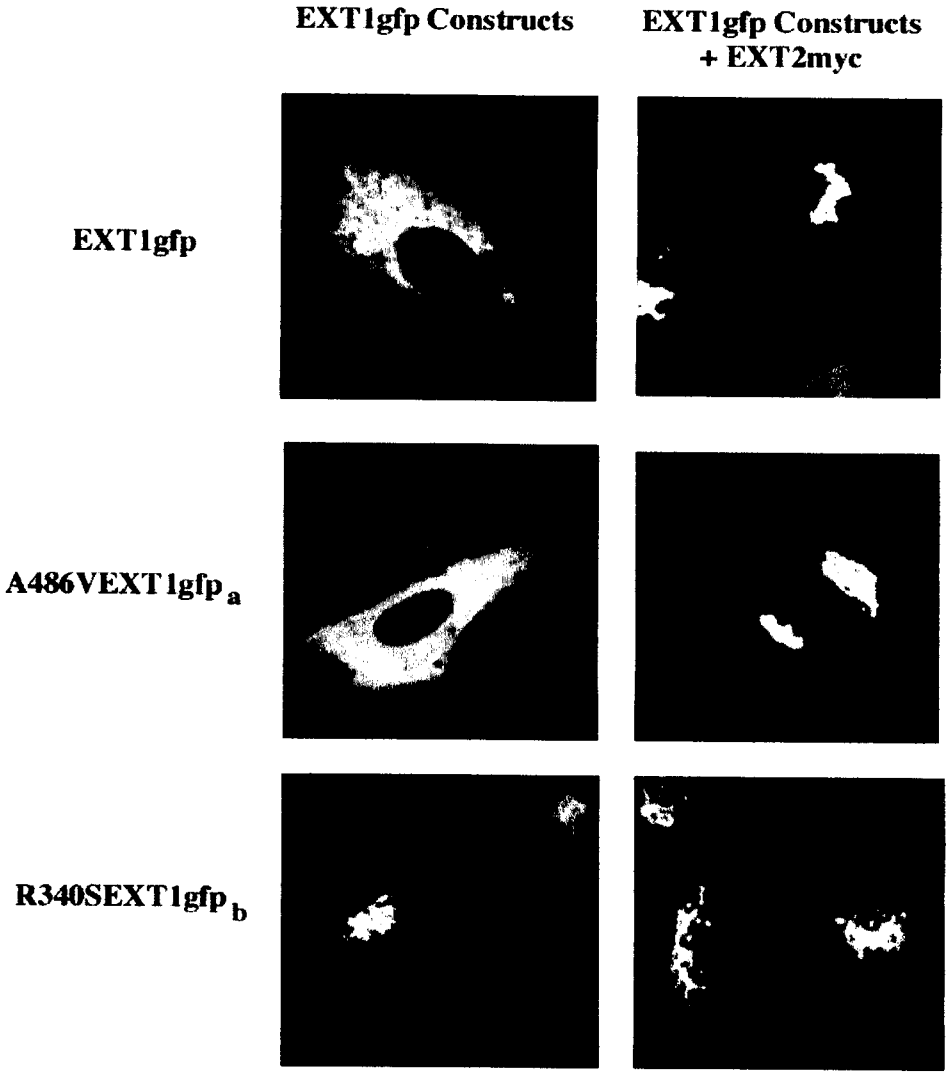


Figure 5.5 Intracellular localization of etiological missense mutations in EXT1.
Monolayers of BHK cells were transfected either with indicated constructs alone (left column) or co-transfected with EXT2myc (right column). The GFP-localization was analyzed by fluorescence confocal microscopy at 488nm.
[a] the following mutations (Q27K, D164H, R280G, R280S, N316S, and P496L) have similar intracellular localization phenotype as observed in A486VEXT1gfp.
[b] R340H and R340L have similar intracellular localization phenotype as observed in R340SEXT1gfp.

5.2.6 Characterization of active EXT1 etiological mutants.

The results so far indicated that four of the putative etiological mutations retained significant HS biosynthesis activity. One possibility to explain these results is that the mutant forms possess partial HS biosynthetic activity compared with wild-type forms. To investigate this possibility further, the four residues in EXT1 were subjected to further alteration. Substitution of the acidic Q27 or N316 residue by alanine, an unrelated aliphatic residue, did not inactivate HS-Pol activity. Likewise, substitution of the aliphatic A486 and P496 residues by a basic histidine residue did not abolish HS biosynthesis. The results are summarized in Table 5.2. Remarkably, HS expression was retained even when the affected amino acid was deleted from the EXT1 protein. Taken together, the data show that these four amino acid residues are not essential for the HS-Pol activity of EXT1.

5.2.7 An aspartate residue required for HS-Pol activity

The inactivating missense mutation D164H, located at the last residue position of a DXD sequence (Fig. 5.7), was also examined further. DXD motifs, and aspartate residues in particular, have been reported to be important components of catalytic sites for glycosyltransferase activity (Busch et al. 1998; Wiggins and Munro 1998). The etiological change from aspartate to histidine represents a severe change from an acidic to a basic amino acid. To determine whether there was an absolute requirement for aspartate, as has been described for other glycosyltransferase enzymes (Griffiths et al. 1998; Wiggins and Munro 1998), the aspartate residue was replaced by the closely related amino acid, glutamate, in the otherwise wild-type EXT1 protein. This mutant form was also defective in HS polymerization, suggesting that EXT1 requires an aspartate residue at position 164 for HS-Pol function (Table 5.2).

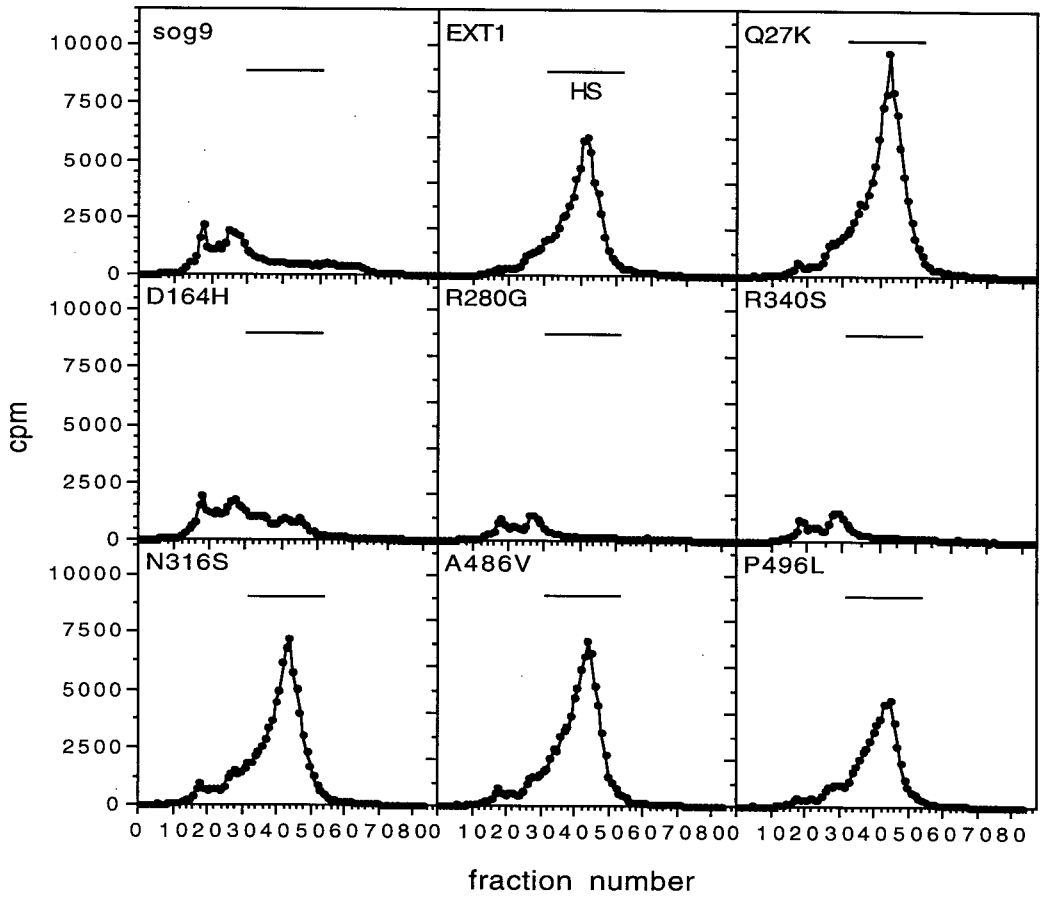


Figure 5.6 Anion-exchange chromatography of cell-surface GAGs.
Sog9 cells stably transfected with the indicated EXT1 forms were grown for 24 hours in the presence of $^{35}SO_4$(60μCi/ml), GAGs were isolated and fractionated by HPLC. The elution position of HS is indicated by a horizontal bar. Samples were normalised by the amount of proteins using Bio-Rad Protein assay. For the two residues affected by multiple etiological missense mutations (R280 and R340), only one mutant form is shown.

Table 5.2 Functional Assay of EXT1 Proteins Bearing Selected Mutations

Amino acid Modification	Nature of Change	HS Biosynthesis [a]
Q27K	Etiological change	+
Q27A	Severe change	+
Q27del	Deletion	+
D164H	Etiological change	-
D164E	Conservative change	-
D164del	Delection	-
N316S	Etiological change	+
N316A	Severe change	+
N316del	Delection	+
A486V	Etiological change	+
A486H	Severe change	+
A486del	Delection	+
P496L	Etiological change	+
P496H	Severe change	+
P496del	Delection	+

[a] + = wild-type EXT1 level of infectivity;

[a] - = background level of infectivity;

del = delection of the indicated amino acid residue.

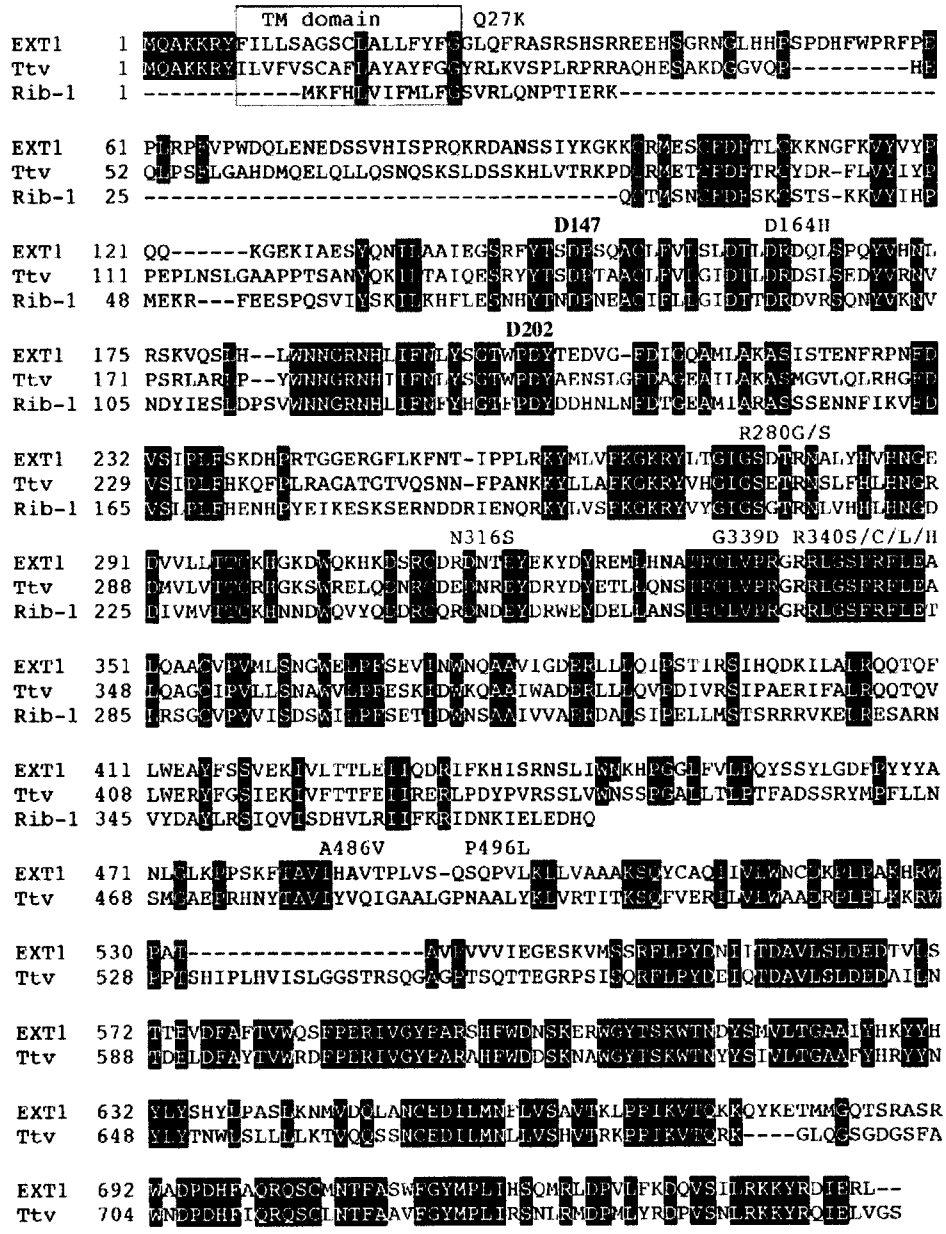


Figure 5.7 Amino acid-sequence alignment for three EXT1 orthologs.
Human EXT1; Ttv, from *Drosophila melanogaster* and Rib-1, from *Caenorhabditis elegans*. The putative transmembrane (TM) domains are labelled, predicted N-linked glycosylation sites are shaded in gray, conserved amino acids are indicated by black boxes, and putative etiological EXT1 missense mutations are highlighted in yellow.

5.3 Discussion

In the present study, I evaluated the effect on HS-Pol activity of HME etiological mutations identified in the EXT1 locus of affected individuals, and I found that not all mutations inactivate HS biosynthesis. These results raise the possibilities that these mutants may not related to disease pathogenesis or, on the other hand, that EXT1 possesses additional functions that are not yet identified. Using the HSV-infectivity assay, I have confirmed that eight previously reported HME-linked missense mutations abolish enzyme function in cell culture. All of these mutations affect amino acid residues that have been evolutionarily conserved, among both vertebrate and invertebrate species (Fig.5.7), suggesting that they are critical for EXT1 function. By contrast, three of the four remaining missense mutations, which do not inhibit HS expression in cell curture, affect residues that are not conserved among distantly related eukaryotes. These amino acids are not critical for EXT1 function in polymerizing HS in cultured cells, as they can be deleted from the protein without incurring loss of HS-Pol function (Table 5.2). The possibility that these active mutants retain partial activity, producing an amount of HS sufficient for HSV entry but insufficient for proper bone development, cannot be excluded. However, it seems unlikely that they possess partial activity because HPLC profiles of the transfected Sog9 cells by active mutants are comparable to that of wild-type EXT1 (Fig.5.6) and deletion of the affected amino acids does not abrogate HS biosynthesis (Table 5.2).

5.3.1 Location of the glucuronosyltransferase domain in the EXT1 protein

HS formation occurs by the polymerization of GlcA and GlcNAc residues. Recent studies have shown that these reactions are catalyzed by a polymerase encoded by EXT1 and possibly EXT2. Interestingly, seven of the twelve reported missense mutations, all of which have the null phenotype, are found in two clusters, at position R280 and around positions G339 and R340 (Fig.5.2), suggesting that these regions may harbor key elements for EXT1 function. Indeed, enzyme assay analysis of two of these missense mutants, G339 and R340, have previously

revealed that mutation of these residues selectively abrogates GlcA-T activity (McCormick et al. 2000). Furthermore, six missense mutations recently found in the same central region, from HS-deficient CHO cell lines, were selectively altered GlcA-T activity (Wei et al. 2000). Taken together, these data suggest that this central region defines the active site of the GlcA transferase domain.

The fact that HME is an autosomal dominant genetic disorder suggests that one mutant copy of the gene should be sufficient to cause disease. However, no dominant negative phenotype was observed, even when mutant and wild-type forms were co-transfected in a ratio of 10:1. Similar results were observed when impaired EXT1 missense mutants were introduced into wild-type CHO cells (Wei et al. 2000). This is a rather surprising result in light of the ability of the EXT proteins, both wild-type and mutant, to form homo-oligomers and hetero-oligomers in cellular level (Kobayashi et al. 2000; McCormick et al. 2000). This implies that oligomer formation may require only one functional copy of EXT1 for the oligomer to retain its function. Alternatively, observed HS-Pol activity may be due to a subset of totally functional oligomers of EXT1 and EXT2 proteins, which likely exist in a small quantity. Taken together, these results suggest that, at the cellular level, one functional copy of the EXT1 gene is sufficient for activity.

CHAPTER 6: The role of furin in the biology of the EXT1 protein

6.1 Introduction

Amino acid residues G339 and R340 of the EXT1 protein are of particular interest in that they seem to represent a "hot-spot" for etiological missense mutation in the *EXT1* gene (Fig. 5.2). A total of five out of twelve missense mutations were found in these two adjacent residues for patients with HME. Moreover, these missense mutations have been repeatedly selected in unrelated HME families (Philippe et al. 1997; Wuyts and Van Hul 2000). Analysis of the amino acid sequence in this region revealed that these two residues are localized in a "$R_{338}G_{339}R_{340}R_{341}$" motif, which is a classic recognition site for cleavage by furin (Lazure et al. 1998).

Furin is a membrane-associated, calcium-dependent, serine endoprotease that cleaves most efficiently at the C-terminal side of an Arg(P4)-Xaa-Lys/Arg-Arg(P1) sequence (Molloy et al. 1992). As listed in Table 6.1, furin catalyses the maturation of a strikingly diverse group of proprotein substrates, ranging from growth factors and receptors to pathogen proteins, in multiple compartments within the trans-Golgi network/endosomal system (Molloy et al. 1999).

It is known that for a number of proteins, such as proalbumin, and insulin pro-receptor, mutation at the furin cleavage site is responsible for a variety of genetic disorders (Nakayama 1997). Most recently, Chen et al. indicated that mutations within a furin consensus sequence block proteolytic release of ectodysplasin-A (EDA) and cause X-linked hypohidrotic ectodermal dysplasia (XLHED) (Table 6.1) (Schneider et al. 2001). This is a human heritable disorder that results in the impaired formation of hair, teeth, and sweat glands during fetal development. In comparison with HME which is caused by mutation in EXT1 and/or EXT2, EDA is also a type II transmembrane protein and missense mutations in the overlapping consensus furin cleavage sites account for around 20% of all known XLHED cases (Chen et al. 2001).

Table 6.1 Proproteins cleaved at furin sites*

Precursors	Cleavage Site					↓			References
	P6	P5	P4	P3	P2	P1	P1'	P2'	
Consensus sequence	X	X	R	X	K/R	R	X	X	
Growth Hormone									
Human pro-parathyroid hormone-related peptide	L	R	R	L	K	R	A	V	(Liu et al. 1995)
Receptor									
Human insulin pro- receptor	P	S	R	K	R	R	S	L	(Bravo et al. 1994)
Plasma Protein									
Human pro-factor X	L	E	R	R	K	R	S	V	(Wallin et al. 1994)
Epithelial Morphogen									
Human Ectodysplasin-A (EDA)	S	R	<u>R</u>	V	<u>R</u>	<u>R</u>	N	K	(Schneider. et al. 2001)
Viral Envelope Glycoprotein									
Human immunodeficiency virus gp160	V	Q	R	E	K	R	A	V	(Hallenberger et al. 1992)
Human cytomegalovirus glycoproteinB	H	N	R	T	K	R	S	T	(Vey et al. 1995)
Bacterial Exotoxins									
Anthrax toxin protective Antigen	N	S	R	K	K	R	S	T	(Klimpel et al. 1992)

*The P6-P2' cleavage site sequences for a selected list of precursors which have been shown to be cleaved by furin are shown above. Mutations of the three underlined arginine residues in EDA protein have been reported to cause X-linked hypohidrotic ectodermal dysplsasia (XLHED). X = any amino acid; Arrow = cleavage site.

6.2 RESULTS

6.2.1 Radioimmunoprecipitation analysis of EXT fusion proteins

To determine whether or not the EXT1 protein is processed by cellular proteases, GFP-tagged functional EXT1 and EXT2 constructs were transfected into Baby Hamster Kidney (BHK) cells, radiolabeled with [^{35}S]methionine, and immunoprecipitated with anti-GFP antibody. As reporteded previously, the molecular weight of the EXT1 protein bearing a C-terminal GFP tag would be ~120kDa by SDS-PAGE, and with a C-terminal MYC tag would be ~85kDa (McCormick et al. 2000; Senay et al. 2000). Using the DNA Strider™ 1.1 program, the predicted molecular weight of the processed form of the EXT1-gfp peptide, which contained 666 amino acid residues, would be ~75.6kDa. Figure 6.1 indicated that SDS-PAGE analysis revealed only the unprocessed, full length forms of the EXT1 in both cell lysate extract and in the media, and no trace of processed peptide was found.

6.2.2 The role of furin in HS biosynthesis

To determine whether furin plays a role in EXT1-mediated HS biosynthesis, a set of three Chinese hamster ovary (CHO) cell lines with different levels of furin expression, generously provided by Dr. Stephen Leppla, were used for this study. Wild-type CHO cells, furin-deficient CHO cells and furin-expressing revertant CHO cells (Gordon et al. 1995) were subjected to HSV-2 infection, and the β-galactosidase expressing HSV-2 infected cells were stained with X-gal. As can be seen in Figure 6.2, no difference in infectivity was observed between all three cell lines. Taken together, these results suggest that furin is not required for HSV infection, and that furin cleavage of EXT1 is not essential for HS biosynthesis in CHO cells.

6.3 Discussion

The recent development in understanding furin and its substrates reveals that for efficient cleavage, a larger specific amino acid recognition sequence is needed. At first, minimal furin

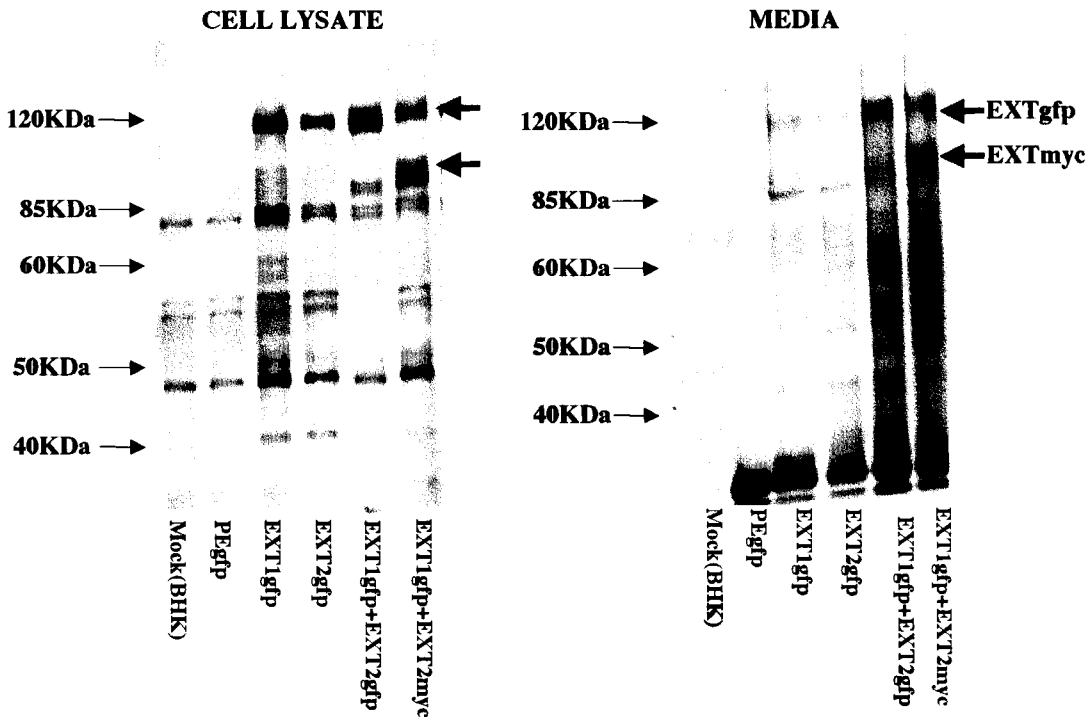
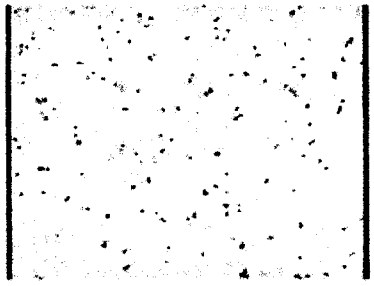


Figure 6.1 Radioimmunoprecipitation analysis of EXT1 protein expression.
BHK cells were transfected with various combinations of myc or GFP tagged EXT1 and/or EXT2 constructs. 30 h post transfection, cells were radiolabeled for 24 h with [^{35}S]methionine and immunoprecipitated with anti-GFP antibody. Immunoprecipitated proteins were separated by SDS-PAGE gel, transferred to Immobilon-P membranes, and exposed to BioMAX MR film. The predicted molecular weight of the processed form of the EXT1-gfp peptide (666 amino acid residues), using the DNA Strider™ 1.1 program, is 75.6kDa.

CHO Clone 6
(Parental)

CHO FD11
(Furin deficient)



CHO FD11 + Furin

Figure 6.2 HSV-2 infectivity of CHO cell lines expressing different levels of furin. Subconfluent CHO cell monolayers were infected with equal amounts of L1BR1, a β-galactosidase expressing HSV-2 strain. At 6 hours post infection, cells were stained with X-gal.

cleavage site Arg(P4)-X-X-Arg(P1), and efficient furin cleavage site Arg(P4)-X-Lys/Arg-Arg(P1), were suggested (Molloy et al. 1992). However, recent findings suggest that the furin cleavage-recognition site actually runs from P6 to P2' ie. including the two amino acid residues upstream and downstream of the tetra-amino acid consensus sequence. For instance, in addition to the P1 Arg, at least two out of the three residues at P2, P4 and P6 are required to be basic for efficient cleavage (Nakayama 1997). Moreover, when an acidic residue was placed at the P3 position, as is the case for one of the EXT1 etiological mutants, G339D, furin cleavage is markedly compromised (Lazure et al. 1998).

In an ongoing study of putative furin cleavage sites naturally occurring in proteins ranging from viruses to bacteria to humans, Dr. Francois Jean from the department of Microbiology and Immunology at UBC also studied the consensus furin cleavage site in EXT1. Using synthetic internally quenched fluorescent peptide derived from EXT1 amino acid sequence residues 336-346 [Val(336)-Pro-Arg(P4)-Gly(P3)-Arg(P2)-Arg(P1)-Leu(P'1)-Gly-Ser-Phe-Gln(346)], he assayed this EXT1 derived peptide as a substrate for furin and interestingly, no cleavage was detected (personal communication). This finding also suggests that EXT1 cannot be cleaved by furin.

Taken together, with the fact that the etiological mutations affecting amino acid residues G339 and R340 are localized in a consensus furin cleavage site, it initially seemed likely that there may be a link between furin, EXT1 and HME disease. However, results from tissue culture studies using furin deficient CHO cells and from Dr. Jean's study, using fluorescent peptide bearing EXT1 sequence, clearly indicated that the putative furin recognition site was not cleaved by furin. These surprising results can perhaps be explained in light of recent findings that P' positions are also important for furin activity. In a study of the furin cleavage site in the human proparathyroid hormone, replacement at the P1' position of Ser by Val in conjunction with replacement of Val by Ala at P2', the sequence becomes an inhibitor for furin cleavage (Lazure et al. 1998). As the EXT1 P1' and P2' amino acid residues, Leu and Gly both differ from the

published inhibitor sequence Val and Ala by only one methyl group at the third carbon position, the EXT1 sequence could also specifically inhibit furin cleavage, despite the "RGRR" consensus motif.

CHAPTER 7: Discussion

7.1 Non-Replaceable EXT1 function in HS polymerization.

The structural complexity of GAGs, in addition to their abundance on the surface of almost all mammalian cells, has made them attractive targets for a number of important pathogenic bacteria and viruses. Over time, many pathogenic microorganisms have evolved to exploit cell surface PGs as receptors for attachment, a process which ultimately facilitates invasion. Herpes simplex virus was the first virus shown to bind cell surface HS GAGs (WuDunn 1989), and to use HS for initial attachment during viral entry into the host cell.

The study presented in Chapter 3 describes the isolation and characterization of a group of nine mammalian mutant cell lines derived from L-EXT1 cells, which exhibit an HSV-1 resistant phenotype due to altered HS GAG biosynthesis. A mutation in any of the genes that are essential for one of the numerous stages in the HS biosynthesis pathway could produce such mutant cell lines. Surprisingly, the enzyme encoded by the EXT1 gene, which polymerizes HS, corrected the HS deficiency of all nine mutant cell lines, even though EXT2 and three other EXT-like proteins have been suggested to contribute to the process of HS biosynthesis (Fig.3.8). Similar results have been published by Esko and colleagues regarding the characterization of a collection of CHO cell mutants that fail to make HS (Wei et al. 2000). A likely explanation may be that some glycosyltransferases, which are involved in HS biosynthesis, are essential for cell survival and/or exist in multiple copies.

Originally, the EXT2 protein had been suggested to harbor both the GlcNAc and GlcA transferase activities involved in HS polymerization, as for EXT1. However, recent data suggests that the low level of activity has seen for EXT2 in mammalian cell systems could be due to contamination by EXT1, given that the enzymes form hetero-oligomeric complexes (Kitagawa et al. 2001). Because none of the nine HS deficient cell lines described in Chapter 3 or from Esko's CHO cell mutants have detectable defects in EXT2, and only EXT1 corrects the HS deficiency, EXT1 could be the sole enzyme that polymerizes HS in mammalian cells.

7.2 The link between EXT1 and HME

Although the pathogenesis of HME was first described by John Hunter in his *Lectures on the principles of surgery* in 1786 (Hennekam 1991), it is only in the past few years that elegant genetic analyses (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996), and intensive biochemical studies (Lind et al. 1998; McCormick et al. 1998) have permitted the identification and functional characterization of the EXT family of putative tumor suppressor proteins. The HSV infectivity assay employed in this study is useful for evaluating the cellular HS-Pol activity as it measures the biosynthesis and cell surface expression of HS. It is highly specific because it was used to show that EXT2 is distinct from EXT1 (McCormick et al. 2000) despite the fact that EXT2 has also been suggested to possess the same glycosyltransferase activities as EXT1, albeit at lower levels (Kitagawa et al. 1999; McCormick et al. 2000; Senay et al. 2000). In the present study I evaluated the effect on HS-Polymerase activity of HME etiological mutations identified in the *EXT1* locus of affected individuals and found that not all mutations were inactivated. These results raise the possibility that not all mutations are related to disease pathogenesis, and some of these reported mutations may be rare genetic polymorphisms.

Interestingly, in many of the genetic studies conducted on HME-affected families, there are reports of individuals and families with no detected mutations in either of the EXT1 or EXT2 coding regions (Dobson-Stone et al. 2000; Francannet et al. 2001; Hecht et al. 1997; Philippe et al. 1997; Wells et al. 1997; Wuyts et al. 1998; Xu et al. 1999). Furthermore, approximately 10% of affected individuals harbor *de novo* germline mutations, for which linkage analysis is not possible (Raskind et al. 1998). As mutational screening by sequencing can not distinguish between rare genetic polymorphisms and disease causing mutations, the HSV infectivity assay could be used to test the diseased chondrocyte EXT1 cDNA for its ability to polymerize HS, the only known function of the EXT1 protein, so far.

For other known glycosyltransferases, domain swaps and site-directed mutagenesis have often proven to be powerful tools for identifying key amino acids involved in enzyme substrate

specificity and catalytic site activity (Hagen et al. 1999; Jing and DeAngelis 2000; Seto et al. 1997; Xu et al. 1996). In the case of the EXT1 protein, it is most likely that naturally occurring etiological missense mutations affect key functional domains, thereby providing us with a subset of candidates for elucidating enzyme function. Indeed, the results of the HSV infectivity assay have confirmed that eight previously reported HME-linked mutations abolish enzyme function in cell culture. All of these missense mutations affect amino acid residues that have been evolutionarily conserved, among both vertebrate and invertebrate species (Fig. 5.6), suggesting that they are critical for EXT1 function. By contrast, three of the four remaining mutations, which do not inhibit HS expression, are not conserved among these distantly related eukaryotes and are not critical for EXT1 function in polymerizing HS in cultured cells. The possibility that these mutants retain partial activity sufficient for HSV entry, but insufficient for proper bone development, cannot be excluded. However, I think it unlikely that they possess partial activity because deletion of the affected amino acids does not abrogate HS biosynthesis.

The one exception, N316S, is conserved (Figure 5.6), and lies directly next to a putative glycosyltransferase catalytic site motif, DXD (Busch et al. 1998; Wiggins and Munro 1998), which is itself, however, not totally conserved (Figure 5.6). The fact that alteration and even deletion of N316 does not eliminate HS biosynthesis is intriguing if this particular DXD motif is the catalytic site for GlcA-T activity, as has been previously proposed (Wei et al. 2000). On the contrary, amino acid residue D164H also lies within a DXD motif (Figure 5.6), which appears to be highly sensitive to mutation, as even a very conserved amino acid substitution was observed to abrogate HS biosynthesis. It has been proposed that DXD motif correspond to nucleotide and cation binding domains critical to catalysis (Busch et al. 1998; Wiggins and Munro 1998), and it is therefore possible that D164 may play a key role in catalyzing HS polymerization. It will be interesting to determine whether mutation of aspartates 162, 164, 313 and/or 315 specifically abolishes either GlcA-T or GlcNAc-T activities.

7.3 The role of furin in HS biosynthesis

A possible molecular mechanism for the functional inactivation of EXT1 was suggested by the missense mutations clustered at amino acid positions 339 and 340 of the protein. In fact, these two residues are localized in a RGRR motif, which is a classic recognition site for cleavage by furin (Lazure et al. 1998). Furin is one of eight proprotein convertases that are known to activate various proproteins by limited proteolysis within the secretory pathway (Jean et al. 1998; Seidah and Chretien 1999).

Most recently, Chen et al. indicated that mutations within a furin consensus sequence block proteolytic release of ectodysplasin-A (EDA) and cause X-linked hypohidrotic ectodermal dysplasia (XLHED) (Chen et al. 2001). This is a human heritable disorder that results in the impaired formation of hair, teeth, and sweat glands during fetal development. In comparison with HME which is caused by mutation in EXT1 and/or EXT2, EDA is also a type II transmembrane protein and missense mutations in the overlapping consensus furin cleavage sites account for around 20% of all known XLHED cases (Chen et al. 2001). It was therefore possible that mutation of G339 and R340 was interfering with furin-mediated activation of EXT1. Our data shows, however, that furin-deficient cells are as sensitive to HSV as their wild-type counterparts (Fig.6.2), indicating that a lack of furin does not inhibit HS expression and transduction to the cell surface. For this reason, it is unlikely that furin-mediated activation of EXT1 is necessary for its HS biosynthetic activity. Thus, etiological missense mutations affecting residues G339 and R340 must inactivate EXT1 by a mechanism other than altering a furin recognition site.

7.4 The paradox of the autosomal dominant phenotype of HME

The autosomal dominant inherited pattern of HME would suggest that one mutant copy of the gene would be sufficient to cause disease. However, no dominant negative phenotype was observed in cultured cells (Fig.5.4), consistent with a previous report (Wei et al. 2000). This is a rather surprising result in light of the ability of the EXT proteins, both wild-type and mutant, to form homo-oligomers and hetero-oligomers in cell culture (Kobayashi et al. 2000; McCormick et

al. 2000). This implies that oligomer formation may require only one functional copy of EXT1 for the oligomer to retain its function. Alternatively, observed HS-Pol activity may be due to a subset of totally functional hetero-oligomers, as it is likely that in any cell there are three types of oligomers – those containing only wild-type copies of EXT1, those containing copies of both wild-type and mutant EXT1 proteins, and those composed of only mutant proteins. Taken together, these results suggest that, at the cellular level, one functional copy of the EXT1 gene is sufficient for activity.

7.5 Model for the formation of EXT related exostoses

As early as the 1920s, a model was proposed in which exostoses form as the result of a lack of coordination between maturation of the chondrocytes, which are the cartilage producing cells, and perichondral bone formation. This model was based on the histologic organization of an exostosis, which showed remarkable similarities with the parent bone from which it arises (Solomon 1963). Current data support such a model in which a localized defect in the molecular signaling pathways could cause a localized defect in perichondral bone formation.

Perichondral bone formation is the process by which bone is formed outside and around the cartilage, a process that results in the formation of a bony collar that surrounds the late hypertrophic chondrocytes. The formation of this bony collar has to be coordinated with the rate of chondrocyte differentiation within the cartilage (Stickens et al. 2000). Based on the observation that prehypertrophic chondrocytes are able to differentiate into osteoblasts, the bone producing cells *in vitro*, it is likely that the same mechanisms that control chondrocyte differentiation also control perichondral bone formation (Bianco et al. 1998; Stickens et al. 2000). Other observations also suggest that interactions between prehypertropic cells, located at the border of the prehypertropic cartilage (borderline chondrocytes), and osteogenic cells in the perichondrium, are required for correct initiation and formation of the bony collar (Bianco et al. 1998).

It is well established that chondrocyte maturation depends on at lease two signaling molecules, Indian hedgehog (Ihh), and parathyroid hormone-related protein (PTHrP). The expression, of Ihh, which is synthesized by chondrocytes in the growth plate, stimulates chondrocyte proliferation by up-regulating the expression of the second signaling molecule, PTHrP, by cells of the periarticular perichondral region (Fig. 7.1). Subsequently, PTHrP binds to the PTH/PTHrP receptor on a subpopulation of proliferating and prehypertrophic chondrocytes, postponing cell death by up-regulating Bcl-2, a well known anti-apoptotic protein (Horton et al. 1998).

More recently, cell surface heparan sulfate proteoglycans (HSPGs) are suggested to be involved in bone formation by shaping bone morphogenetic proteins (BMPs) gradients (Paine-Saunders et al. 2001; Paine-Saunders et al. 2000). Although HSPGs do not bind BMPs directly, they regulate the cellular distribution of a BMP antagonist, Noggin, and therefore offer one potential mechanism through which HSPGs could regulate cellular responsiveness to BMPs (Paine-Saunders et al. 2001). Important to this hypothesis is the discovery that the binding of Noggin to HSPGs is independent of its activity as a BMP antagonist, inasmuch as Noggin bound to cell surface HSPGs retains its ability to bind BMP (Paine-Saunders et al. 2001). The binding of Noggin to the cell surface HSPGs might therefore establish gradients of BMP antagonism, resulting in turn in inverse activity gradients of BMPs.

Studies with the *Drosophila* homolog of EXT1, Ttv, have shown that this protein is necessary for the diffusion of Hedgehog (Hh), a homolog of mammalian Ihh (Bellaiche et al. 1998). As mentioned above, Ihh appears to play a key regulatory role in chondrocyte differentiation (Lanske et al. 1996; Vortkamp et al. 1996). It is therefore possible that mechanisms which control chondrocyte differentiation and perichondral bone formation may be disrupted in EXT1 or EXT2-defective HME patients. Specifically, a defect in HS biosynthesis

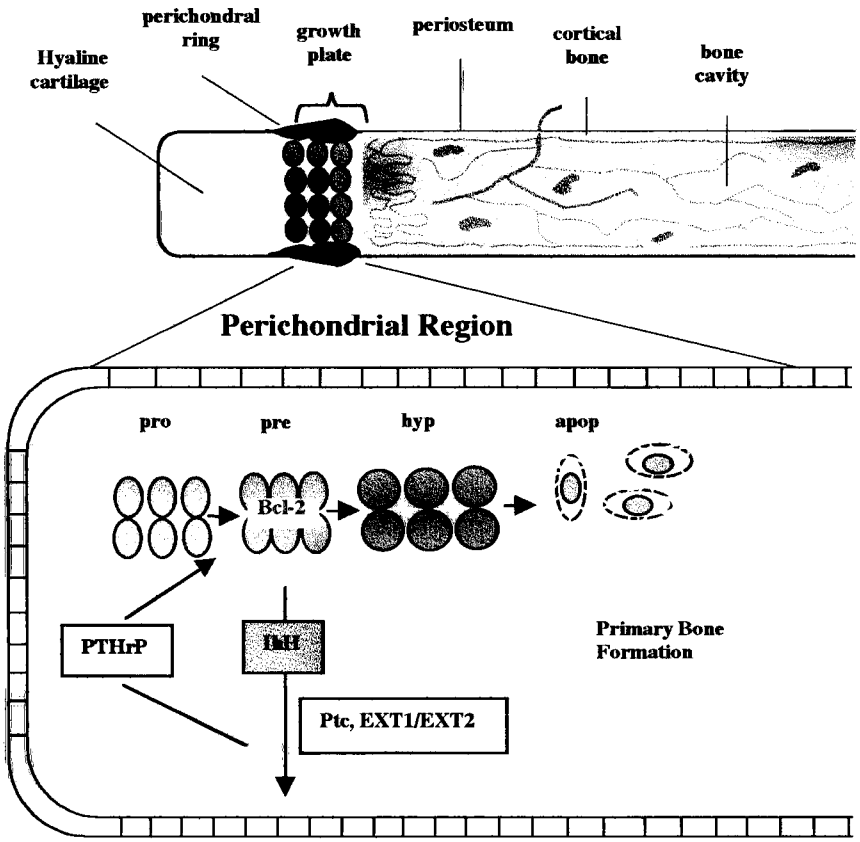


Figure 7.1 A model for the regulation of chondrocyte differentiation.
Indian hedgehog (Ihh) is produced by chondrocytes within the growth plate that are committed to hypertrophy. Either directly or indirectly, Ihh stimulates the production of parathyroid hormone-related protein (PTHrP) in the periarticular perichondrium, which diffuses to the prehypertrophic chondrocytes and induces production of Bcl-2, a well-known anti-apoptotic protein. Proper diffusion of Ihh depends on the cell surface transmembrane receptor patched (Ptc), and perhaps EXT1 and EXT2 via their heparan sulfate proteoglycan products. Later in the maturation process, chondrocytes differentiate into hypertrophic chondrocytes, which undergo apoptosis and are then replaced by bone-formating osteoblasts. Abbreviations: Pro, proliferating chondrocytes; Pre, prehypertrophic chondrocytes; Hyp, hypertrophic chondrocytes. Apop, hypertrophic chondrocytes undergoing apoptosis. Adapted from (Ingham 1998).

could result in a defect in the cross-talk, by altering the BMPs gradients, between the borderline chondrocytes and the osteogenic perichondrium cells which are responsible for perichondral bone formation. As a result of this localized defect, the formation of the bony collar would be temporarily and spatially interrupted, leaving chondrocytes the possibility of escape from further negative growth regulation (Fig.7.2) (Stickens et al. 2000). This model of exostosis formation would require a second somatic mutation in the borderline chondrocyte at the perichondrium in patients genetically heterozygous for either a mutant EXT1 gene or a mutant EXT2 gene. My findings support such a model since no dominant negative effect has been observed in the case of EXT1; both EXT1 genes have to be impaired for exostosis formation. Furthermore, no other tested glycosyltransferases including EXT2, could replace the HS polymerization activity of EXT1, a defect in EXT1 alone could therefore abolish HS biosynthesis and cause exostosis formation.

7.6 Prospects for the future

The results presented in this thesis provide evidence for the non-replaceable identity of the EXT1 gene as the HS polymerase. Detailed analysis of all the known EXT1 etiological missense mutations in this study have helped to further the fine mapping of the glycosyltransferase domains and the intermolecular contact points of EXT1 and EXT2. As in most other investigations, this study also raises other important unsolved issues, including the question of why germline defects in either EXT1 or EXT2 manifest only in bone tissue. Furthermore, if the alteration of HS expression causes HME, why then is it that mutations in loci encoding other glycosyltransferases involved in HS biosynthesis were not found in patients with HME. Finally, perhaps a more important question is whether the EXT1 protein harbors additional functions besides polymerizing HS.

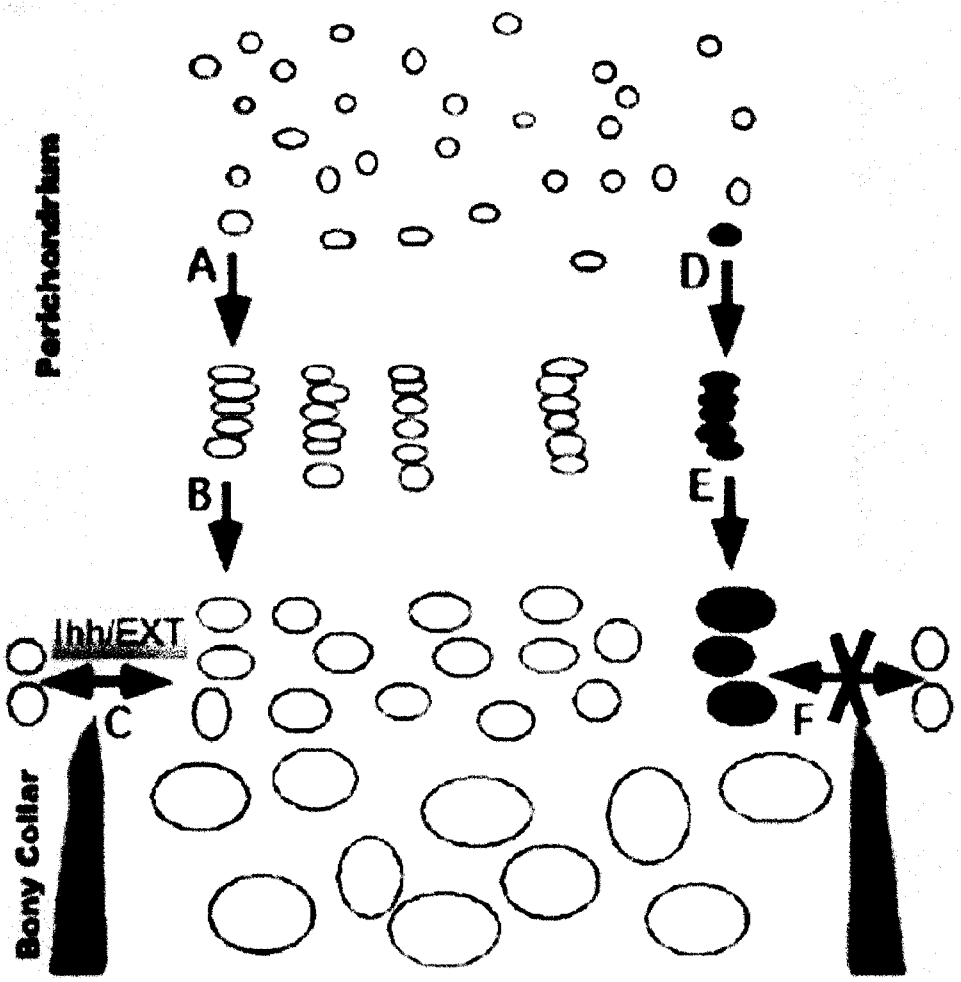


Figure 7.2 Model for EXT-derived pathogenesis in hereditary multiple exostoses.
Resting chondrocytes will start the path of maturation by entering the proliferative stage (arrow A). After proliferation, chondrocytes will enter the stage of prehypertrophy (arrow B), a progression believed to be controlled by the Ihh/PTHrP pathway.The cross-talk between borderline prehypertrophic chondrocytes and osteogenic cells located in the perichondrium initiates the process of bony collar formation. This cross-talk would be established by an Ihh/EXT pathway and by shaping BMP gradients (arrow C). When a chondrocyte with an inactivated EXT1 or EXT2 gene (red cell) starts to proliferate (arrow D), all daughter cells will have an inactivated EXT1 or EXT2 gene, resulting in EXT1 or EXT2 inactivated prehypertrophic chondrocytes (arrow E). Inactivation of EXT1 or EXT2 would impair the cross-talk necessary for initiation of perichondral bone formation (arrow F). This would result in a temporary disruption of the bony collar and leave a path for chondrocytes to escape further negative growth control. Adapted from (Stickens et al. 2000).

APPENDIX 1 Defining the spectrum of HSV-2 TK genotypic lesions in eight Acyclovir resistant clinical isolates

8.1 Introduction

Genital HSV infections are a major public health problem (Fleming et al. 1997). In the immunocompetent host, recurrences are self-limiting, but infections in untreated immunocompromised individuals can become chronic and progressive (Whitley et al. 1984). Acyclovir (ACV) is a potent and selective antiviral nucleoside which remains the "gold standard" of treatment and suppression in both normal and immunocompromised hosts (Shepp et al. 1985; Wade et al. 1984). Clinically significant resistance to ACV has been almost exclusively seen in the immunocompromised population, where it occurs in up to 5% of patients (Erlich et al. 1989; Wade et al. 1983). The mechanism responsible for the majority of ACV-resistant (ACV_r) isolates is a lack of viral thymidine kinase (TK), the enzyme required for initial phosphorylation of ACV before it undergoes further phosphorylation by cellular kinases to generate its active triphosphate form (Hill et al. 1991). Occasionally, ACV resistance can be due to an alteration of the TK protein such that the usually promiscuous spectrum of TK activity excludes ACV (Hill et al. 1991; Kost et al. 1993), or, alternatively, a mutation may occur in the viral DNA polymerase (*pol*) gene allowing it to exclude ACV (Collins et al. 1989; Sacks et al. 1989). The aim of this work was to sequence a collection of ACV_r clinical isolates and thus define the spectrum of TK genotypic lesions within such isolates. We found that five of eight isolates contained mutations at two homopolymer stretches, suggesting the presence of two mutational hot spots, which produce nonfunctional, truncated TK proteins. The repair of one homopolymer frameshift mutation (the isolate with an extra G insertion in a run of 7Gs) restored TK activity, demonstrating that this mutation is responsible for the observated of TK deficiency.

8.2 Result

8.2.1 TK gene sequencing

In an attempt to identify the genetic basis of the ACV resistant phenotype in eight of the clinical isolates, the TK genes of all eight isolates were sequenced. To do this, primers were produced for amplification of the TK region of the genome and the resulting PCR products were purified and sequenced. Results indicated that five of eight isolates contained mutations at two homopolymer stretches found in the TK nucleotide sequence. Three of the isolates demonstrated a single G insertion in a homopolymer stretch of 7G residues between nucleotides 433 and 439 of the open reading frame, suggesting the presence of a mutational hot spot (Figure 8.1). Of the remaining two isolates containing mutations in the two homopolymer runs, both contained frameshift mutations (a deletion and an insertion), but these occurred in a downstream stretch of six cytosines between nucleotides 550 and 555. These data suggested the presence of a second homopolymer hot spot (Figure 8.1). For the rest of the remaining three isolates, no mutation was found within the coding region of TK.

8.2.2 Western blot analysis.

The sequencing data indicated that several of the isolates had suffered mutations that would lead to frameshifts and result in truncated proteins. To test this hypothesis, Western blot assays were performed on extracts of cells infected with plaque isolates, using a monoclonal antibody to HSV-2 TK. HSV-2 control wild type strain G produced the expected full-length 40-kDa protein. By contrast, analysis of the protein products formed by isolates containing the G insertion within the homopolymer stretch of 7G residues, showed that a 28-kDa truncated protein product was produced. Isolates containing frameshift mutations within the downstream homopolymer stretch of 6 C residues also produced truncated TK products of 32kDa for the single G deletion and 28 kDa for the single G insertion mutations (Figure 8.1).

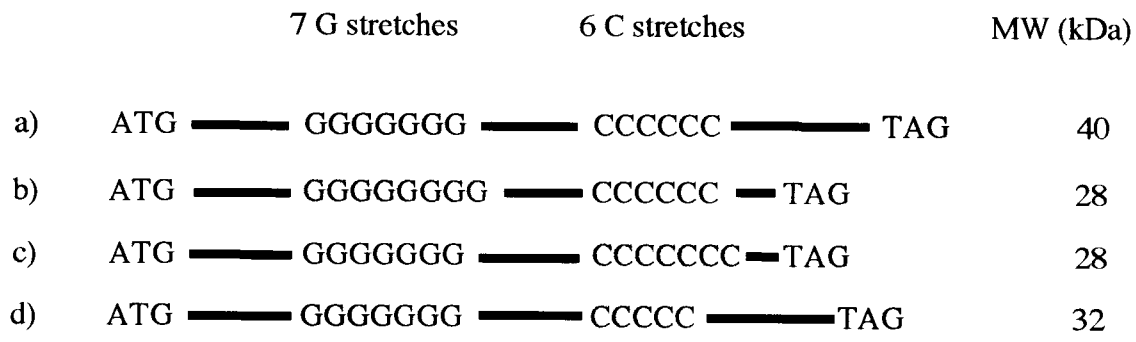


Figure 8.1 Diagram depicting the locations of mutations at the two identified hot spots, and their effects on protein product size.
(a) Diagram of wild-type TK, depicting sites of homopolymer runs of 7 G's and 6 C's, producing a full-length 40-kDa TK. (b) G insertion within the stretch of 7 G's which alters the amino acids within the nucleoside-binding site and codes for a premature stop codon which results in a truncated 28-kDa protein lacking amino acid 336. This mutation was detected in three of the eight isolates. (c) C insertion within the stretch of 6 C's which codes for a premature stop codon at the same site as in panel b and also results in a truncated 28-kDa product lacking amino acid 336. This was detected in one of the isolate. (d) C deletion within the same string of 6 C's depicted in panel c but coding for a premature stop codon further downstream than that of panels b and c, resulting in a 32-kDa truncated protein also lacking amino acid 336. This was detected in one of the isolate.

8.2.3 Mutation repair

To determine whether the single base change could account for the loss of TK activity in these virus isolates, the extra G in the homopolymer string of 8 G residues in one of the isolate was repaired so that it contained 7 G's. To this end, a two-step PCR procedure was devised to generate a plasmid containing the extra G *tk* gene with a targeted deletion of a single G in the homopolymer stretch. This fragment was then cloned into the mammalian expression vector pRC/CMV and introduced into TK-deficient mouse L cells by transfection. Following selection in HAT medium to kill cells that remained TK deficient, colonies of TK-expressing cells arose in the samples transfected with the repaired gene but not in control cells transfected with the mutant *tk* gene (Figure 8.2). Taken together, these results indicate that the single base change introduced to repair the mutant *tk* gene restored its TK activity. Thus, the loss of TK activity in the mutant virus could be accounted for solely by this mutation.

8.3 Discussion

This study identifies several *tk* mutations in clinical isolates which result in resistance to ACV. Moreover, the data show that frameshift mutations at a limited number of long homopolymer nucleotide stretches underly the majority of defects in TK activity and appear to be mutational hot spots. This is significant as the identification of a hot spot may suggest a mechanism for the appearance of ACV resistance. The results demonstrated that of the eight ACV_r isolates sequenced, three contained identical G insertions within a stretch of 7 G residues and two contained C frameshift mutations at a second downstream stretch of 6 C's. These two homopolymer stretches are the longest in the gene. The predicted consequences of such mutations would be that the frameshifts would alter every amino acid beyond the mutation and would code for a premature stop codon, resulting in a truncated protein devoid of activity (Figure 7.1). The restoration of TK activity in one isolate following repair of its homopolymer mutation demonstrated that these frameshift mutations were responsible for TK deficiency (Figure 7.2).

Figure 8.2. Selection of TK-expressing cells in HAT medium.
DNAs containing the mutant extra G *tk* gene (A) and the repaired gene (B) were transfected into TK-deficient mouse L cells growing on 10-cm plates by use of lipofectamine. Following transfection, cells were allowed to grow for several days and then selected in HAT medium to kill cells that remained TK deficient. Dishes were stained after 7 days and photographed.

Homopolymer nucleotide stretches have been shown to be particularly susceptible to frameshift mutations in other systems. Sites of homopolymer nucleotides have been shown to be mutational hot spots in the T4 bacteriophage (Streisinger and Owen 1985) and polyomavirus (Wilson et al. 1986) genomes, as well as within eucaryotic DNA, such as the mouse immunoglobulin heavy chain locus (Baumann et al. 1985). Such hot spots have been shown to be susceptible to both spontaneous and mutagen-induced mutations, with the rate of mutation being proportional to the number of identical nucleotides. Given that the two putative mutation hot spots found in the *tk* gene are the longest nucleotide homopolymer stretches and that the HSV *pol* gene has been shown to have a high mutation frequency (Hwang and Chen 1995), the viral DNA polymerase may preferentially slip or stutter at homopolymer residue stretches. Furthermore, as ACV is a guanosine analog, it may influence the viral DNA polymerase to selectively introduce mistakes in regions containing G-C homopolymers and induce frameshift mutations.

APPENDIX II

Chemical reagents and laboratory supplies

Acetic acid	Fisher
Acrylamide	Canadian Life Technologies
Agarose	Canadian Life Technologies
Ammonium persulfate(APS)	Canadian Life Technologies
Ammonium acetate	Fisher
Ampicillin	Sigma
Bis-acrylamide	BioRad
Bovine serum albumin (BSA)	Roche
Bromophenol Blue	VWR
Chloroform	Fisher
Chondroitin ABC lyase	Sigma
Chondroitin sulfate C	Sigma
Coomassie Brilliant Blue	Bio-Rad
DEAE-Sephacel	Pharmacia
Dextrose	Fisher
Dimethyl sulfoxide (DMSO)	Fisher
Dulbecco's Modified Eagle Media (DMEM)	Canadian Life Technologies
DMEM without glutamine, methionine, cystine	ICN
Dithiothreitol (DTT)	Canadian Life Technologies
EDTA	Fisher
Endoglycosidase F	Roche
Endoglycosidase H	Roche
Ethidium Bromide	Sigma

Fetal Bovine Serum (FBS)	Canadian Life Technologies
Geneticin (G418)	Canadian Life Technologies
Glycerol	Fisher
^{3}H Glucosamine hydrochloride	Amersham
Ham's F-12 media	Canadian Life Technologies
HAT supplement	Canadian Life Technologies
Heparan sulfate	Sigma
Heparin	Sigma
HEPES	Fisher
Hydrochloric acid (HCl)	Fisher
Isoamyl alcohol	Fisher
Kodak BioMax ML film	Interscience
Kodak BioMax MR film	Interscience
L-glutamine	Canadian Life Technologies
LipofectAMINE	Canadian Life Technologies
LipofectAMINE PLUS	Canadian Life Technologies
Lithium Chloride	ICN
LumiGLO chemiluminescent substrate kit	KPL
Luria broth base	Canadian Life Technologies
2-mercaptoethanol	Sigma
Methanol	Fisher
Methylene Blue	Sigma
N, N'-methylene-bis-acrylamide	Sigma
N-octyl-b-glucopyranoside	Sigma
Nonidet P-40 (NP-40)	Fisher
Paraformaldehyde	Fisher

Paraformaldehyde (16% EM-grade)	Canemco
Phenol, buffer-saturated	Canadian Life Technologies
Phenylmethylsulfonyl fluoride (PMSF)	Sigma
Polyethylene glycol 8000 (PEG)	BDH
Potassium Chloride	Fisher
Potassium Ferricyanide	Sigma
Potassium Ferrocyanide	Sigma
Potassium Phosphate (monobasic)	Fisher
Potassium Phosphate (dibasic)	Fisher
Pre-stained Protein Molecular Weight Markers	Canadian Life Technologies
Pronase	Sigma
2-Propanol	Fisher
Protease	Sigma
Protein G-Sepharose-4 Fast Flow	Pharmacia
Saponin	Sigma
Scintillation cocktails (Ready Safe)	Beckman
Select Agar	Canadian Life Technologies
Sepharose CL4B	Pharmacia
^{35}S-methionine	NEN
Sodium Acetate	Fisher, Mallinckrodt
Sodium Chloride (NaCl)	Fisher
Sodium Deoxycholate	Sigma
Sodium Dodecyl Sulfate (SDS)	Bio-Rad
Sodium Hydroxide	Fisher
Sodium Phosphate (monobasic)	Mallinckrodt
Sodium Phosphate (dibasic)	Fisher

^{35}S-sulfate	ICN
Sucrose	Fisher
TEMED	Fisher
Tissue culture flasks & dishes	Canadian Life Technologies
Trichloroacetic acid	Fisher
Tris	Canadian Life Technologies
Triton X-100	Sigma
Tween-20	JT Baker
Ultracentrifuge rotors & tubes	Beckman
3mm Whatman filter paper	VWR
X-gal	Canadian Life Technologies
Zwittergent 3-12	Calbiochem

List of Suppliers

Supplier	Location
Amersham	Oakville, Ontario
BDH	Toronto, Ontario
Beckman	Palo Alto, California
Becton Dickinson	Mississauga, Ontario
Bio-Rad	Mississauga, Ontario
Calbiochem	San Diego, California
Canadian Life Technologies	Burlington, Ontario
Canemco	Lachine, Quebec
Dupont-NEN	Mississauga, Ontario
Fisher	Edmonton, Alberta
ICN	St. Laurant, Quebec
Interscience	Markham, Ontario
JT Baker	Hayward, California
Kirkegaard & Perry Laboratories (KPL)	Gaithersburg, Maryland
Millipore	Mississauga, Ontario
New England Biolabs	Mississauga, Ontario
Pharmacia	Baie d'Urfe, Quebec
Sigma	Mississauga, Ontario
VWR	Burnaby, British Columbia

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