THE UNIVERSITY OF CALGARY

Alternative Splicing of the G3 Domain of Aggrecan in Osteoarthritic Canine Articular Cartilage

by

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A DISSERTATION

SUBMITTED TO FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

AUGUST, 1997

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ABSTRACT

The G3 domain of aggrecan, which is subject to alternative splicing, is composed of one or two EGF-like modules (E1 or E2), a lectin-like module (L), a complement regulatory protein-like module (C) and a C-terminus. The purposes of this project were to study the G3 domain of canine aggrecan by cloning and sequencing the cDNA of the G3 domain and portion of the 3' chondrointin sulfate rich region II (CS-II) up to a putative "aggrecanase" cleavage site; defining and quantifying the G3 isoforms expressed in articular cartilage with osteoarthritis (OA); and producing recombinant peptides of the G3 isoforms.

The cDNA sequence of the canine G3 domain has 91% to 74% homologous with the G3 domain of human, bovine, rat and chicken aggrecan. A putative "aggrecanase" cleavage site (VSQELAQR) exists in the CS-II region and is very similar to the site found at leucine residue 1939 (VSQE↓LGQR) in human aggrecan. Both the LC and ELC (with the E2-like module) were expressed in the articular cartilage of normal young, normal adult and adult dogs in the hypertrophic stage of experimental osteoarthritis.

The G3 isoforms were quantified by RT-PCR using total RNA from cartilage of adult stifles in the hypertrophic stage of experimental OA and the contralateral non-operated control (NOP). Expression of the LC isoform was significantly increased when comparing the OA to the NOP by Student's t-test ($p \leq 0.0078$), with $1.8 \times 10^{10} \pm 0.66 \times 10^{10}$ (copies/mg DNA; n=5) in the NOP and $5.7 \times 10^{10} \pm 2.6 \times 10^{10}$ (copies/mg DNA; n=5) in the OA. Expression of the ELC isoform was also significantly increased when comparing the OA to the NOP ($p \leq 0.0281$), with $0.24 \times 10^{10} \pm 0.12 \times 10^{10}$ (copies/mg DNA; n=5) in the NOP and $0.54 \times 10^{10} \pm 0.36 \times 10^{10}$ (copies/mg DNA; n=5) in the OA. Finally, recombinant peptides for these two G3 isoforms were successfully produced in pMAL-c2 expression system.

In conclusion, the G3 domain of aggrecan is conserved in five species compared. The same types of the G3 isoforms were expressed in all the canine articular cartilage

investigated. The LC isoform was predominantly expressed in all the cartilage samples tested. The putative G3-containing peptide cleaved by "aggrecanase" and the increase of the ELC isoform in OA may be physiological and pathological significant in the normal and osteoarthritic cartilage.

ACKNOWLEDGMENTS

I sincerely wish to thank my supervisor, Dr. Mark E. Adams, for his constant encouragement, trust, patience, commitment, and guidance during this project. Without his support, I would not have been able to finish this degree. I also wish to express my appreciation for Dr. Admas for his help in editing this dissertation. I thank my co-supervisor Dr. D.A. Hart, my PhD supervisor committee members, Drs. L. Gedamu, M. D. Hollenberg, G. A. Schultz. I also thank Dr. D. Edwards for his serving as an external examiner during my candidacy exam and Dr. T. T. Glant of the Rush University (Chicago, IL) for his commitment to be the external examiner for my thesis defense. Their comment, guidance and vision proved to be extremely helpful for my success in this project.

I offer further special thanks to my colleague Dingqiu Huang who taught me the basic techniques of molecular biology and always gave me valuable advice whenever I came across problems in experiments. Dingqiu also significantly contributed his talent to this project in screening the cDNA library, sequencing part of the G3 domain and PCR to identify some of the G3 isoforms. My special thanks also goes to Dr. John Matyas who had conducted most of the surgeries to induce experimental osteoarthritis in dogs and gave me lots of help in the statistical analyses. I wish to thank Ajay Bhatia who helped me to isolate the EGF2-like module by PCR. I thank Dr. Mayi Y. Arcellana-Panlilio for her expertise advise on quantitative RT-PCR; my friends Drs. Mao Long Lu, Gang Yu, and Marie-Pierre Gastineau, for their generous help while I did expression of recombinant proteins. Many people such as Liangsu Wang, Steven Lenz, Carol Reno, Pauline Zhao, Glenn Dourado, Paul Sciore, Sandy Peterson-Jones, Linda Marchuk, Joan Miller, Dr. Phyllis LuValle, Dr. Frank Beier, and many more, have given me help, friendship, and support, whenever I needed. I wish to thank them all. The financial support from the Medical Research Council of Canada is gratefully appreciated.

A very special thanks goes to my family, my husband Datong, my parents and my son Diwen. They supported me to finish my PhD and had put up with my leaving

family responsibilities to them.

DEDICATION

To: Datong, Diwen, Dishi and my parents

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LIST OF ABBREVIATIONS

aa	Amino acid
ACL	Anterior cruciate ligament
AMV	Avian myoblastosis virus
bp	Base pair
bFGF	Basic fibroblast growth factor
CAPAGE	Composite agarose-polyacrylamide gel electrophoresis
cDNA(s)	Complementary deoxyribonucleaic acid(s)
CD spectrum	Circular dichroism spectrum
C-like	Complement regulatory protein-like
COL2	Type II collagen
CPM or cpm	Counts per minute
CRD	Carbohydrate recognition domain
CS	Chondroitin sulfate
CSRR	Chondroitin sulfate-rich region
CsTFA	Cesium trifluoroacetate
C-terminus	Carboxyl terminus (= carboxyl terminal end or side)
2D	Two dimensional
ddH_2O	Distilled deionized water
DEPC	Diethyl pyrocarbonate
DNA(s)	Deoxyribonucleic acid(s)
DS	Dermatan sulfate
E-like	Epidermal growth factor-like
E. coli	*Escherichia coli*
EDTA	Ethylenediamine-tetraacetic acid (disodium, dihydrate)
EGF	Epidermal growth factor
EthBr	Ethidium bromide
F.	Fluorescence unit

FRR	Formaldehyde RNA running buffer
G1 domain	The first globular domain or HA-binding domain (of aggrecan)
G2 domain	The second globular domain (of aggrecan)
G3 domain	The third globular domain (of aggrecan)
GAG	Glycosaminoglycan
GIT	Guanidine isothiocyanate
GST	Glutathione S-transferase
HA	Hyaluronic acid
HABR	Hyaluronic acid binding region
HPLC	High performance liquid chromatography
ID	Identification
IGD	The interglobular domain (of aggrecan)
IGF-I	Insulin growth factor-like I
IGF-II	Insulin growth factor-like II
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-6	Interleukin-6
IPTG	Isopropyl β-D-thiogalacto-pyranoside
kb	Kilobase pair
kD	Kilodalton
KS	Keratan sulfate
KSRR	Keratan sulfate-rich region
LDL	Low density lipoprotein
L-like	Lectin-like
LMP	Low melting point
MBP	Maltose binding protein
MOPS	3-(N-morpholino)propane sulfonic acid
mRNA	Messenger ribonucleic acid
M.W.(s)	Molecular weight

NOP	Non-operated control
NR	Non-translated region
nt	Nucleotide
N-terminus	Amino terminus (= amino terminal end or side)
OA	Osteoarthritis
OD	Optical density
PCR	Polymerase chain reaction
pfu	Plaque forming units of bacteriophage
PG(s)	Proteoglycan(s)
PIPES	Piperazine-N, N'-bis(2-ethanesulfonic acid)
PNK	Polynucleotide kinase
PRELP	Proline arginine-rich end leucine-rich repeat protein
PMSF	Phenyl methyl-sulfonyl fluoride
PVDF	Polyvinylidene difluoride
RNA(s)	Ribonucleic acid(s)
RNase	Ribonuclease
RT	Reverse transcription
RT-PCR	Reverse transcription and polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLRPs	Small leucine-rich proteoglycans
snRNA	Small nucleotide ribonucleic acid
SP	Signal peptide
SPC(s)	Subtilisin-like proprotein convertase(s)
ssDNA	Salmon sperm deoxyribonucleic acid(s)
T	The C-terminus (of aggrecan)
TdT	Terminal deoxynucleotide transferase
TEMED	N,N,N',N'-tetra- methylethylenediamine

TGF-beta	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinase
tPA	Tissue plasminogen activator
Tris	Tris(hydroxymethyl) aminomethane
tRNA	Transfer ribonucleic acid
uPA	Urokinase plasminogen activator
5'UTR	5' Untranslated region
UV	Ultraviolet

CHAPTER 1.

1. INTRODUCTION

1.1 BACKGROUND

Osteoarthritis (OA) is the most common and costly form of arthritis. It is clear from both clinical and experimental studies that a variety of complex mechanical, morphological, and metabolic changes occur during the development and progression of this disease in all the joint tissues, particularly in the articular cartilage (Meachim & Brooke, 1984; Sokoloff & Hough, 1985; Howell *et al.* 1986; Adams, 1987; Hamerman, 1989). Mechanically, the articular cartilage involved in OA is less resilient and less slippery than normal (Kempson *et al.* 1970). Macroscopically, some areas of articular cartilage in OA appear hypertrophied and swollen while others are fibrillated and eroded; microscopically, some areas of articular cartilage have reduced cellularity and diminished histochemical staining for proteoglycans (PGs) while others have increased cellularity and intensified pericellular staining for PGs (Meachim & Brooke, 1984). Thus, as in many states of repair and remodeling, the metabolic balance can favor anabolism or catabolism in different areas of osteoarthritic cartilage at the same time. Because the maintenance of the normal material properties of articular cartilage depends on normal chondrocyte metabolism, any changes in chondrocyte metabolism could alter the material properties of the cartilage. Characteristic changes in the material properties of articular cartilage and in chondrocyte metabolism are known to occur in OA (Mankin & Lippiello, 1970; Palmoski & Brandt, 1979; Santer *et al.* 1981; Adams *et al.* 1987; Pelletier *et al.* 1988; Murphy *et al.* 1990), and it seems likely that alterations in both are central to the pathogenesis of OA. The most dramatic metabolic changes that occur in the structural macromolecules of articular cartilage in OA involve the PGs and the collagens, which are the most abundant macromolecules in articular cartilage (Adams, 1989).

In the late stages of OA in humans, the articular cartilage is disintegrated and the underling bone becomes eburnated (Adams, 1987). However, it is very difficult to study the early stages of OA in humans due to both difficulties in making an early diagnosis and the lack of availability of early and matched control specimens. Fortunately, the early stage of OA can be seen in animal models. For example, anterior cruciate ligament (ACL) transection of the canine stifle is an animal model that can be studied in its early stage. ACL transection in canine stifle results in an arthropathy that progresses to true OA (Adams & Brandt, 1991; Brandt *et al.* 1991a; Brandt *et al.* 1991b; Adams, 1994). (The proper veterinary term is cranial cruciate ligament because of the different anatomical position of the dog; the human term ACL will be used in this study.) In this project, we focused on the study of the G3 domain at the C-terminus of aggrecan in articular cartilage of normal and an early (the hypertrophic) stage of experimentally osteoarthritic dogs.

1.1.1 Canine articular cartilage in normal stifles and in the anterior cruciate ligament transection model of experimental OA

Normal stifle (or knee) articular cartilage is composed of hyaline cartilage. (Stifle is the proper veterinary term for the joint that is equivalent to the human knee). Articular cartilage is aneural, avascular and alymphatic with only one type of cell, chondrocytes. The biochemical composition of the extracellular matrix is complex, being composed of collagens (type II, VI, IX, XI, and others), proteoglycans (PGs) (aggrecan, biglycan, decorin, and fibromodulin), hyaluronan (also called hyaluronate or hyaluronic acid), link protein, fibronectin, growth factors, *etc.* (Heinegård & Oldberg, 1989; Eyre *et al.* 1991). The predominant macromolecules by mass are type II collagen and aggrecan. The physical properties of articular cartilage can be attributed largely to the macromolecular milieu of these two molecules: a balance exists between the swelling pressure of the hydrated large proteoglycan (aggrecan) and the tensile restraining forces of three-dimensional meshwork of fibrillar type II collagen. This composition endows articular cartilage with the unique viscoelastic material properties that are essential for its normal biomechanical function. Should the concentration or the molecular structure of either the collagens or

proteoglycans change, cartilage may alter its response to mechanical load and may not function optimally.

Animal models of OA, such as those induced by immobilization of a joint, by supraphysiological external forces applied to a joint, by surgical manipulation to create instability, and by other techniques, have been used in the studies of experimental OA (Adams & Billingham, 1982; Troyer, 1982). In the model induced by surgical transection of the ACL in the canine stifles, the natural history of experimental OA is very well defined (Figure 1.1): After ACL transection, the cartilage goes through a short lag phase, followed by a growth (or hypertrophic) phase. At approximately 20 to 24 weeks post surgery, it reaches a stable compensated (plateau) phase, which can last for 3 to 4 years. After that, cartilage enters a degeneration phase (Adams & Brandt, 1991; Brandt *et al.* 1991a; Brandt *et al.* 1991b; Adams, 1994). It is this latter phase which resembles human clinical OA. These observations suggest that injured cartilage passes through a distinctly anabolic phase, *i.e.*, when the net metabolic balance favors anabolism, prior to developing endstage OA (Adams & Brandt, 1991). Osteophyte formation at the margin of the articular cartilage, capsular thickening and subchondral bone sclerosis are often seen in the ACL-transected joint (Hardingham *et al.* 1991). In the hypertrophic stage of the experimental OA *(e.g.*, 10 weeks after ACL transection), chondrocyte metabolism is very active in response to the injury. The cartilage is thicker than that of the contralateral non-operated control; the turnover rate of proteoglycan and the rate of biosynthesis of proteoglycan and collagen are also increased (Eyre *et al.* 1980; Adams & Brandt, 1991; Ratcliffe *et al.* 1992). Fiber disruption of the midzone perilacunar collagen is seen and collagenlytic activity is higher in the operated joint (Pelletier *et al.* 1983). There is a much greater increase in the expression of type II collagen mRNA (8-fold increase) than that of aggrecan (2-fold increase) (Matyas *et al.* 1995). There is an increase in the water content in the cartilage (McDevitt & Muir, 1976). In this project, osteoarthritic cartilage at the hypertrophic stage of experimental OA was derived from skeletal mature male canine stifles 10 weeks after ACL transection (Adams & Pelletier, 1988; Adams, 1994).

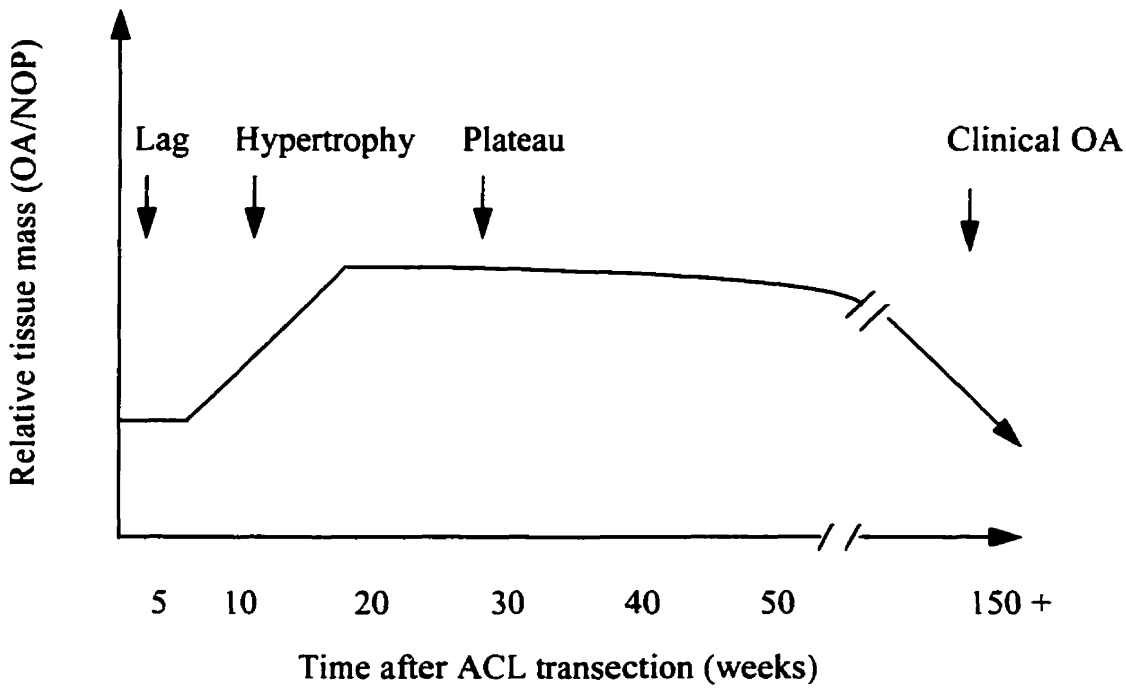

Figure 1.1 Schematic diagram illustrating the natural history of experimental osteoarthritis.

This is a diagram of the natural history of the animal model of experimental OA induced by ACL transection of the canine stifles in skeletal mature male young dogs (Adams, 1994). Time after ACL transection is plotted on the abscissa and the relative cartilage mass (mass of cartilage in the osteoarthritic joint divided by the NOP joint) is plotted on the ordinate. NOP= the contralateral non-operated control.

1.1.2 Proteoglycan structure and metabolism in cartilage

PGs in the extracellular matrix can be divided into two major classes: the small leucine-rich PGs (SLRPs) and the modular PGs (Iozzo & Murdoch, 1996). All PGs consist of a protein core with one or more covalently bound glycosaminoglycan (GAG) side chains such as chondroitin sulfate (CS), keratan sulfate (KS) or dermatan sulfate (DS); they may have O-linked and N-linked oligosaccharides as well (DeLuca *et al.* 1980; Lohmander *et al.* 1980; Thonar *et al.* 1983; Carney & Muir, 1988; Heinegård & Oldberg, 1989). Several

SLRPs are present in articular cartilage: decorin, biglycan, fibromodulin, and at least two proteins, chondroadherin and proline arginine-rich end leucine-rich protein (PRELP), which have structures like the SLRPs but without GAG side chains, have been identified in articular cartilage (Heinegård *et al.* 1981; Stanescu & Sweet, 1981; Rosenberg *et al.* 1985; Heinegård *et al.* 1986b; Neame *et al.* 1994; Bengtsson *et al.* 1995; Grover *et al.* 1995).

The modular PGs (*e.g.*, aggrecan, versican and variants, neurocan, brevican, testican, *etc.*) are structural macromolecules, but also have modules that exert various biological functions. By mass, the most abundant PGs in articular cartilage is the modular PG aggrecan, which is also a hyalectin, binding hyaluronan at its N-terminus. Aggrecan was the primary subject of the research in this study.

1.1.2.1 Aggrecan structure: an overview

The core protein of aggrecan has three distinct globular domains (G1, G2, and G3) and two interglobular domains (Figure 1.2) (Hardingham *et al.* 1986). The G1 domain (or HABR for hyaluronic acid binding region) is located at the N-terminus of the core protein and is known to interact non-covalently with hyaluronan and link protein in the formation of proteoglycan-hyaluronan aggregates (Paulsson *et al.* 1987). The interaction of the G1 domain with hyaluronan and link protein helps to anchor the aggrecan in the tissue. The formation of aggrecan-hyaluronan aggregates is essential for the biomechanical function of articular cartilage. A relatively short interglobular domain (IGD) spans between G1 and G2 and has no known function, but this domain contains a number of protease cleavage sites (Sandy *et al.* 1991b). Although G2 has sequence homology with G1, the G2 domain of aggrecan does not associate with hyaluronan, and its specific biological function remains obscure (Morgelin *et al.* 1988). A large interglobular domain separates G2 from G3 and serves as the site of attachment of KS and CS side chains, which give aggrecan the hydrophilicity that contributes to its mechanical function. Most of the KS chains are attached to the KS-rich region (KSRR), which is located adjacent to the C-terminal side of G2 (Antonsson *et al.* 1989); the CS chains are attached to a very large chondroitin sulfate-rich region (CSRR) which is subdivided into CS-I and CS-II regions, that are located on

the C-terminal side of the KS-rich region (Oldberg *et al.* 1987) and extend to the N-terminal side of the G3 domain. They are formed from one large exon. The CS-I has more dense chain packing than the CS-II. The G3 domain is located in the C-terminal region of aggrecan. The exact function of the G3 domain is as yet unknown, but it has complex structure composed of several modules that suggests that it may have biological activity in cartilage (see section 1.1.3 below).

The cDNA structure of the G3 domain of human aggrecan is presented in Figure 1.3 (Baldwin *et al.* 1989; Doege *et al.* 1991; Fülöp *et al.* 1993; Valhmu *et al.* 1995). There are 19 exons in the genomic DNA of human aggrecan. The 5' untranslated region (5' UTR) is coded by the exon No. 1; the signal peptide (SP) is coded by the exon No.2; the G1 is coded by exons No. 3 to 6; the IGD is coded by the exon No.7; the G2 is coded by the exons No. 8 to 10; the N-terminal side of KS is coded by exon No. 11; the C-terminal side of KS and the whole CS (including CS-I and CS-II) is coded by exon No. 12; the G3 domain is coded by the exons No. 13 to 19. There are two epidermal growth factor-like modules (E1 and E2) that can be present in human aggrecan. The E1 is coded by the exon No. 13 and the E2 is coded by the exon No. 14. The lectin-like module (L) is coded by exons No. 15 to 17; the complement regulatory protein-like module (C) is coded by the exon No. 18; and finally, the C-terminus (T) and part of the 3' non-translated region (NR) is coded by the exon No. 19.

The biochemical profile of aggrecan extracted from articular cartilage is both polydisperse and heterogeneous (Gibbons, 1972): polydisperse largely because of extensive glycosylation; heterogeneous in that two large forms have been observed by composite agarose-polyacrylamide gel electrophoresis (CAPAGE) (McDevitt & Muir, 1971; Heinegård *et al.* 1985a; Heinegård *et al.* 1985b; Stanescu & Pham, 1986; Adams *et al.* 1987). Biochemical analysis reveals that the larger of the two forms has a core protein of 208 kD with 97 CS chains (average size is 19.7 kD) and 30 KS chains (average size is 5.9 kD); the smaller form has a core protein of 94 kD with 41 CS chains (average size is 16.4 kD) and 16 KS chains (average size is 8.3 kD) (Heinegård *et al.* 1985b). Molecular biological studies suggest that the aggrecan core protein is the product of a single gene

(Antonsson *et al.* 1989); *in vivo* and *in vitro* radiolabelling studies suggest that the larger form of the aggrecan may be the precursor of the smaller form (Heinegård *et al.* 1986a). Electron microscopy reveals that the G3 domain is absent from 50-70% of aggrecan molecules extracted from various cartilages (Flannery *et al.* 1992). This result suggests that the smaller form of aggrecan is generated due to cleavage of a C-terminal portion of the larger form of aggrecan including the G3 domain. However, one study suggested that post-translational cleavage of G3 domain may not be the sole explanation of the differences in the electrophoretic behavior between these two forms of aggrecan, but a more recent study (may be the methodology is more specific) supports the hypothesis that the faster migrating band is lacking the G3 domain (Dudhia *et al.*, 1996).

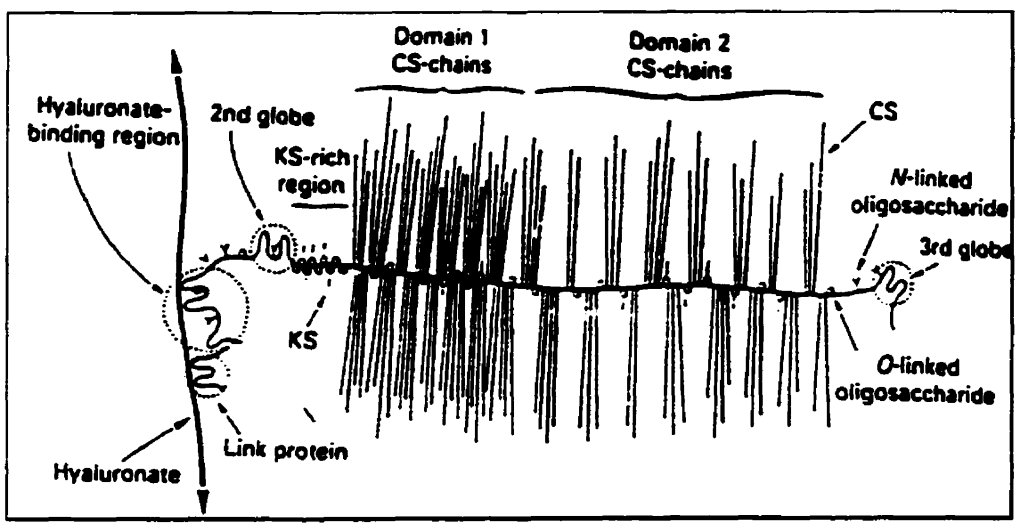

Figure 1.2 Schematic illustration of the structural features of aggrecan.

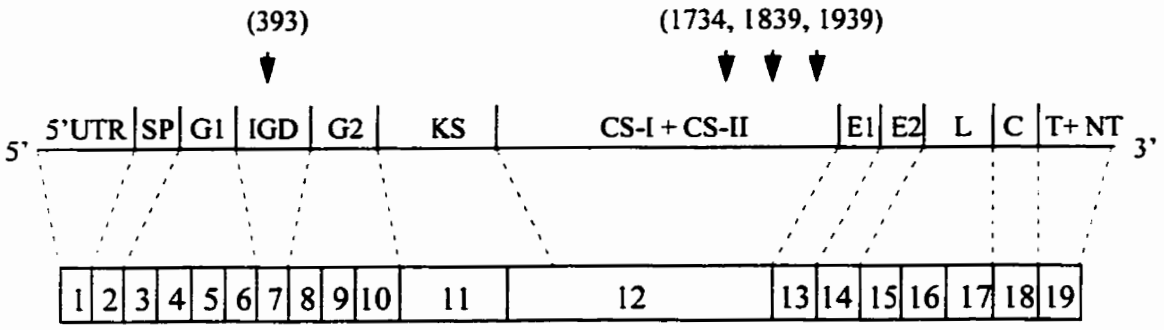

Figure 1.3 The complete mRNA structure for the protein domains and modules and the corresponding exons of the cDNA of human aggrecan.

This is a schematic diagram of the complete mRNA structure (the solid horizontal line with vertical lines that indicate the protein domains and modules) of human aggrecan and their corresponding exons (the boxes) (Baldwin *et al.* 1989; Doege *et al.* 1991; Fülöp *et al.* 1993; Valhmu *et al.* 1995). The dashed lines indicate the corresponding coding exons for the protein domains and modules. The arrows (with amino acid numbers which begin at the SP) indicate the putative "aggrecanase" cleavage sites (Ilic *et al.* 1992).

Note:

(1) 5'UTR = 5' untranslated region, SP = signal peptide, G1 = the first globular domain, IGD = the interglobular domain, G2 = the second globular domain, KS = keratan sulfate-rich region, CS-I and CS-II = chondroitin sulfate-rich regions I and II, T= the C-terminal peptide, NT= 3' non-translated region.

(2) The G3 domain is composed the E1-like module, the E2-like module, the L-like module, the C-like module, and the C-terminal peptide.

(3) The cleavage site at 393 aa is identical to the site numbered 374 in other reports because in those reports the numbering of the amino acids started at the first amino acid of the mature core protein after removal of the signal peptide (at the first 19 aa) (Sandy *et al.*, 1992; Lohmander *et al.*, 1993).

(4) The sizes of the exons, protein domains and modules in this figure are not proportional to their actual length.

While not substantial enough to account for the difference in size between these two forms of aggrecan that are seen on CAPAGE, "microheterogeneities" also exist in the protein structure of aggrecan. For example, the G3 domain of aggrecan has been sequenced in a number of different species including chicken (Tanaka *et al.* 1988), human (Baldwin *et al.* 1989; Doege *et al.* 1991) , bovine (Oldberg *et al.* 1987), rat (Doege *et al.* 1987; Doege *et al.* 1986), dog (Fülöp *et al.* 1993; also see Chapter 2) and mouse (Walcz *et al.* 1994), and sequence heterogeneity has been seen in the G3 domain of aggrecan that is brought about by alternative mRNA splicing (Baldwin *et al.* 1989). The G3 domain contains one or two epidermal growth factor (EGF)-like modules (E1 and E2), each of which has 114 nucleotides (nt), a lectin-like module (L, 387 nt) which is also referred to as the "carbohydrate recognition domain" (CRD), a complement regulatory protein-like module (C, 183 nt) and a C-terminal peptide (Figure 1.3) (Baldwin *et al.* 1989; Fülöp *et al.* 1993). These modules and their possible biological functions in cartilage are described in more detail below (section 1.1.3). Alternative splicing of the mRNA coding for the G3 domain may involve the EGF-like modules and the C-like module.

The catabolism of the cartilage matrix is controlled, at least in part, by neutral metalloproteinases (*e.g.*, collagenases, gelatinases, and stromelysins) and their inhibitors (Docherty & Murphy, 1990). Different regions of the aggrecan core protein are susceptible to enzymatic degradation. The short IGD between G1 and G2, for instance, is vulnerable to proteolysis by neutral metalloproteinases (Fosang *et al.* 1991; Hollander *et al.* 1991; Sandy *et al.* 1991a; Sandy *et al.* 1991b; Ilic *et al.* 1992; Loulakis *et al.* 1992; Mok *et al.* 1992; Sandy *et al.* 1992; Van de Loo *et al.* 1992). However, for this study, it is especially noteworthy that fragments containing the G3 are cleaved from aggrecan (Loulakis *et al.* 1992; Ilic *et al.* 1992) suggesting that an as yet unidentified proteinase (known as "aggrecanase") is involved in this proteolysis.

Substantial changes in structure and the biochemistry of aggrecan occur during skeletal growth, with aging, and in OA. In advanced human OA, the total PG content of articular cartilage is reduced (Mankin & Lippiello, 1970; Meachim & Brooke, 1984; Sokoloff & Hough, 1985; Howell, 1986). In older animals and with progression of end-

stage OA, aggrecan is progressively fragmented (Sandy *et al.* 1992; Cs-Szabó *et al.* 1995) and is decreased in hydrodynamic size, carbohydrate to protein ratio, and CS chain length (Inerot *et al.* 1978). This likely results from the elaboration of interleukin-1 (IL-1) and other catabolic cytokines that can both stimulate the secretion of proteinases and suppress PG synthesis (Campbell *et al.* 1986; Ratcliffe *et al.* 1986; Dingle *et al.* 1987). In contrast to this loss of PGs from cartilage in advanced OA, *in vivo* studies using animal models of early stage of OA, or *in vitro* culture of osteoarthritic cartilage show that PG synthesis, as measured by uptake of $[^{35}S]O_4$ (Collins & McElligott, 1960; Mankin & Lippiello, 1970; Mankin *et al.* 1971; McDevitt *et al.* 1977; Thompson & Oegema, 1979; Moskowitz *et al.* 1981; Palmoski & Brandt, 1983; Sandy *et al.* 1984) and the expression of aggrecan mRNA (Matyas *et al.* 1995) are both increased about 2-fold. The hexuronate content (a measure of CS) and the CS/KS ratio is increased even in morphologically normal articular cartilage taken from joints with experimental OA (Thompson & Oegema, 1979). The increased ratios of galactosamine to glucosamine and chondroitin 4-sulfate to chondroitin 6-sulfate, the longer CS chains and more highly glycosylated protein core and higher G3 containing form of aggrecan are consistent with the hypothesis that the aggrecan in OA is more like the aggrecan from younger tissues (Mankin & Lippiello, 1970; McDevitt & Muir, 1976; McDevitt *et al.* 1977; Thompson & Oegema, 1979; Cox *et al.* 1985; Adams, 1994; Cs-Szabó *et al.*, 1995). Thus, in the early stages of OA the overall metabolic balance favors aggrecan anabolism because the matrix becomes enriched in aggrecan (Vignon *et al.*, 1983; Adams, 1989). This increase in the amount of aggrecan (and mass of cartilage) is called hypertrophic repair.

Finally, it is important to note that the amount of the larger form of aggrecan is increased substantially both in juvenile cartilage and in osteoarthritic adult cartilage (Inerot & Heinegård, 1983; Adams, 1994). OA cartilage is enriched in a population of aggrecan that includes the G3 domain. Thus, the biological function of the G3 domain, whatever it may be, may be exaggerated in articular cartilage at least during the hypertrophic stage of OA.

1.1.3 The possible biological functions of the G3 domain of aggrecan

1.1.3.1 The G3 domain of aggrecan

As mentioned above, in addition to the C-terminal peptide, the G3 domain of aggrecan core protein may be composed of three different modules, though not necessarily all at once, because of alternative mRNA splicing. Whereas the nucleic acid sequence homologies between the modules of the G3 domain (E, L and C) and the corresponding proteins from which the names of the modules are derived are imperfect, it is possible that the protein structure of the G3 domain may mimic some of the activities ascribed to some or all of these bioactive proteins. It is both curious and intriguing that a mosaic architecture consisting of these same three modules occurs in many proteins other than aggrecan, though the order and number of modules may vary. For example, the C-terminal portion of versican, a PG secreted by fibroblasts that resembles aggrecan, contains two epidermal growth factor-like modules, a lectin-like module, and a complement regulatory protein-like module (Zimmermann & Ruoslahti, 1989). Recombinant lectin-like module of the C-terminal domain of versican produced in mammalian cells was reported to bind specifically and in a calcium-dependent manner to tenascin-R which is an extracellular matrix glycoprotein and a cell adhesion protein (Aspberg *et al.*, 1995). However, the recombinant lectin-like module of versican lost its ability to bind to the chemically deglycosylated tenascin-R (Aspberg *et al.*, 1995). Furthermore, the selectins, which bind cell surfaces *via* carbohydrates, contain these same three (E, L and C-like) modules, though the order and number of the modules are not the same (Bevilacqua *et al.* 1989; Bowen *et al.* 1989; Johnston *et al.* 1989; Lasky *et al.* 1989; Siegelman *et al.* 1989).

While it is difficult to generalize about the functions of these individual modules, they all have in common the ability to bind to cells, proteins, or carbohydrates. Thus, these modules of the G3 domain of aggrecan may have unique binding properties that allow them to attach to chondrocytes and/or extracellular matrix proteins, and affect their function.

It has been demonstrated by the nanomelic chick that the G3 domain is crucial to aggrecan (Li *et al.* 1993). This fatal genetic mutant has a premature stop codon, and lacks the terminal portion of the CS-II region and the whole G3 domain. The animal makes no cartilage; the abnormal aggrecan is not translocated through the Golgi (Vertel *et al.* 1993), which result suggests that the G3 domain helps in the transport of aggrecan to the extracellular milieu. This hypothesis was supported by the evidence that a G3 construct transfected into Chinese Hamster Ovary cells was processed by the cells much faster than was a G1 construct (Luo *et al.* 1996). It is not clear what part of the G3 domain is responsible for this. The G3 domain has also been shown to bind to hyaluronan (Yang *et al.* 1995), and does have at least one BX_7B hyaluronic acid binding consensus sequence (B=basic amino acids, X= other amino acids) (Yang *et al.* 1994). The strength of this binding is not known, but it presumably is less than that of the G1 of aggrecan, as aggrecan is seen on electron microscopic spread films with G1 bound to hyaluronan, but not with the G3 bound. The biological significance of this putative hyaluronan binding of the G3 is not clear.

1.1.3.2 The C-terminal peptide

This last 25 amino acid segment is well conserved among mammalian species. Its sequence contains many basic amino acids and three RXXR (R= arginine, X=other amino acids) subtilisin-like proprotein convertase (SPC, such as furin) cleavage sites (Molloy *et al.* 1992). As SPCs often act in the trans-Golgi and on the cell surfaces, it is highly likely that this C-terminal peptide is absent in secreted aggrecan. An evidence supported that this segment is cleaved was: an antibody directly against rat C-terminus (with epitope TYKHP) can recognize aggrecan from rat chondrosarcoma (presumably defective in the SPCs), but not mature human aggrecan or any other species (J.D. Sandy, personal communication). Alternatively, this may also be due to slight differences in protein structure (change from H to R in human aggrecan) that may change the epitope sufficiently.

1.1.3.3 The complement regulatory protein-like module

The complement regulatory protein-like module of aggrecan core protein obviously shares homology with complement regulatory protein, but also with several other proteins, including protein C, protein H, factor B, haptoglobin, the IL-2 receptor, and factor XIII (Rhodes *et al.* 1988; Doege *et al.* 1991). Homology with the complement regulatory protein was first described by Patthy (Patthy, 1987) who analyzed the cDNA sequence data. Again, the exact functions of the C-like module in aggrecan is unknown.

1.1.3.4 The lectin-like module

The lectin-like module of aggrecan, also called the carbohydrate recognition domain (CRD), has homology with modules found in hepatic asialoglycoprotein receptors, the human Fc receptor for IgE, thrombomodulin, tetranectin, selectins, and many other proteins (Fuhlendorff *et al.* 1987; Drickamer, 1988; Petersen, 1988; Kluft *et al.* 1989; Atoda *et al.* 1991; Hoyle & Hill, 1991). The lectin-like protein modules have been classified into two types, the C-type, to which the aggrecan lectin-like module belongs, and the S-type (Drickamer, 1988). In the S-type lectins, the cysteine residues are conserved and the cysteine residues are not present as disulfides but as sulfhydryl groups. The binding of S-type lectins to sugar is Ca^{2+}-independent (Drickamer, 1993). The C-type lectins have disulfide bonds, are calcium dependent, and bind to sugars, particularly galactose and fucose. Indeed, it is known that the lectin-like module of G3 binds to fucose and galactose, and this binding is Ca^{2+}-dependent (Halberg *et al.* 1988; Saleque *et al.* 1993). There has been one report that says the most abundant isoform of the G3 domain expressed in human fetal cartilage contains only the lectin-like module (Doege *et al.* 1991). However, the exact biological function of the lectin-like module in aggrecan is unknown.

1.1.3.5 The epidermal growth factor-like module

The epidermal growth factor-like module of aggrecan core protein shares homology with EGF and similar modules that are found as single or multiple repeats in proteins such as

EGF precursor (Bell *et al.* 1986), uPA (Appella *et al.* 1987), tPA (Gerard *et al.* 1986), laminin B1 (Panayotou *et al.* 1989), LDL receptor (Murdoch *et al.* 1992), and many others (Cool *et al.* 1985; Hartley *et al.* 1987; Ryan *et al.* 1989). Many of these proteins bind calcium which can affect their function (Yamamoto *et al.* 1986; Mayhew *et al.* 1992; Selander-Sunnerhagen *et al.* 1992; Clarke *et al.* 1993; Hewett *et al.* 1993; Rezaie *et al.* 1993; Handford *et al.* 1995). Human aggrecan has two different epidermal growth factor-like modules, E1 and E2, which resemble those of versican (Zimmermann & Ruoslahti, 1989), and which may be expressed together, independently, or not at all (Fülöp *et al.* 1993). All species studied today can express the E2-like module, but the expression of the E1-like module varies. Bovine chondrocytes express a very low level of the E1-like module, and canine chondrocytes do not express it at all (Fülöp *et al.* 1993). A deletion of a polypyrimidine tract in the intron located upstream from the exon encoding the E1-like module may contribute to the lack of expression of the E1-like module in dog (Fülöp *et al.* 1996). Presence of the E2-like module was not described in the G3 genomic DNA sequence from chicken aggrecan by the authors (Tanaka *et al.* 1988). However, an E2-like sequence was found in the No. 389-502 nucleotide region of the same genomic DNA sequence with conserved splicing donor and acceptor sites.

Based on their disulfide bonding and amino acid structure, E-like modules have been classified into three types using the E-like module of tPA as a prototype (Appella *et al.* 1988): The amino acid consensus sequence of the type I EGF-like module is C1PXnC2LN(H)GGTC3XXXXXXXDXXAC4XC5XXGF(Y)GXRC6E(Q), with the left first amino acid residue starting at the N-terminal side. Furthermore, three disulfide bonds are formed between C1 and C3, C2 and C4, C5 and C6, respectively. The EGF-like module can be divided into three regions: region A, between C2 and C3, region B between C3 and C4, and region C between C5 and C6. In type I EGF-like modules, the C1 is N-terminal to C2, with 1 to 7 residues between C1 and C2. Region A is highly conserved in length (5 to 6 residues) and sequence. Region B has 10 to 11 amino acids and is highly divergent in sequence. Region C is conserved in length and has 4 to 5 specific amino acids. Type II EGF-like modules have similar structure as type I, except that region B is three

residues shorter but are more conserved in amino acid sequence than type I. Although type III EGF-like modules maintain many identifiable sequence similarities with respect to each other or type I and type II, the three regions are less conserved in spacing, and the disulfide bonds are quite different from type I and type II.

Many type I E-like modules and some type II E-like modules were reported to have some biological activities, including EGF-like activity, binding to calcium ion, protein-protein interaction, protein-cell interaction, mediating cell attachment, migration and receptor binding, whereas no type III E-like module was reported to have such activities (Appella *et al.* 1988). Both E1-like and E2-like modules in aggrecan core protein are predicted to be of the type II class (Appella *et al.* 1988; Fülöp *et al.* 1993; Baldwin *et al.* 1989) and, thus, may possess some EGF activity. However, the ability of the type II E-like modules to bind to a receptor or a ligand may be influenced by the sequence adjacent to the E-like module (Appella *et al.* 1988), and this might change with different patterns of alternatively splicing.

In proteins other than aggrecan, E-like modules have been reported to be involved in receptor interactions, *e.g.*, the receptor binding specificity of urokinase lies within the E-like module of uPA (Appella *et al.* 1987). Some E-like modules, such as those in laminin, have been demonstrated to have growth factor activity (Timpl *et al.* 1983; Panayotou *et al.* 1989). Deletion of the first two E-like modules from the LDL-receptor strongly interferes with the binding capacity and specificity of this receptor (Yamamoto *et al.* 1986). Even synthetic peptides that contain E-like repeats can mediate cell attachment, migration and receptor binding despite the lack of normal N-linked glycosylation (Appella *et al.* 1987; Graf *et al.* 1987; Ryan *et al.* 1989).

In reporting the presence of an E-like module in the G3 domain, Baldwin, *et al.* (1989) speculated that this module might affect cartilage metabolism by stimulating anabolism after the proteolytic release of the G3 domain from the aggrecan core protein. While the vast majority of E-like modules in other proteins do not exhibit growth factor activity, the one in aggrecan might because it is a type II E-like module. Cleavage of aggrecan at the most C-terminal "aggrecanase" cleavage site would release a G3-

containing fragment into the tissue. The N-terminal portion of this cleavage peptide had been identified in cartilage treated with IL-1 (Ilic *et al.* 1992). Thus, breakdown of the G3-containing form of aggrecan could release a C-terminal fragment bearing the E-like module that might initiate and stimulate cartilage repair. Notably, Larsson *et al.* (1989) showed that intact aggrecan and globular domains of aggrecan stimulated chondrocyte biosynthesis in culture, particularly type II procollagen and aggrecan itself. HA and CS alone were not stimulatory, whereas, reduced aggrecan and aggrecan with the globular domains removed were inhibitory. Interestingly, fetal cartilage was less stimulatory than adult cartilage. However, the specific globular domain(s) which were stimulatory were not identified.

Although it is unknown if the E-like modules of aggrecan have any EGF activity, it is important to note that cartilage does respond to a variety of growth factors including EGF. For instance, TGF-β and IGF-I increase overall PG anabolism and at the same time impede cartilage catabolism by promoting the secretion of proteases inhibitors such as TIMP (Chandrasekhar & Harvey, 1988; Morales & Roberts, 1988; Morales & Hascall, 1989; Schalkwijk *et al.* 1989). For this proposal, it is noteworthy that cartilage has EGF receptors and is responsive to EGF (Osborn *et al.* 1989; Kinoshita *et al.* 1992).

A decrease either in anabolic factors, as occurs with age (Copeland *et al.* 1990), or in the responsiveness of chondrocytes (Joosten *et al.* 1989), or perhaps, a reduction in growth factor-like activity derived from extracellular matrix proteins could conceivably contribute to the development of OA. The prevalence of OA increases dramatically with age and there is some evidence suggesting that patients with OA have lower levels of some growth factors such as IGF-I compared to age-matched controls (Denko *et al.* 1990). It seems likely that controlling the anabolic response to joint injury could be therapeutically important in OA. Indeed, blocking the intrinsic "repair" response with some types of drug therapy is believed to account for the worsening seen with such therapies in many types of experimental OA (Palmoski & Brandt, 1979; Palmoski *et al.* 1980; Carreno *et al.* 1986; Howell *et al.* 1986; Brandt, 1987; Ghosh, 1988; Pelletier *et al.*, 1988; Pelletier *et al.* 1989).

In summary, the G3 domain of aggrecan might affect cartilage metabolism because (a) it has at least one E-like module which is a type II E-like module; (b) a G3 domain fragment of aggrecan with the N-terminal sequence of the last "aggrecanase" cleavage site has been identified in cartilage; (c) chondrocytes have EGF-receptors and can response to EGF; and (d) fragments with intact globular domains of aggrecan can stimulate cartilage metabolism. If osteoarthritic cartilage is enriched in a population of aggrecan that includes the G3 domain, the biological function of G3, whatever it may be, may be exaggerated in articular cartilage, at least during the anabolic stages of OA.

1.1.4 Alternative mRNA splicing in connective tissue repair

The regulation of the expression of structurally distinct, developmentally regulated, and cell type-specific protein, is a fundamental characteristic of eukaryotic cell differentiation. Although the molecular mechanisms responsible for generating this protein diversity are presently poorly understood, they can be categorized into two systems: those that select one gene among the members of a multigene family for expressing in a particular cell, developmental stage, or physiological condition; and those that generated several different protein isoforms from a single gene. The later comprises DNA rearrangement and alternative pre-mRNA splicing, each leading to the differential expression of genomic sequences and so producing multiple protein isoforms from a single gene.

Most eukaryotic genes that code for proteins contain the sequences present in the corresponding mature mRNA in discontinuous DNA segments (exons) interspersed among sequences (introns) that do not form a part of mature mRNA (Sharp, 1988). The primary gene transcript, which is capped at its 5' end and polyadenylated at its 3' end, constitutes the substrate for the pre-mRNA splicing. Introns are demarcated by invariant consensus sequences at their 5' boundaries (donor site, with conserved dinucleotide GU at the splice junction) and 3' boundaries (acceptor site, with conserved dinucleotide AG at the splice junction) and these intron sequences are precisely excised by a nuclear multistep process known as pre-mRNA splicing (Sharp, 1988). The pathway of splicing involves the dynamic assembly of a spliceosome complex containing the 5' and 3' splice sites, cleavage

at the donor site, formation of lariate branchpoint, and cleavage at the acceptor site with concomitant ligation of the 5' and 3' exons. This spliceosome complex contains the highly conserved small nuclear RNA (snRNAs) U2, U4, U5 and U6, and the most abundant snRNA, U1, is required to form the spliceosome and may be a part of the spliceosome (Rio, 1992). *In vivo*, this complex is rapidly disassembled and the spliced intron-RNA degraded. The spliced exon-RNAs are released free of snRNAs. The consensus intron sequences of a branch site is typically within 20 to 100 nucleotides of the 3' splice site. Another conserved sequence in the intron includes a 14 nucleotide polypyrimidine stretch immediately upstream of the 3' splice site. The polypyrimidine stretch in the intron near the 3' splice site, an intact 5' splice site, a 3' splice site and a branch point are absolutely required for splicing complex formation (Frendewey and Keller, 1985; Ruskin & Green, 1985).

There are two types of pre-mRNA splicing (Breitbart *et al.* 1987): (1) constitutive splicing, which yields a single gene product from each transcriptional unit with each and every one of the exons present in a gene incorporated into the unique mature mRNA through the invariant ligation of consecutive pairs of donor and acceptor splice sites removing every intron; and (2) alternative splicing, in which non-consecutive exons (or splice sites) are joined in the processing of some but not all of the exons from a gene. This alternative pattern of pre-mRNA splicing can exclude individual exon sequences from the mature mRNA in some transcripts but include them in others.

The use of differential splicing patterns in transcripts from a single gene yields mRNAs with different primary structures. When the exons involved contain translated sequences, these alternatively spliced mRNAs will encode related but distinct protein isoforms. The capacity to generate different but closely related protein isoforms by alternative mRNA splicing increases significantly the phenotypic variability that can be obtained from a single gene. The existence of alternative mRNA splicing raises important questions concerning the function of different protein isoforms and the mechanism of splicing *per se*.

The mechanism involved in the regulation of alternative splicing pathways are presently not well understood. Primary transcript sequences acting in *cis* are likely to be involved; specific *trans*-acting factors are essential for regulating alternative splicing of a single transcript (Breitbart *et al.* 1987). The central problem in pre-mRNA splicing, both constitutive and alternative, is the selection of the correct pairs of donor and acceptor sites to be joined. Exon "compatibility" may play a fundamental role in exon selection in alternatively spliced transcripts.

Alternative mRNA splicing is commonly used by eukaryotic cells to generate different protein isoforms from a single gene to meet different physiological functions, such as occurs during development and healing (Breitbart *et al.* 1987; Ffrench-Constant *et al.* 1989; Magnuson *et al.* 1991; Sandell *et al.* 1991). In tissue repair, the isoforms expressed are often those that are expressed in juvenile tissues. For example, during wound healing in adult rat, an embryonic fibronectin, *i.e.*, the isoform containing the modules EIIIA and EIIIB, reappears in cells present in the base of the wound (Ffrench-Constant *et al.* 1989). Conversely, all three exons that can be alternatively spliced in fibronectin (EIIIA, EIIIB and V) are more frequently spliced out in aged tissue and cells (Magnuson *et al.* 1991). Alternative splicing also occurs in cartilage. For example, type II collagen (COL2) may undergo alternative mRNA splicing (Ryan & Sandell, 1990). The expression of different isoforms of COL2 mRNA and protein have been used to identify different cell types during chondrogenesis: COL2B (without exon 2) is expressed by mature chondrocytes and COL2A (with exon 2) is expressed by chondroprogenitor cells (Sandell *et al.* 1991). The alternatively spliced form of type II collagen, COL2A, is increased in developing osteophytes in experimental OA (Matyas *et al.*, 1997).

As was mentioned above, the G3 domain of aggrecan can undergo alternative splicing. However, prior to this study, it was not known which isoforms and how much of each of the isoform were expressed in normal and experimentally osteoarthritic cartilage. Thus, the focus of this project was on the expression of alternatively spliced isoforms of the G3 domain of aggrecan in canine articular cartilage from normal juveniles, normal adults, and adults in hypertrophic stage of experimental OA. We hypothesized that

different isoforms of the G3 domain of aggrecan may have different effects on the metabolism of chondrocytes in normal and osteoarthritic cartilage. Since the metabolism of chondrocytes is dramatically altered in osteoarthritic cartilage (Malemud & Hering, 1992), it is important to define the alternatively spliced isoforms of the G3 domain qualitatively and quantitatively.

1.1.5 Quantification of messenger RNA by reverse transcription and polymerase chain reaction

There are several different approaches to quantification of a specific messenger RNA. Traditional methods include Northern hybridization and ribonuclease (RNase) protection assay. Northern hybridization is usually not as sensitive as the RNase protection assay for mRNAs with a low level of expression. However, due to the reusability of the RNA samples blotted onto a membrane, Northern hybridization could use less RNA than the RNase protection assay if many different genes were being studied. Reverse transcription and polymerase chain reaction (RT-PCR) is the most sensitive method for detecting specific mRNAs. However, RT-PCR is also sensitive to error. Therefore, care must be taken when using this technique for quantitative work. Quantitative RT-PCR may be classified into three major categories: methods with no standard, methods with an external standard, and methods with an internal standard (Wang *et al.* 1989; Gilliland *et al.* 1990; Yu *et al.* 1991; Wiesner, 1992; Arcellana-Panlilio & Schultz, 1993). There is no ideal method for quantitative RT-PCR and each of these methods has its advantages and disadvantages. Some of these methods related to this project are discussed below.

For the methods with external standards, the advantages are: one can use the authentic gene as a standard, less total RNA is needed and the procedure is much simpler than the one with the internal standard methods. The major disadvantage with external standard methods is that it is difficult to control sample-to-sample variation. For better control of the error caused by sample-to-sample variation in the RT-PCR, internal standard methods are superior to external standard methods. There are several internal standard methods. One can add as an internal standard a cDNA or mRNA with a different size than

the gene being quantified. If one can ensure that the PCR efficiency of the internal standard is similar to that of the gene being quantified (by measures such as controlling the GC ratio and the size of the cDNA as close to the sample gene as possible, yet maintaining an ability to differentiate it after PCR from the mRNA of interest), then, an exogenous gene or modified authentic gene (deletion or addition of a segment of cDNA) with different size than the sample gene can be used as an internal standard. Another internal standard method involves using site-directed mutagenesis of the authentic gene to make an internal standard. The mutant usually has only a few nucleotides changed from the authentic gene but with an added unique restriction site that is different from the sample gene. The efficiency of this kind of internal standard in RT-PCR should be identical to the sample gene being quantified. However, the major disadvantage of the later method is that it is often difficult to achieve 100% digestion with the unique restriction enzyme. This digestion is critical for calculating accurately the amount of sample gene being quantified. In this project, we used both RT-PCR with an external standard (with the authentic cDNA as the standard) (Arcellana-Panlilio & Schultz, 1993) and competitive RT-PCR with an internal standard (with site-directed mutagenesis of the authentic gene) (Ho *et al.* 1989; Gilliland *et al.* 1990) for quantifying the G3 isoforms (see Chapter 3 for details).

1.1.6 Production of recombinant peptides for the G3 domain of aggrecan

There are four major systems commercially available for producing recombinant peptides and proteins. They are bacterial (e.g., *E. coli)*, yeast, insect cell (e.g., baculovirus), and mammalian cell expression systems (Ausubel *et al.*, 1989b; Goeddel *et al.*, 1991). Each of these systems has its advantages and disadvantages. To choose an expression system, one needs to consider the properties of the peptides or proteins to be produced, the purpose of producing the recombinant peptides and proteins, and the previous knowledge and experience of the investigators with the expression system.

Bacterial cell systems based on *E. coli* are among the most extensively studied expression systems. Many fusion protein expression systems that use *E. coli* are available commercially. *E. coli* cells are easily manipulated and grow quickly in inexpensive

medium. The fusion protein expression systems can ensure good initiation of translation and often overcome the instability problem associated with producing small sized recombinant peptides. Recombinant peptides and proteins with fusion proteins from *E. coli* system usually are produced with a high yield and thus, it is a preferred method for generating immunogens. Bacterial fusion protein expression systems also have the great advantage that purification of the recombinant peptides and proteins is often simplified and can be tailored to the fusion protein. Major disadvantages of the *E. coli* expression systems are: (1) it is difficult to form correct disulfide bonds and make other post-translational modifications of the recombinant proteins, such as glycosylation, phosphorylation, *etc*; (2) the recombinant proteins are often produced intracellularly as insoluble inclusion bodies.

Yeast systems offer many important advantages of both the prokaryotic and the eukaryotic systems. Yeast cells can grow rapidly in inexpensive medium and achieve high cell densities easily. The yield of the recombinant peptides and proteins are often as high as those achieved in an *E. coli* expression system. They are able to make the post-translational modifications that bacterial systems are unable to perform. Thus, *E. coli* or yeast expression systems have been an initial choice to produce recombinant peptides and proteins for many genes.

Production of recombinant proteins can be made using an insect cell system such as baculovirus. The insect cells are eukaryotic and can be maintained in suspension culture with high titers. The yield of recombinant proteins are usually high and soluble. These systems have a higher chance of forming correct post-translational modifications for higher eukaryotic proteins than do the yeast expression systems, though this result is not guaranteed. One of the disadvantages of the insect cell system is that the experimental procedures are more complicated and it is also more expensive than the bacterial and the yeast expression systems.

Mammalian expression systems are often used to express genes obtained from higher eukaryotes because the signals for post-translational processing and secretion of these proteins are readily available in the host cells. However, the experimental procedures using a mammalian expression system are more complicated. It usually takes longer time

to produce a recombinant peptide or protein than with the bacterial and yeast expression systems, especially when one tries to establish a stably transfected cell line. Culturing mammalian cells is usually more expensive than culturing bacterial and yeast cells. The yield of the recombinant peptides or proteins from a mammalian expression system is usually much lower than that from the other three expression systems, and thus, it is the most expensive expression system to use.

Because each cDNA or gene may have its own particular problems to overcome in order to accomplish a high-level of expression, none of the expression systems can guarantee success *a priori* in producing a recombinant peptide or protein; and, thus, production of foreign peptides and proteins in an expression system is still largely empirical. The purpose of producing a recombinant peptide or protein may be to verify that the correct cDNA sequence has been isolated, to use it as an antigen for preparing antibody, to study the biological function, or to use it for structural determination. Historically, *E. coli* expression systems have been used with great success for identifying mammalian proteins, often yielding biological active proteins and nearly always, immunoreactive recombinant proteins.

To produce the recombinant peptides for the isoforms of the G3 domain of canine aggrecan for the purpose of generating polyclonal antibodies, we first chose two *E. coli* expression systems (pGEX-3x, from Pharmacia and pMAL-c2, from New England Biolabs). We also tried a yeast expression system (*Pichia* from Invitrogen) and a mammalian expression system (pCEP/d4-7, a modified vector derived from pCEP of Invitrogen) (Mayer *et al.*, 1993) to produce recombinant peptides for the isoforms of the G3 domain of canine aggrecan (See Chapter 4 for details).

1.2 HYPOTHESES

For this project, we hypothesized:

(1) the types of mRNA isoforms of the G3 domain of aggrecan core protein expressed are the same in the canine articular cartilage of both normal and hypertrophic stage of experimentally osteoarthritic dogs; and

(2) the isoform(s) with an E-like module is (are) expressed more abundantly in the OA cartilage than in the normal adult cartilage.

1.3 SPECIFIC AIMS OF THE STUDY

There were four specific aims in this project:

(1) to clone and sequence the cDNAs of canine aggrecan for the G3 domain and part of 3' end of CS-II region to the last putative "aggrecanase" cleavage site,

(2) to identify which of the theoretically possible G3 mRNA isoforms are actually expressed in the articular cartilage of normal young dogs, normal adult dogs, and adult dogs in the hypertrophic stage of experimental osteoarthritis,

(3) to quantify the relative amounts of the alternatively spliced G3 mRNA isoforms comparing the contralateral non-operated control with the hypertrophic stage of experimentally osteoarthritic articular cartilage in adult dogs, and

(4) to initiate the production of recombinant peptides for the G3 isoforms that are expressed in canine articular cartilage, in preparation for future studies of the structure and biological function of the G3 domain and for generating antibodies to the G3 isoforms.

1.4 SUMMARY OF THE PROJECT

1.4.1 Summary of the strategies of the study

To accomplish these objectives, most of the cDNA sequence of the G3 domain of canine aggrecan was obtained by screening a cDNA library derived from normal adult canine articular cartilage; a small cDNA fragment (with the E2-like module) was obtained from direct cloning of an RT-PCR product. Cartilage with experimental OA in the hypertrophic

stage was obtained from canine stifles 10 weeks post-ACL transection in skeletally mature male dogs. Total RNA was extracted from the cartilage of stifles of normal young dogs (10 days old), normal adult dogs, and adult dogs in the hypertrophic stage of experimental osteoarthritis and their contralateral non-operated control. Using the cDNA sequence information of the G3 domain of canine aggrecan, and the published human cDNA and chicken genomic DNA sequence information for aggrecan, a series of oligonucleotides that cross the theoretical exon boundaries were designed and synthesized. These oligonucleotides were used as primers for the investigation of the alternatively spliced mRNA isoforms of the G3 domain of aggrecan in the canine articular cartilage by RT-PCR. cDNAs amplified by PCR were cloned using a TA Cloning™ kit (Invitrogen) and analyzed by DNA sequencing. Verification of the isoforms obtained from RT-PCR was attempted using Northern hybridization, ribonuclease protection assay, and re-screening the cartilage cDNA library. The G3 isoforms that were identified and verified were quantified in the articular cartilage of contralateral non-operated control and the hypertrophic stage of experimentally osteoarthritic adult dogs by quantitative RT-PCR. Production of the recombinant peptides for the canine G3 isoforms was accomplished by using the pMAL-c2 prokaryotic expression system.

1.4.2 Rational and significance of the study

While the function of the G3 domain of aggrecan is unclear, it is known that the G3 domain undergoes alternative mRNA splicing and that fragments of aggrecan containing the G3 domain are present in articular cartilage. Aggrecan is enriched in cartilage from early OA, and activities of the protease such as collagenase, stromelysin and, particularly aggrecanase are increased, so it is expected that there would be an increase in the G3-containing fragments in OA, which, in turn, might show different biological activity because of the different modules present in these G3 domains.

At the beginning of this project, the exact proportion of G3 isoforms expressed in OA was not known. However, in general, the cartilage in the hypertrophic stage of osteoarthritis exhibits active metabolism. Therefore, we felt that it was important to define

which isoforms are expressed and how their quantities changed during development of OA.

The canine ACL transection model of experimental OA was chosen for these studies because it has a well defined natural history so that the stage of OA can be carefully controlled as a function of time after surgery. The biochemistry and the biomechanics of the articular cartilage are also relatively well characterized in this model. Furthermore, we already had a cDNA library of normal canine articular cartilage and the bovine G3 cDNA probe, both of which were needed to pursue successfully the work put forth in this project. Finally, our laboratory had extensive experience with the surgical induction of OA in this animal model and the RNA extraction procedure from articular cartilage. Numerous experimental studies had suggested that cartilage changes in this animal model closely resemble those observed in human OA. Thus, the canine ACL transection model of experimental OA was the model system of choice for the proposed studies.

The powerful amplification ability of PCR was needed to use most efficiently the small amounts of RNA available from cartilage samples for this investigation, and to minimize time, cost, and the number of animals needed. Recombinant DNA techniques are necessary to produce sufficient peptides of G3 domain of aggrecan for *in vitro* studies of the structure and function of the G3 domain; as they would be difficult to obtain from *in vivo* sources in sufficient quantities for such studies.

From this project, we expected to define the expression of the alternatively spliced G3 isoforms in articular cartilage of adult dogs in the hypertrophic stage of experimental osteoarthritis qualitatively and quantitatively. It is very important to understand whether the expression of the G3 isoforms are changed in the early stage of OA because the changes of the expression of G3 isoforms may provide some clues as to whether the G3 isoforms undergo metabolic changes during osteoarthritis, and whether the different isoforms of the G3 domain have different biological functions in normal and osteoarthritic cartilage. We have initiated the production of the recombinant peptides for the G3 isoforms

in this study, which should be very useful in facilitating future studies of the potential biological functions of the G3 domain.

CHAPTER 2.

2. ALTERNATIVELY SPLICED ISOFORMS OF THE G3 DOMAIN OF CANINE AGGRECAN

2.1 INTRODUCTION

Aggrecan, the largest and most abundant proteoglycan in cartilage, contains three globular domains, a short interglobular domain (IGD) and a large glycosaminoglycan attachment domain. As this later domain is heavily glycosylated dominantly in specific regions either with keratan sulfate (KS) or chondroitin sulfate (CS) side chains to varying degree locally, it is further divided into KS-rich region and CS-rich regions I and II (Heinegård & Oldberg, 1989; Upholt *et al.* 1993) (see Chapter 1 for more details). The third globular domain (G3) located in the C-terminal end of aggrecan is composed of up to three different modules with homologies to other bioactive proteins: one or two epidermal growth factor-like modules (E1 or E2), a lectin-like module (L), and a complement regulatory protein-like module (C) (Patthy, 1987; Drickamer, 1988; Baldwin *et al.* 1989; Fülöp *et al.* 1993), as well as a C-terminal peptide (Doege *et al.* 1991). Three alternatively spliced mRNA isoforms of the G3 domain of aggrecan containing L, LC, ELC had been reported in human cartilage (Baldwin *et al.* 1989; Doege *et al.* 1991). In addition, human aggrecan has two different E-like modules, E1 and E2, which resemble those of versican (Zimmermann & Ruoslahti, 1989), and which may be expressed together, independently, or not at all (Fülöp *et al.* 1993). The E1-like module of aggrecan was not expressed at a significant level in the non-human species examined due to a truncated polypyrimidine track upstream from the exon (Fülöp *et al.* 1996).

Alternative mRNA splicing is a common mechanism of gene regulation in eukaryotic cells used to generate different protein isoforms with different physiological functions from a single gene (Breitbart *et al.* 1987; Ffrench-Constant *et al.* 1989; Magnuson *et al.* 1991; Sandell *et al.* 1991). The exact function of the C-like module in

aggrecan is unknown. The L-like module of the G3 domain binds to fucose and galactose (Halberg *et al.* 1988; Saleque *et al.* 1993). Single or multiple repeats of E-like modules may represent putative calcium-binding motives (Yamamoto *et al.* 1986; Mayhew *et al.* 1992; Selander-Sunnerhagen *et al.* 1992; Clarke *et al.* 1993; Hewett *et al.* 1993; Rezaie *et al.* 1993). It has not been reported, however, which or how many isoforms of the G3 domain are expressed in normal and osteoarthritic canine articular cartilages.

Four specific cleavage sites in aggrecan, preserved in all species examined to date, have been identified by N-terminal sequencing and a putative proteinase named "aggrecanase" is postulated to cleave at these sites (Ilic *et al.* 1992; Loulakis *et al.* 1992). A major "aggrecanase" cleavage site of aggrecan is located in the IGD and the catabolism of aggrecan at this site was increased in interleukin-1 treated cartilage explant (Lark *et al.* 1995; Sandy *et al.* 1991b). It has been proposed that the G3 domain can be released from the aggrecan core protein by the putative "aggrecanase" as one of the initial steps of degradation of aggrecan (Ilic *et al.* 1992).

Although chondrocyte metabolism and the overall composition of aggrecan is dramatically altered in osteoarthritic cartilage (Malemud & Hering, 1992), we hypothesized that the type of the isoforms of the G3 domain of aggrecan expressed may be the same in the osteoarthritic cartilage. Because anterior cruciate ligament (ACL) transection in dog is one of the most relevant animal models of human osteoarthritis, we used this animal model to simulate *in vivo* events which may occur during the development of the disease.

The aims of this study were (1) to clone and sequence the cDNA of the complete G3 domain and C-terminal end of the CS-II region (including the last putative "aggrecanase" cleavage site at the C-terminal end) of canine aggrecan and (2) to identify the alternatively spliced mRNA isoforms of the G3 domain of canine aggrecan.

2.2 MATERIALS AND METHODS

2.2.1 Reagents and solutions

A cDNA clone of the bovine G3 domain of aggrecan (PG-LA5) (Oldberg *et al.* 1987) was generously provided by Professor Dick Heinegård, University of Lund, Sweden. Radioactive isotopes were from Amersham Life Science (Oakville, ON) and Du Pont, NEN® (Mississauga, ON). Most of the chemicals used in the studies were Molecular Biology grade and were from Sigma (St. Louis, MO) or GIBCO/BRL (Burlington, ON) unless otherwise stated. Cesium trifluoroacetate (CsTFA) and RNase free DNase-I were from Pharmacia (Baie D'Urfé, PQ). XL1-Blue stock cells, R408 helper phage, Duralon-UV™ nylon membranes and the *in vitro* transcription kit were from Stratagene (La Jolla, CA). Random Primer DNA Labelling System, terminal deoxynucleotide transferase (TdT) and an RNA ladder (0.24 to 9.5 kb) were from GIBCO/BRL (Burlington, ON). The Taq DNA polymerase was from Perkin Elmer Cetus (Mississauga, ON). Oligo (dT) cellulose, cDNA Cycle™ kit and TA Cloning™ kit were from Invitrogen (San Diego, CA). Geneclean® II kit was from Bio/Can (Mississauga, ON). X-ray films (X-OMAT, Kodak) were from Sigma (St. Louis, MO). Sequenase® version 2.0 was from United States Biochemical (Cleveland, OH). Taq DyeDeoxy™ Terminator Cycle Sequencing kit was from Applied Biosystems Canada Inc. (Mississauga, ON). Biotrans™ nylon membranes were from ICN Biochemicals Inc. (Costa, Mesa, CA). Nitrocellulose membranes (S&S) were from Schleicher & Schuell (Keene, NH). Promega's P2020 *In Vitro* Transcription kit and a low molecular weight RNA ladder (0.155 to 1.77 kb) were from Promega (Madison, WI). RNase A and RNase T1 were from Boehringer Mannheim Canada (Laval, PQ).

Preparation of the following solutions or reagents was based on "Molecular Cloning: A Laboratory Manual" (Sambrook *et al.* 1989): stock ampicillin (50 mg/ml), LB broth, LB-ampicillin agar plate, 50×TAE buffer, 20×SSC, 20×PIPES, 20×SSPE, 50×Denhardt's solution, TE buffer, 5×TBE buffer, 20×PE buffer, 6×DNA loading buffer, 5×formaldehyde RNA running buffer (FRR), RNA loading buffer (see Appendix 7.1 of the thesis for details). NZY agar plate, SM buffer and YT medium were prepared according to

the manufacturer's manual for screening a cDNA library with Duralon-UV™ nylon membranes (Stratagene, La Jolla, CA) (see Appendix 7.1 of the thesis for details). Stock PE buffer (5×) was prepared as described in the manual of the *in vitro* transcription kit (Stratagene, La Jolla, CA) (see Appendix 7.1 of the thesis for details).

2.2.2 Extraction of total RNA

Normal cartilages were from stifles of puppies (10 days old) and skeletally mature male dogs (Husky x Shepherd). Experimentally osteoarthritic cartilages were from skeletally mature male dogs (Husky x Shepherd) 10 weeks post-ACL transection of the stifles (Adams & Pelletier, 1988). Fresh cartilage samples from these sources were harvested and immediately immersed in liquid nitrogen. They were transferred to a -70°C freezer for longer storage.

Cartilage chips were weighed and separated into fractions of 1 g or less for each pulverization (1 g per min at maximum setting) in a Spex™ freezer Mill (Spex Industries, Inc, Edison, NJ, USA) in liquid nitrogen. The cartilage powder from the same joint was mixed well and a sample (approximately 1% of the cartilage powder) was used for measurement of total DNA content. The remainder of the cartilage powder was used to extract and purify total RNA using a previously described method (Adams *et al.* 1992; Matyas *et al.* 1995). One gram of the cartilage powder was mixed with 10 ml of 4 M GIT extraction solution containing 4 M guanidium isothiocyanate (GIT), 25 mM sodium citrate, pH 7.0, 0.5% sodium dodecyl sarcosine and with a fresh addition of 0.1% (v/v, final concentration) of 2-mercaptoethanol and then gently mixed at room temperature for 4 hours. Thirty ml of 77% water-saturated phenol / 8% (v/v) sodium acetate, pH 4 / 15% chloroform:isoamyl alcohol (49:1) was added to the sample and mixed by vigorously shaking for 10 seconds. It was chilled on ice for 15 min and then centrifuged at 10,000*g* for 20 min at 4°C.

The aqueous phase was saved and extracted sequentially once with each of the following three reagents:

(1) with 1/2 volume of water-saturated phenol and 1/2 volume of chloroform: isoamyl alcohol (24:1);

(2) with 1/2 volume of buffer-saturated phenol and 1/2 volume of chloroform: isoamyl alcohol (24:1);

(3) with 1 volume of chloroform:isoamyl alcohol (24:1);

After each extraction, the sample was centrifuged as above and the aqueous phase was saved. DNA was sheared by passing the sample 10 times through an 18 gauge needle with a syringe. The volume of the sample was adjusted to 18 ml with 4 M GIT extraction solution. It was loaded on the top of a 19 ml CsTFA cushion in a polyallomer ultracentrifugation tube for a SW 28 rotor (Beckman, Mississauga, ON) and centrifuged at 25,000 rpm at 15°C for 24 hours.

After centrifugation, the supernatant was carefully removed from the tube and the tube was inverted on a piece of paper towel at room temperature for 5 min. The pellet, which was barely visible, was dissolved in 0.5 ml 4 M GIT extraction solution by repeated pipetting and RNA was re-precipitated from it by adding 0.5 ml of 100% isopropanol and then incubating it at -20°C for 30 min. The sample was centrifuged at 14,000 rpm in an Eppendorff™ microfuge at 4°C for 15 min. The pellet was washed with 0.5 ml 75% cold ethanol and incubated at room temperature for 15 min to dissolve any residual GIT. It was again centrifuged for 10 min in the microfuge as above. The pellet was air dried at room temperature for 10 min and then redissolved in 50 µl of autoclaved distilled deionized water (ddH_2O). A small sample was used for measurement of OD_{260nm} and OD_{280nm} in a spectrophotometer (Ultrospec 3000 UV/Visible Spectrophotometer, Pharmacia Biotech in Canada, Baie D'Urfé, PQ). The remaining samples were stored at -70°C until use.

2.2.3 Preparation of the cDNA library

Messenger RNA (mRNA) was isolated by oligo (dT) affinity chromatography of pooled total RNA that had been extracted from cartilages of normal adult canine stifles (Adams *et*

al. 1992). A customised cDNA library was constructed using this pooled mRNA by Clontech Laboratories Inc. (Palo Alto, CA). The cDNAs were cloned at the *EcoR*I sites in Lambda Zap® II. The sizes of the cDNAs varied from 0.6 to 4.0 kb (average 1.5 kb). The mRNA of canine aggrecan is about 8000 nucleotide (nt) long when it was detected by Northern hybridization with the PG-LA5 probe (Adams *et al.* 1992). Although this cDNA library may contain little or may not have any intact cDNA of canine aggrecan, it should not affect this project because our main focus was on the C-terminal end of canine aggrecan. The size of the interested cDNA of the C-terminal end of canine aggrecan for the studies is about ≤2 kb long.

2.2.4 Screening the cDNA library with the bovine G3 domain cDNA probe to obtain canine specific G3 clones

A single colony of the bacterial host cell (XL1-Blue) was inoculated in 50 ml LB broth supplemented with 0.2% maltose and 10 mM $MgSO_4$. The cells were cultured overnight at 30°C with shaking at 250 rpm. The cells were pelleted by centrifugation at 2000 rpm (IEC Centra-7R refrigerated centrifuge, International Equipment Company, a Division of DAMON, Needham Hts, MA) and resuspended in 10 mM $MgSO_4$ to an optical density of 0.5 at 600 nm (OD_{600}). To prepare 10 plates each with 50,000 plaque forming units of bacteriophage (pfu) from the amplified cDNA library, 10 aliquots of 50,000 pfu were suspended in tubes each containing 0.6 ml of the host cells at $OD_{600} = 0.5$. The tubes were incubated at 37°C for 15 min. Each sample was mixed with 7.5 ml of melted top agarose (48-49°C) and spread evenly onto a 2-day old NZY agar plate (150 mm diameter). The plates were incubated at 37°C for about 8 hours (until pinhead-sized plaques were visible). The plates were chilled at 4°C for 2 hours. The plaques were transferred to Duralon-UV™ nylon membranes for 2 min at room temperature. The membranes with the transferred plaques were submerged in denaturing solution (0.5 M NaOH / 1.5 M NaCl) for 2 min, then transferred into neutralisation solution (1.5 M NaCl / 0.5 M Tris·HCl, pH 8.0) for 5 min, and finally rinsed with a solution containing 0.2 M Tris·HCl, pH 7.5 / 2×SSC for 30 seconds. The excess buffer was blotted off the membranes using a piece of Whatman 3

MM filter. The DNA in the plaques was fixed onto the damp membranes using the "autocrosslink" function (1200 × 100 μ Joules) in the UV Stratalinker™ 1800 (Stratagene, La Jolla, CA). The plates were stored at 4°C.

A cDNA probe (PG-LA5) was labelled with α-[^{32}P]-dATP (specific activity = 3000 Ci/mmole) using the Random Primers DNA Labelling System. It was purified by gel-filtration chromatography using a 1-ml Sephadex™ G-50 spun column (Sambrook *et al.* 1989). The membranes prepared as above were incubated with prehybridization solution containing 2xPIPES / 50% deionized formamide / 1.0 % SDS / freshly denatured salmon sperm DNA (ssDNA) (100 μg/ml) at 50°C for 4 hours. The PG-LA5 cDNA probe, which was freshly labelled and denatured (by boiling for 3 min and chilled on ice for 5 min), was added into the hybridization bag at a final concentration of $1x10^5$ cpm/ml. Incubation was continued at 40°C overnight. After hybridization, the membranes were washed once with 1×SSC / 0.1 % SDS for 10 min at room temperature, then with three washes of 0.1×SSC / 0.1 % SDS for 15 min at 40°C. The membranes were exposed to X-ray films (X-OMAT, Kodak). Positive plaques were collected and each was dissolved in 0.5 ml of SM buffer and 20 μl of chloroform. They were re-screened with the same probe at reduced pfu density until single positive plaques were obtained.

For *in vitro* excision, 200 μl of the positive phage particles suspended in SM buffer was incubated with 200 μl of XL1-Blue cells at OD_{600} = 1.0 and 1 μl of R408 helper phage (final concentration = 1 x 10^6 pfu/ml) at 37°C for 15 min. To each sample, 5 ml of 2×YT medium was added to the tube which was further incubated at 37°C for 3 hours. The samples were heated at 70°C for 20 min and then centrifuged at 4000*g* for 5 min. Ten to 20 μl of the supernatant which contained the pBluescript phagemid packaged as filamentous phage particles was incubated with 200 μl of OD_{600}=1.0 XL1-Blue cells at 37°C for 15 min. One to 100 μl of the sample could be plated on LB-ampicillin (50 μg/ml) agar plates and incubated at 37°C overnight. Some clones were used for preparing plasmid DNA which were analyzed by digestion with restriction enzyme (*EcoR*I) and DNA sequencing (see section 2.2.8).

2.2.5 Oligonucleotides

Oligonucleotides for PCR primers or probes were designed based on the cDNA sequence of canine aggrecan. The exon boundaries for the L-like and C-like modules were defined from chicken genomic DNA sequence of the G3 domain of aggrecan (Tanaka *et al.* 1988) and cDNA sequences of human aggrecan (Doege *et al.* 1991; Baldwin *et al.* 1989). The exon boundary of CS-II region and E2-like module was defined according to the cDNA sequence of canine G3 domain obtained in this study and from Fülöp *et. al.* (1993).

For defining the alternatively spliced isoforms of the G3 domain of canine aggrecan, various oligonucleotides as primers were designed using Primer Designer™ (Scientific & Educational Software, State Line, PA) and synthesized (The Calgary Regional DNA Synthesis Laboratory, University of Calgary, AB). Their sequences and schematic locations on aggrecan are listed in Table 2.1 and Figure 2.1. The theoretical RT-PCR products of various combinations of the primer pairs are listed in Table 2.2. To screen further the cDNA library by PCR, three oligonucleotides (CSIIR, poly1F and poly2F) were synthesized. The CSIIR (complementary strand) was located in the 3' end of the CS-II region. The other two primers were located in the polylinker region of the pBluescript plasmid vector at the 5' (for poly1F) and 3' (for poly2F) ends of *EcoR*I sites where the cDNA library was inserted (Table 2.1). In an attempt to verify a putative "C-alone isoform" identified by reverse transcription and polymerase chain reaction (RT-PCR), an oligonucleotide called RCSIIC (complementary to another oligonucleotide called CSIIC) which is located in the putative exon boundary of CS-II and "C-like module alone" (Table 2.1) was synthesized. RCSIIC was used as an oligoprobe (3' end labeling) in Northern hybridization and rescreening the cDNA library (see below).

Table 2.1 DNA sequences of synthetic oligonucleotides.

Name	DNA Sequence		Tm [f]	Location[g]
FCSII5	5'-dCCAGAATCTAGCAGCGAGACA-3'	(S[d])	71	202-222
L1	5'-dGAGTCAGCACCAGCCATTC-3'	(S)	69	733-751
L2[a]	5'-dGAGGTGTCCGCACCAGCCATT-3'	(S)	73	730-750
L3	5'-dAGTGCCTCTCAAGTCCTTGT-3'	(S)	66	764-783
CSIIE	5'-dGCCATTCCAG| [c]ACATTGACG-3'	(S)	69	745-754/755-763
CSIIL	5'-dACCAGCCATTCCAG| ACCAGG-3'	(S)	75	741-754/869-874
CSIIC	5'-dCATTCCAG| TGGCCTGC-3'	(S)	65	747-754/1256-1265
R1	5'-dCTGCGCTTGTAAGTGGAGG| -3'	(A[c])	70	1439-1457
R2	5'-dCCTTCGATGGTCCTGTCATTC-3'	(A)	72	1059-1079
R2.1	5'-dCTCACACAGCTCCTGGT| CGAT-3'	(A)	73	865-868/869-885
R3	5'-dTCGTAGCGGTCCTTCTTCTG-3'	(A)	71	1303-1322
LT	5'-dTTGTAAGTGGAGG| CTGTGC-3'	(A)	67	1250-1255/1439-1451
ET	5'-dAAGTGGAGG| CGATCTCACA-3'	(A)	69	856-868/1439-1477
CT	5'-dGTAAGTGGAGG| GGTCTGTGC-3'	(A)	70	1430-1438/1439-1449
RCSIIC	5'-dGCAGGCCA| CTGGAATG-3'	(A)	65	747-754/1256-1263
CSIIR	5'-dCTGGTGCTGACTCATCTGT-3'	(A)	63	727-745
poly1F[b]	5'-dACCATGATTACGCCAAGCTC-3'		71	
poly2F[b]	5'-dATAAGCTTGATATCGAATTCC-3'		64	

(a) Three synthetic errors (underlined) were found in the L2 primer. However, this primer was able to prime the canine aggrecan mRNA at the correct location and so was still used in the experiment;

(b) The primers poly1F and poly2F are in the polylinker region of pBluescript plasmid and are not shown in Figure 2.1. Except for these two oligonucleotides, all those listed in this table are specific to canine aggrecan;

(c) The vertical lines in the DNA sequences indicate the putative exon boundaries;

(d) S = sense strand;

(e) A = antisense strand;

(f) Tm = melting temperature (°C) of the oligonucleotides;

(g) The locations of the oligonucleotides specific to canine aggrecan refer to Figure 2.3 in the section 2.3.1. The slashes (/) separate the putative exon boundaries.

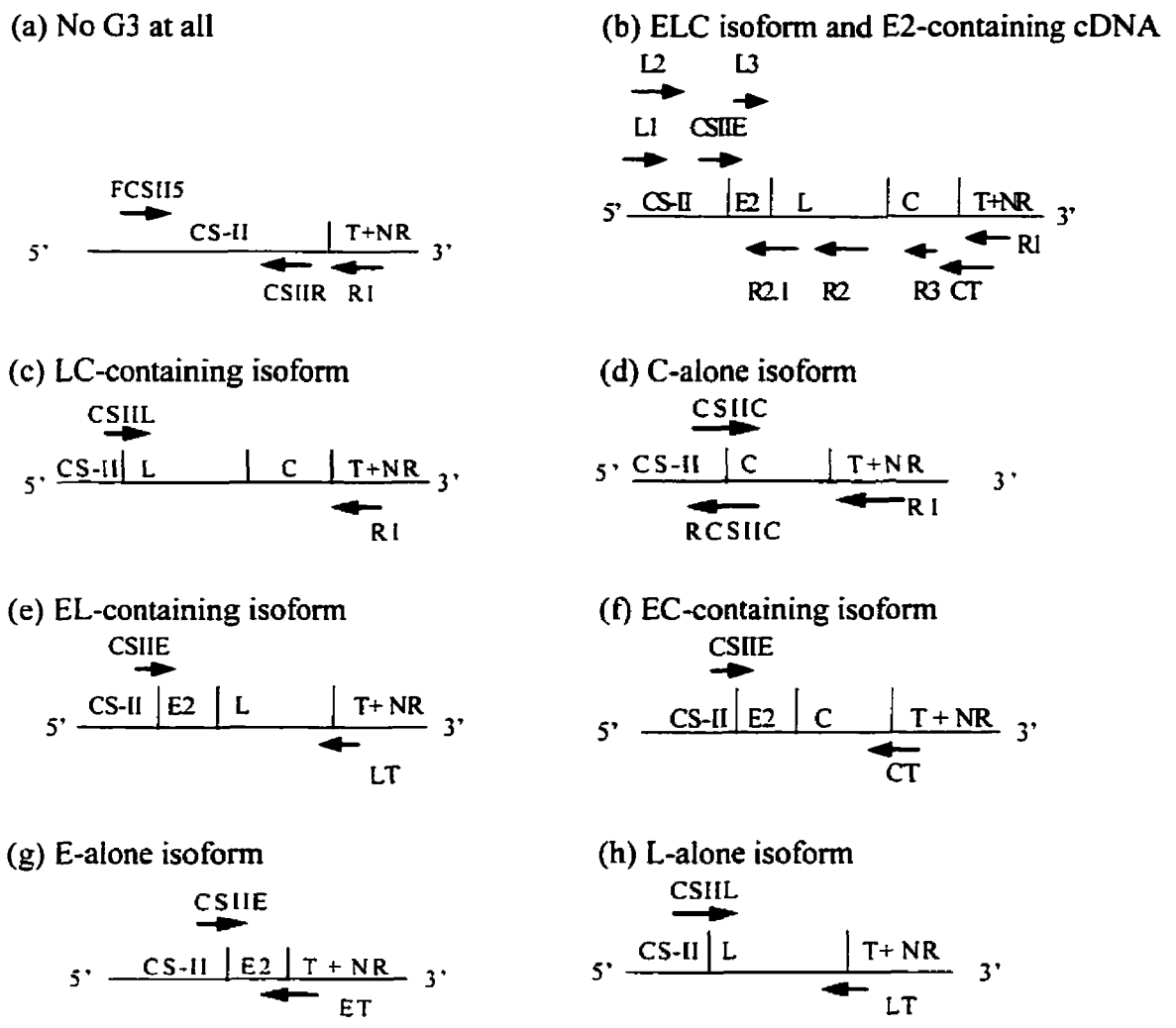

Figure 2.1 Schematic diagrams of the possible spliced isoforms and the locations of the synthetic oligonucleotides in the cDNA of the C-terminus of aggrecan.

Vertical lines indicate the putative exon boundaries of protein domains and modules. Arrows indicate the 3' ends of primers (Table 2.1). T=C-terminus coded by the last exon of aggrecan; NR=3' non-translated region.

Table 2.2 The predicted RT-PCR products with no G3 domain and with complete or partial G3 isoforms using various combinations of primer pairs.

RT-PCR Primer pairs	cDNAs with no G3 or with complete G3 isoforms (bp)								Others (bp) Partial isoforms
	No G3	ELC	EL	EC	E	LC	L	C	
FCSII/R1	572	1257	1074	869	686	1143	960	755	—
L1/R1	41	725	543	338	155	611	429	224	—
L2/R2	—	—	—	—	—	—	—	—	350(EL'),236 (L')
L2/R2.1	—	—	—	—	—	—	—	—	152 (EL')
L3/R3	—	—	—	—	—	—	—	—	559 (E'LC')
CSIIE/R1	—	714	531	326	143	—	—	—	—
CSIIE/LT	—	—	525	—	—	—	—	—	—
CSIIE/CT	—	704	—	318	—	—	—	—	—
CSIIE/ET	—	—	—	—	133	—	—	—	—
CSIIL/R1	—	—	—	—	—	603	421	—	—
CSIIL/CT	—	—	—	—	—	595	—	—	—
CSIIL/LT	—	—	—	—	—	—	415	—	—
CSIIC/R1	—	—	—	—	—	—	—	210	—
CSIIC/CT	—	—	—	—	—	—	—	202	—

(1) The "—" sign indicates that there is no PCR product for the isoform.

(2) The E', L' and C' indicate the partial sequence of each module.

(3) The sizes of the complete cDNAs for the E2-, L-, C-like modules and the C-terminus are 114, 388, 183, and 75 bp, respectively. The size of the cDNA for the CSII region is >1000 bp.

(4) The different sizes for the PCR products that contain the same G3 isoform are due to the different locations of the primer pairs used (see Table 2.1 and Figure 2.1 for details).

(5) The sizes of all the predicted PCR products in this table included a portion of the CSII region (except for the primer L3) and a portion of the C-terminus (except for the primers R2, R2.1 and R3).

(6) The PCR product of the primer pairs of FCSII/R1 contained 553 bp of partial CSII cDNA and 19 bp of C-terminus cDNA.

2.2.6 Cloning of the E2-like module by RT-PCR

Reagents and the protocol for RT-PCR were from the cDNA Cycle™ Kit, except otherwise specified. For reverse transcription (RT), 5 μg of total RNA from articular cartilage of normal puppies was mixed with a specific primer R1 (10 pmole) and ddH_2O in a total volume of 12.5 μl. The sample was incubated at 65°C for 10 min and then at room temperature for 2 min. After briefly centrifuging the sample in an Eppendorff™ microfuge, the following reagents were added sequentially:

RNase Inhibitor (10 units/μl)	1 μl
5×RT buffer	4 μl
dNTPs (25 mM each)	1 μl
80 mM sodium pyrophosphate	1 μl
AMV reverse transcriptase (10 units/μl)	0.5 μl

The reaction was performed at 42°C for 1 hour. It was terminated by heating to 95°C for 2 min followed by chilling on ice for 2 min. After briefly centrifuging as above, 0.5 μl of the AMV (avian myoblastosis virus) reverse transcriptase was added to the tube and the reaction was repeated once as above. The sample was again denatured by boiling at 95°C for 3 min and then on ice for 5 min.

The PCR reaction contained:

The RT sample	2 μl
10×PCR buffer	5 μl
dNTPs (25 mM each)	1 μl
L2 (10 pmole/μl)	6 μl
R2.1 (10 pmole/μl)	6 μl
Taq DNA polymerase (5 unit/μl)	0.8 μl
ddH_2O	29.2 μl

The primer L2 was located at the 3' end of the CS-II and the primer R2.1 was located at the exon boundary of E2-like module and L-like module of canine aggrecan (see panel b of Figure 2.1). PCR conditions were 94°C for 3 min, followed 30 cycles of 94°C

for 1 min, 55°C for 2 min and 72°C for 3 min in a thermocycler (Perkin Elmer Cetus, model 9600, Mississauga, ON). The PCR product and a 1 kb DNA ladder were fractionated in a 2% low melting point (LMP) agarose gel containing 0.36 μg/ml ethidium bromide (EthBr). The cDNA band corresponding to the E2-like module and small parts of the CSII and L in size (152 bp) was excised from the gel. The cDNA was extracted from the LMP agarose gel as follows:

The volume of the LMP agarose gel that contained the cDNA was estimated after brief centrifugation in an Eppendorff™ microfuge, and 1/10 sample volume of 3 M sodium acetate, pH 5.2 was added to the sample. The gel was melted at 68°C for 10 min. One volume of Tris-buffer-saturated phenol was added to it and the sample was further incubated at 68°C for 5 min. It was chilled on ice for 10 min and centrifuged in an Eppendorff™ microfuge at 12,000*g* for 15 min at room temperature. The aqueous phase was saved and the cDNA in the sample was precipitated with 1 ml of cold 100% ethanol. The pellet was washed with 1 ml of cold 70% ethanol and then dried in a SpeedVac (Model SVC100, Savant Instruments, Inc. Farmingdale, NY). The gel-purified cDNA was cloned into the pCR™II vector in the TA Cloning™ kit as described in the manufacturer's manual.

2.2.7 Cloning of the 3' end of the CS-II region by PCR screening the cDNA library

Two pairs of primers (poly1F/CSIIR; poly2F/CSIIR, Table 2.1) were used to amplify the canine cartilage cDNA library by PCR to obtain a cDNA clone with a partial 3' end of the CS-II region up to a putative "aggrecanase" cleavage site. Briefly, 5 to 10 μl of the canine cartilage cDNA library was mixed with primers and PCR reagents and PCR was performed as described in section 2.2.6. The PCR products and a 1 kb DNA ladder were fractionated in a 1% agarose gel containing 0.36 μg/ml EthBr in 1×TAE buffer. Because only one primer (CSIIR) that was specific to the canine aggrecan was used in each PCR reaction (the other primers were located in the polylinker region of the pBluescript), the size of the

specific PCR products could not be estimated. Therefore, one of the strongest cDNA bands (about 800 bp) visualized under UV light was randomly chosen for cloning and was sliced out of the gel. The cDNA in the gel slice was purified using the Geneclean® II kit. The gel-purified PCR cDNA sample was cloned into the pCR™II vector as above.

2.2.8 Sequencing the G3 domain of canine aggrecan and partial CS-II region

The cDNA clones P1 and P9 were obtained by conventional library screening; 21E, 22E, 23E, 24E and 25E (containing the E2-like module) were obtained by RT-PCR of canine total RNA; and CSII5 (the partial CS-II region) was obtained by PCR screening the library. Plasmid DNAs for all these clones were purified by a standard miniprep protocol (Sambrook *et al.* 1989). To prepare the plasmid DNA for sequencing, plasmid DNA in TE buffer containing DNase-free RNase A (from the miniprep step) was incubated at 37°C for 1 hour. It was extracted once with 1 sample volume of Tris-buffer-saturated phenol. The concentration of the plasmid DNA was measured using a spectrophotometer at 260_{nm} (OD_{260}). Five μg of the plasmid DNA was incubated with 0.4 M of NaOH (final concentration) at 65°C for 10 min and then chilled on ice for 10 min. The DNA was precipitated with 0.3 M sodium acetate (final concentration), pH 5.2 and 3 volumes of cold 100% ethanol at -70°C for 30 min. The sample was centrifuged in an Eppendorff™ microfuge at 12,000***g***, 4°C for 30 min. The pellet was washed with 1 ml of cold 70% ethanol once and it was then recovered by centrifugation at 12,000***g***, 4°C for 5 min. The plasmid DNA pellet was air dried and then dissolved in 7 μl of autoclaved ddH_2O.

Except clone CSII5, which contained part of the CS-II region and was sequenced by automatic DNA sequencing using Taq DyeDeoxy™ Terminator Cycle Sequencing kit, the other clones (P1, P9, 21E, 22E, 23E, 24E, and 25E) were sequenced by a manual dideoxy-termination method (Sanger *et al.* 1977). The sequencing reaction (labelled with α-[^{35}S]-dATP, specific activity = 1000-1500 Ci/mmole) followed the manufacturer's protocol from the Sequenase® version 2.0. The sequences were resolved by gel

electrophoresis (6% polyacrylamide gel containing 8 M urea in 0.5×TBE buffer, 0.4 mm thickness) at 65 Watts for 1 to 4 hours. The gel was fixed in a solution containing 5% methanol / 5% acetic acid for 15 min and then lifted onto a piece of 3MM Whatman paper. The gel was dried in a gel dryer (Model 483, Bio-Rad) and then exposed to an X-ray film (X-OMAT, Kodak) at room temperature overnight.

2.2.9 RT-PCR for defining alternatively spliced mRNA isoforms in the G3 domain of canine aggrecan

To define the alternatively spliced isoforms of the G3 domain of canine aggrecan, 1 to 5 μg of total RNA were reverse transcribed into cDNA using oligo (dT) as primer, as described in the protocol of the cDNA Cycle™ kit. After phenol:chloroform (1:1) extraction, the reverse transcribed sample was re-dissolved in 20 μl of autoclaved ddH_2O. The PCR reactions with various combinations of primer pairs (Figure 2.1 and Table 2.2) were performed as described in section 2.2.6. The PCR products were fractionated by electrophoresis in 1% regular agarose gels or 2 % LMP agarose gels. The fractionated cDNA was subjected to Southern blotting, or purification from the gel for cloning into the pCR™II vector in the TA Cloning™ kit (see below).

2.2.10 Cloning and sequencing of the mRNA isoforms of the G3 domain by RT-PCR

The RT-PCR products obtained (see section 2.2.9) were fractionated by electrophoresis in agarose gels. The expected cDNA bands that were amplified by RT-PCR and with sizes greater than 300 bp were purified from 1% agarose gels using Geneclean® II kit. The expected cDNA bands that were amplified by RT-PCR and with sizes less than 300 bp were purified from 2% LMP agarose gels by heating the gel at 68°C plus phenol extraction as described in Section 2.2.6.

The gel-purified cDNA fragments of the RT-PCR products were cloned into the pCR™II vector, as described in the TA Cloning™ kit. Plasmid DNAs containing the clones

of the RT-PCR products for the G3 domain of canine aggrecan were purified by miniprep of the plasmid DNA and analyzed by restriction enzyme digestion. Positive clones were prepared and sent to the Calgary Regional DNA Synthesis Laboratory for automatic DNA sequencing using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit. cDNAs for the ELC (primer pairs CSIIE/CT), LC (primer pairs L1/R1 and CSIIL/R1), and C (primer pairs CSIIC/R1) were correct in sequences. The partial G3 sequences (EL' and L') seen in RT-PCR using primer pairs L2/R2.1 were not cloned. The rest cDNA bands were non-specific sequences due to mispriming.

2.2.11 Identification of the G3 isoforms of canine aggrecan in the RT-PCR products by Southern hybridization

After agarose gel electrophoresis of the RT-PCR product (reaction done with primer pairs L1/R1), the gel was denatured in 0.5 M NaOH / 1.5 M NaCl for 30 min, neutralised in 0.5 M Tris·HCl, pH 8.0 / 1.5 M NaCl for 30 min and briefly rinsed with 20×SSC. The gel was equilibrated with 20×SSC for 20 min and the PCR products were transferred by capillary blotting onto a Biotrans™ membrane in 20×SSC overnight. After Southern blotting, the samples were fixed onto the membrane by the UV Stratalinker™ 1800 as described above.

To prepare radioactive antisense riboprobe, clones of the P1 in pBluescript, 21E in pCR™II and C in pCR™II were linearized by digestion with the restriction enzyme *BamH*I. They were then digested with proteinase K and purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction (Sambrook *et al.* 1989). The P1 clone contains the LC sequence of canine aggrecan (Figure 2.2). The 21E clone contains the E2-like module of canine aggrecan and was obtained from direct cloning of the RT-PCR product using L2/R2.1 primers. The C clone contained the "C-like module alone" cDNA which was obtained from cloning of RT-PCR product using primer pairs CSIIC/R1. Using T7 RNA polymerase, the linearized P1/pBluescript, 21E/pCR™II or C/pCR™II were labelled with α-[^{32}P]-UTP (specific activity = 800 Ci/mmole) by *in vitro* transcription, as described in the Stratagene kit. Each radioactive riboprobe was separated from the free isotope by passage through a 1-ml Sephadex G-50 spun column (Sambrook *et al.* 1989). The Southern

blot was incubated with prehybridization solution containing 50% deionized formamide / 5×SSC / 1×PE / 150 μg/ml freshly denatured ssDNA at 48°C (for the P1 riboprobe), or at 40°C (for the 21E) for 4 hours. For the C riboprobe, the Southern blot was incubated with prehybridization solution containing 25% deionized formamide / 5×SSC / 1×PE / 150 μg/ml freshly denatured salmon sperm DNA at 40°C for 3 hours. Each radioactive riboprobe was added into the corresponding prehybridization bag (about 1×10^6 to 1.5×10^6 cpm/ml) and incubation continued as above overnight.

The post-hybridization conditions for the P1 probe were washing the membrane in 2×SSC / 0.1 % SDS at 70°C for 15 min twice and then in 0.1×SSC / 0.1 % SDS at 65°C for 10 min once. The membrane was exposed to an X-ray film (X-OMAT, Kodak) for 2 hours at -70°C.

The post-hybridization conditions for the 21E probe were washing the membrane with 2×SSC / 1% SDS at 40°C for 15 min twice, then in 0.1×SSC / 0.1 % SDS at 40°C for 1 hour three times, at 60°C for 30 min once, and finally at 70°C for 10 min once. The membrane was exposed to an X-ray film (X-OMAT, Kodak) overnight at -70°C.

The post-hybridization conditions for the C riboprobe were washing the membrane with 2×SSC / 0.1% SDS at 40°C for 15 min twice, then in 0.1×SSC / 0.1 % SDS at 40°C for 30 min twice, and finally in 0.1×SSC / 0.1 % SDS at 60°C for 1 hour. It was exposed to an X-ray film (X-OMAT, Kodak) for 4 hours at -70°C. Because the background of the blot was still too high, the blot was further washed with a higher stringency solution: in 0.1×SSC / 0.1 % SDS at 70°C for 30 min. The membrane was exposed to an X-ray film (X-OMAT, Kodak) at -70°C for 24 hours.

2.2.12 Northern hybridization

Each aliquot of total RNA (5 to 10 μg) from articular cartilages of normal puppies, normal adult dogs, and adult dogs with experimental OA, or 5 μg of RNA ladder (0.24 to 9.5 kb) was mixed with 100% deionized formamide to a final volume of 14.5 μl. It was mixed with 3.7 μl of 37% (v/v) formaldehyde and 2 μl of 5×FRR; incubated at 65°C for 10 min

and chilled on ice for 5 min. It was incubated with 1 μl of EthBr (1 μg/μl) on ice for 3 min. Each sample was mixed with 2.1 μl of RNA loading buffer and fractionated by electrophoresis in two 1% agarose gels containing 6.6% formaldehyde run at 45 V for 4 hours. The gels were soaked in 0.5 N NaOH for 20 min at room temperature and then in 20×SSC for 45 min at room temperature. RNAs were transferred from the gels on to nitrocellulose membrane (S&S) or BioTrans™ nylon membrane by capillary blotting, and fixed on to the nitrocellulose membrane by baking at 80°C for 2 hours, or to the nylon membrane by the UV Stratalinker™ 1800 as described in above.

Northern hybridization with the 21E riboprobe (using the Northern blot on the nylon membrane) was performed as follow: prehybridization the blot with 25% deionized formamide / 5×SSC / 1×PE / 150 μg/ml freshly denatured ssDNA at 40°C for 3 hours, then addition of 2×10^6 cpm/ml of the 21E riboprobe and incubation continued overnight. The post-hybridization conditions for the 21E riboprobe were washing the membrane with 2×SSC / 0.1% SDS at 40°C for 15 min twice, then in 0.1×SSC / 0.1 % SDS at 40°C for 30 min twice, and finally in 0.1×SSC / 0.1 % SDS at 60°C for 1 hour. The membrane was exposed to an X-ray film (X-OMAT, Kodak) at -70 °C for 48 hours.

Northern hybridization with the P1 riboprobe (using the Northern blot on the nylon membrane) was performed as follow: prehybridization the blot with 50% deionized formamide / 5×SSC / 1×PE / 150 μg/ml freshly denatured ssDNA at 60°C for 3 hours, then addition of 2×10^6 cpm/ml of the P1 riboprobe and incubation continued as above overnight. The post-hybridization conditions for the Northern hybridization with the P1 riboprobe were washing the membrane in 0.1×SSC / 0.1 % SDS at 67°C for 1 hr. The membrane was exposed to an X-ray film at -70°C for 16 hours.

For Northern hybridization using the oligoprobe RCSIIC, the RNA containing nitrocellulose blot was incubated with prehybridization solution containing 20% formamide / 1x Denhardt's solution / 5×SSC / 0.5 mg/ml tRNA / 25 mM of 1:1 Na_2HPO_4:NaH_2PO_4 at 24°C for 4 hours. The oligonucleotide (RCSIIC) was labelled with α-[^{32}P]-dATP at 3' end by terminal deoxynucleotide transferase (TdT): mix 5 μl 5×TdT

buffer, 10 µl of RCSIIC (1.5 pmole/µl), 8 µl of α-[^{32}P]-dATP (10 µCi/µl) and 3 µl TdT (15 units/µl). The reaction was performed at 37°C for 90 min and 74 µl of TE buffer was then added to it. The sample was extracted once with one volume of Tris-buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1). The supernatant was further purified by passing it through a TE-buffer-saturated Sephadex-G 25 column as follows:

Sterile glass wool was placed at the end of a baked Pasteur pipette and the pipette was packed with TE-buffer-saturated Sephadex G-25. The column was washed first with 500 µl of TE buffer and then with 500 µl of sterile ddH_2O. The sample was loaded onto the column and 400 µl of ddH_2O was immediately added onto the column for washing the column. DEPC-treated ddH_2O was added onto the column ten times (100 µl/each) sequentially and each fraction was collected into a microfuge tube (100 µl/fraction). One µl of each fraction from the column was used for counting the radioactivity in a scintillation counter. Fractions containing the highest radioactivity in the labelled probe were pooled and used for hybridization.

The labelled probe was added to the prehybridization solution (approximately 1×10^6 cpm/ml) and incubation continued at 24°C for 18 hours. After hybridization, the membrane was washed with 4xSSPE buffer at room temperature three times (10 min for each wash). The membrane was exposed to an X-ray film as described above.

2.2.13 Ribonuclease protection assay

For the ribonuclease (RNase) protection assay, a standard method described in "Current Protocols in Molecular Biology" was used (Ausubel *et al.* 1987a). To prepare a radioactive antisense riboprobe, clone 7-8<b>1/pCR™II, which contains the ELC isoform, was linearized by digestion with the restriction enzyme *EcoR*V. It was then digested with proteinase K and purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction. One µg of the linearized plasmid DNA was transcribed *in vitro* with SP6 RNA polymerase, as described in Promega's P2020 *In Vitro* Transcription kit and labelled with α-[^{32}P]-UTP (specific activity = 800 Ci/mmole) (final volume = 20 µl). To this *in vitro* transcribed

product, 30 µl of DNase digestion buffer (40 mM Tris·HCl / 6 mM $MgCl_2$ / 10 mM NaCl, pH 8.0) and 7.5 units of RNase free DNase-I was added and incubated at 37°C for 15 min. Transfer RNA (tRNA, 20 µg) as carrier was added into the tube and the riboprobe was extracted once with one volume of Tris-buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1). The probe was purified from free isotope by passing it through two 1-ml Sephadex G-50 spun columns (Sambrook *et al.* 1989). The probe was diluted to $2x10^5$ cpm/µl in RNA hybridization buffer (80% deionized pure formamide / 10 mM PIPES / 1 mM EDTA / 0.4 mM NaCl, pH 6.4). Preparation of a riboprobe with the E2-like module (with clone 21E/pCR™II linearized with *BamH*I) was described in Section 2.2.10.

Five µg of total RNA (from cartilage of the stifle of a normal puppy) was mixed with $2x10^5$ cpm of the [^{32}P]-labelled ELC or the 21E riboprobe in 50 µl (total volume) of RNA hybridization buffer. The sample was incubated at 68°C for 5 min. It was immediately transferred into a 45°C water bath and incubation was continued overnight. Five µg of tRNA (as a control) was incubated with the same probe using the same conditions as with the total RNA sample.

After hybridization, the samples and control were digested with ribonucleases, by incubating each with 396 µl of RNase digestion buffer (10 mM Tris·HCl / 5 mM EDTA / 300 mM NaCl, pH 7.5) and RNase (5 µg/ml RNase A / 250 units/ml RNase T1) at 15°C for 30 min. They were then digested with proteinase K. Carrier (40 µg of tRNA) was added into the sample or control and they were extracted once with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) as above. They were then precipitated with 2 volumes of cold 100% ethanol. The pellets were washed with 1 ml cold 70% ethanol once and dried in a SpeedVac™ for 10 min. The sample, control and an RNA ladder (from 0.155 to 1.77 kb) were fractionated in a 1.5% agarose gel containing 6.6% formaldehyde. They were transferred by capillary blotting onto a Biotrans™ nylon membrane (ICN) in 20×SSC (Sambrook *et al.* 1989) and fixed onto the membrane by UV crosslinking as above. The membrane was exposed to an X-ray film (X-OMAT, Kodak) at -70°C for various periods of time.

2.2.14 Screening the cDNA library with an oligonucloetide probe in order to obtain a clone for the canine specific G3 domain with the "C-like module alone" isoform

The oligonucleotide (RCSIIC) (Table 2.1) was labelled with α-[^{32}P]-dATP at 3' end with TdT and isolated from free isotope as described in the section 2.2.12. In order to isolate a clone with the putative "C-like module alone" isoform, 17,950 pfu of the unamplified cDNA library (equivalent to approximately 1.8×10^7 pfu in the amplified cDNA library) was plated on NZY agar plates as described in section 2.2.4. The plaques were lifted onto Duralon-UV™ nylon which were then treated as in section 2.2.4. They were screened with the RCSIIC oligoprobe. Briefly, the membranes were incubated with prehybridization solution (10 ml/bag) containing 6×SSC / 20 mM NaH_2PO_4 / 1% SDS / 5×Denhardt's solution / 100 μg/ml freshly denatured ssDNA at 25°C for 2 hours. About half of the prehybridization solution was removed from the bag and pooled oligonucleotide probe (about 1×10^6 cpm) was added into the prehybridization bag. Incubation was continued at 25°C overnight. In the post-hybridization, the membranes were washed with 6×SSC / 0.1% SDS at room temperature for 15 min and exposed to the X-ray films (X-OMAT, Kodak) at -70°C overnight. Few plaques with weakly hybridized signals were rescreened with the same probe once more as above.

2.3 RESULTS

2.3.1 Cloning and sequencing of the complete G3 domain and partial CS-II region of canine aggrecan

Two overlapping clones (P1 and P9) which hybridized with the bovine G3 cDNA probe (Oldberg *et al.* 1987) were isolated from the cDNA library (Figure 2.2). On comparison with other cDNA sequences for aggrecan, it was found that the total 819 nt sequence of the two clones includes 73 nt of the C-terminal portion of the CS-II region, the complete L-

like module, the C-like module, a last exon of the C-terminus, a stop codon and a part of the 3' non-translated region (Figure 2.3). These two clones lack any E-like module.

Clone CSII5 (754 nt) was obtained by PCR screening the cDNA library using primers poly1F/CSIIR and poly2F/CSIIR (Table 2.1). It contains the CS-II region overlapping the last putative "aggrecanase" cleavage site at the 3' end of aggrecan (Figures 2.2 and 2.3).

Based on the above cDNA sequence information and the cDNA sequence of canine E2 (Fülöp *et al.* 1993), the E2-like module was cloned directly from RT-PCR products of normal young canine articular cartilage using primers L2 and R2.1 (Tables 2.1 and 2.2, Figures 2.1b, 2.2 and 2.3). Analysis of the cDNA sequences for five random clones (21E, 22E, 23E, 24E and 25E) showed that they were identical to the cDNA sequence of the E2-like module of canine aggrecan described by Fülöp *et al.* (1993).

Comparison of the cDNA and the deduced amino acid sequences of the C-terminal end of canine aggrecan was performed by computer software programs PC/GENE (IntelliGenetics, University of Geneva, Switzerland) and the BLAST server (Altschul *et al.* 1990). The results are summarized in Table 2.3. The deduced amino acid sequence from the CS-II cDNA sequence contains a VSQELAQR sequence (the underlined amino acid sequence in Figure 2.3), which is very similar to the putative "aggrecanase" cleavage site at leucine residue 1939 of cDNA in human aggrecan (VSQE↓LGQR) (Ilic *et al.* 1992).

In the G3 domain, the numbers and locations of all the cysteine residues are highly conserved among canine, human (Baldwin *et al.* 1989; Doege *et al.* 1991; Fülöp *et al.* 1993), bovine (Oldberg *et al.* 1987), rat (Doege *et al.* 1987), and chicken (Tanaka *et al.* 1988) aggrecan. This results suggested that the disulfide bonds of the G3 domain were conserved. The results implied that the overall three dimensional structure of the G3 domain was conserved in canine aggrecan.

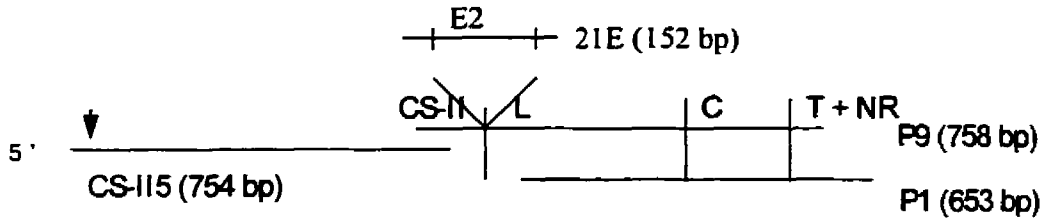

Figure 2.2 Schematic diagram of four cDNA clones of the C-terminus of canine aggrecan obtained from this study.

The vertical lines indicate the exon boundaries of protein domains and modules. The arrow indicates the putative "aggrecanase" cleavage site. T= C-terminus of aggrecan coded by the last exon; NR= 3' non-translated region.

Table 2.3 Homology of nucleotide and deduced amino acid sequences of the C-terminal end of canine aggrecan to the corresponding regions in other species.

canine aggrecan	cDNA				Amino acid sequence identity with human aggrecan
	Bovine	Human	Rat	Chicken	
CSII5	80%	81%	68%	53%	73%
E2	97%	94%	93%	89%	97%
L	90%	92%	90%	78%	90%
C	81%	88%	85%	81%	94%
C-terminus	88%	91%	79%	47%	88%

The canine aggrecan cDNA or deduced amino acid sequence was compared to that of bovine (Oldberg *et al.* 1987), human (Baldwin *et al.* 1989; Doege *et al.* 1991; Fülöp *et al.* 1993), rat (Doege *et al.* 1987) and chicken (Tanaka *et al.* 1988).

GGACTTTTAGAAGTGACTTTAATCACTTCCGAGTTTGTGGAGGGTGTCACTGAACCAACT 60
G L L E V T L I T S E F V E G V T E P T
GTTTCCCAGGAACTTGCCCAAAGACCCCCTGTGACACACACCCCTCAGCTTTTTGAGTCC 120
V S Q E L A Q R P P V T H T P Q L F E S
AGTGGAGAAGCCTCTGCATCCGGGGAAATTAGTGGAGCTACACCAGCGTTCCCCGGGTCT 180
S G E A S A S G E I S G A T P A F P G S
GGGCTGGAAGCGTCATCAGTCCCAGAATCTAGCAGCGAGACATCTGACTTTCCTGAGCGT 240
G L E A S S V P E S S S E T S D F P E R
GCAGTGGGGGTGTCTGCTGCCCCTGAGGCCAGTGGAGGAGCTTCTGGTGCCCCTGATGTA 300
A V G V S A A P E A S G G A S G A P D V
AGCGAAGCCACCTCCACCTTCCCTGAAGCCGATGTGGAGGGAGCCTCAGGCTTGGGAGTG 360
S E A T S T F P E A D V E G A S G L G V
AGTGGTGGCACCTCAGCCTTTCCTGAAGCCCCCAGGGAGGGCTCAGCCACCCCAGAAGTC 420
S G G T S A F P E A P R E G S A T P E V
CAGGAGGAGCCAACCACATCCTATGATGTGGGCCGAGAGGCGTTGGGCTGGCCTTCAGCC 480
Q E E P T T S Y D V G R E A L G W P S A
ACTCCAACAGCATCTGGAGACAGGATTGAAGTCAGTGGAGACCTGTCTGGTCACACGTCG 540
T P T A S G D R I E V S G D L S G H T S
GGGCTGGATGTTGTCATCAGCACTAGCGTCCCAGAGTCTGAGTGGATCCAGCAGACCCAG 600
G L D V V I S T S V P E S E W I Q Q T Q
CGCCCTGCAGAGGCGCATTTAGAAATCGAGGCCTCAAGCCCCTTGCACTCAGGAGAGGAG 660
R P A E A H L E I E A S S P L H S G E E
ACCCAAACAGCCGAGACAGCCACCTCCCCTACAGATGATGCTTCCATCCCAACTTCTCCA 720
T Q T A E T A T S P T D D A S I P T S P
TCAGGGACAGATGAGTCAGCACCAGCCATTCCAGACATTGACGAGTGCCTCTCAAGTCCT 780
S G T D E S A P A I P D I D E C L S S P
TGTTTGAATGGAGCCACCTGCGTGGACGCCATCGACTCTTTCACATGCTTATGCCTTCCC 840
C L N G A T C V D A I D S F T C L C L P
AGCTACCGAGGGGACCTGTGTGAGATCGACCAGGAGCTGTGTGAGGAGGGCTGGACCAAG 900
S Y R G D L C E I D Q E L C E E G W T K
TTCCAGGGCCACTGTTACCGCTACTTCCCCGACCGTGAGAGCTGGGTGGATGCGGAGAGC 960
F Q G H C Y R Y F P D R E S W V D A E S
CGGTGTCGGGCACAACAGTCACACCTGAGCAGCATTGTCACCCCTGAGGAACAGGAGTTT 1020
R C R A Q Q S H L S S I V T P E E Q E F
GTCAACAACAATGCCCAAGACTACCAGTGGATTGGCCTGAATGACAGGACCATCGAAGGG 1080
V N N N A Q D Y Q W I G L N D R T I E G
GACTTCCGCTGGTCAGATGGACACTCCTTGCAATTTGAGAACTGGCGCCCCAACCAGCCC 1140
D F R W S D G H S L Q F E N W R P N Q P

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GACAACTTCTTTGTCAGCGGAGAGGACTGTGTGGTGATGATCTGGCACGAGAAGGGAGAG  1200
D  N  F  F  V  S  G  E  D  C  V  V  M  I  W  H  E  K  G  E
TGGAATGACGTGCCTTGCAATTACTACCTGCCCTTCACGTGTAAAAAGGGCACAGTGGCC  1260
W  N  D  V  P  C  N  Y  Y  L  P  F  T  C  K  K  G  T  V  A
TGCGGAGACCCCCCTGTGGTGGAACACGCCAGGACCTTCGGCCAGAAGAAGGACCGCTAC  1320
C  G  D  P  P  V  V  E  H  A  R  T  F  G  Q  K  K  D  R  Y
GAGATCAACTCGTTGGTACGGTACCAGTGCACTGAGGGTTTCGTCCAGCGGCATGTGCCG  1380
E  I  N  S  L  V  R  Y  Q  C  T  E  G  F  V  Q  R  H  V  P
ACCATCCGGTGCCAGCCTAGCGGCCACTGGGAAAAACCTCGCATCACCTGCACAGACCCC  1440
T  I  R  C  Q  P  S  G  H  W  E  K  P  R  I  T  C  T  D  P
TCCACTTACAAGCGCAGACTACAGAAGCGGAGCTCACGGGCCCCACGGAGGAGCCGCCCC  1500
S  T  Y  K  R  R  L  Q  K  R  S  S  R  A  P  R  R  S  R  P
AGCACAGCCCACTGAGAGGAGCTTCCAGGACGCACCCAGGATGCTGAGCCCAGGAGCCCC  1560
S  T  A  H
GCCAGGCTGACATCCCACATGGATGGTGTCCTCTTCTTGCCGCTTTCTGTCATATAA     1617
```

Figure 2.3 The cDNA sequence (starting at the 5' end) and the deduced amino acid sequence of the C-terminal end of canine aggrecan.

The sequences of the isolated clones contain a partial CS-II region (1-754 nt), the G3 domain with an E2-like module (755- 868 nt), a L-like module (869-1255 nt), a C-like module (1256-1438 nt), the last exon of aggrecan coded the last 25 amino acids of the C-terminus (1439-1512 nt) followed by a stop codon (underlined cDNA, 1513-1515 nt) and a part of the 3' non-coding region (1516-1617 nt). The polyA site is not included in this sequence. The amino acid sequence with a single-underline indicates the putative "aggrecanase" cleavage site. The cysteine residues are double-underlined.

2.3.2 Identification of alternatively spliced mRNA isoforms of the G3 domain in canine aggrecan by RT-PCR and Southern hybridization

To determine which isoforms of the G3 domain were actually expressed in canine articular cartilage, total RNA was reverse transcribed with oligo (dT) and PCR with canine aggrecan specific primer pairs CSIIE/CT, CSIIE/R1, CSIIE/LT, CSIIE/ET, CSIIL/LT, CSIIL/R1, CSIIC/CT, CSIIC/R1, L3/R3 and FCSII5/R1 (Tables 2.1 and 2.2, Figure 2.1a-h). All PCR products with sizes in the range of the theoretical G3 isoforms were cloned into the pCR™II vector and identified by DNA sequencing. Only three distinct clones were obtained; their cDNA sequences were identical to the G3 isoforms of ELC (using CSIIE/CT primers), LC (using CSIIL/R1 primers) and a putative new isoform with "C-like module alone" (using CSIIC/R1 primers) (Note: no EL, EC, L or E were found in these RT-PCR products). These three cDNAs were detected in the RT-PCR products of all RNA samples from articular cartilages of normal young, normal adult and the adult osteoarthritic dogs (Figure 2.4a, 2.4b, and 2.4c).

Using primers L2/R2 for RT-PCR (Table 2.2 and Figure 2.1b), two cDNAs were amplified with expected sizes: a L' (the partial L-like module) and an EL' (the complete E-like module plus a partial L-like module) (Figure 2.5a). The ratio of these two cDNAs was estimated to be about 5:1 (L':EL') by scanning the Polaroid film of the agarose gel (Figure 2.5b).

In another RT-PCR reaction, total RNA was reverse transcribed with oligo (dT) and PCR with L1 and R1 primers (Figure 2.1b and Table 2.2). Theoretically, this pair of primers should amplify all of the G3 mRNA isoforms. Electrophoresis of the products in an EthBr stained agarose gel revealed only one 611 bp band, which corresponds to the LC isoform (Figure 2.6, lane 1). Southern hybridization of this sample with the P1 riboprobe (containing the LC cDNA, Figure 2.2) also confirmed that this 611 bp band was the LC isoform (Figure 2.6, lane 2). However, when the membrane was re-hybridized with a 21E/pCR™II riboprobe (including 21 nt of the CS-II region, a complete E2-like module of 114 nt and 17 nt of the L-like module), a 725 bp band corresponding to the ELC isoform

was identified (Figure 2.6, lane 3). When washing the Southern blot that hybridized with the C alone riboprobe in low stringency (at 60°C), a 224 bp band corresponding to the "C-like module alone" in size, the 611 bp (LC) and other non-specific bands were seen the in the X-ray film after 4 hour's exposure (data not shown; Note: This 224 bp cDNA band was sbucloned into pCR™II vector and analyzed by DNA sequencing. It was an unspecific cDNA due to RT-PCR artifact). When further washing this Southern blot in higher stringency (*i.e.*, the same washing solution as above but at 70°C), the 224 bp cDNA did not show; only the 611 bp (LC) cDNA was seen in the X-ray film (Figure 2.6, lane 4). Because different hybridization and post-hybridization conditions were used for these three riboprobes, the length of the exposure time for each probe did not directly reflect their relative amounts of RT-PCR products in the Southern blots. Therefore, the intensity of the signals in the X-ray films can not be compared among these three probes.

From the RT-PCR using different combination of primer pairs and Southern hybridization using three different riboprobes, the results suggested that the level of expression of the LC isoform was higher than that of the ELC in canine articular cartilage. However, whether the newly discovered putative isoform with the "C-like module alone" is a true native isoform was not known.

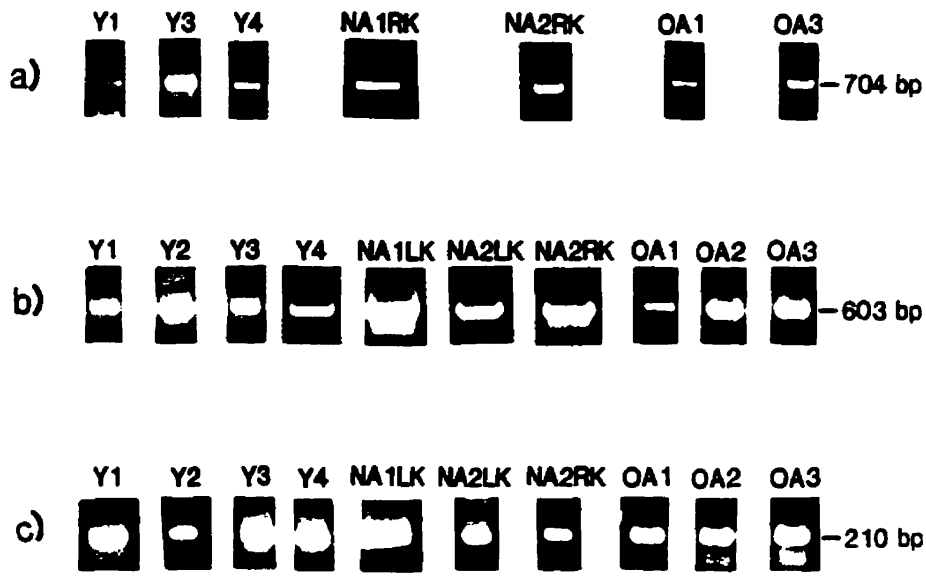


Figure 2.4 Electrophoresis of RT-PCR products for three G3 cDNAs.

This figure shows 1% agarose gels stained with EthBr for identifying the G3 isoforms of aggrecan in articular cartilage from samples tested: normal young (Y1, Y2, Y3 and Y4), normal adult (NA1LK, NA1RK, NA2LK and NA2RK), and osteoarthritic adult (OA1, OA2 and OA3) dogs. LK=left knee, RK=right knee.

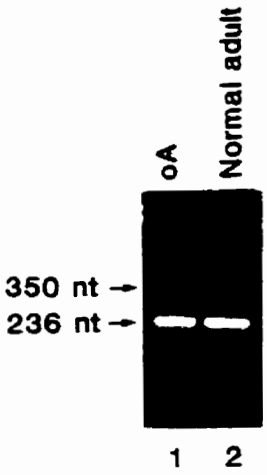
(a) The ELC isoform (704 bp; primers used: CSIIE/CT);

(b) The LC isoform (603 bp; primers used: CSIIL/R1);

(c) The "C-like module alone" isoform (210 bp; primers used: CSIIC/R1).

Note: In the panel (a), the cDNA above the 704 bp in the sample Y3 was a non-specific cDNA. In panel (c), lanes OA1, OA2 and OA3: the EthBr stained bands below the 210 bp cDNA were primer dimers which would disappear from the gel if the gel was run longer by electrophoresis.

(a)



(b)

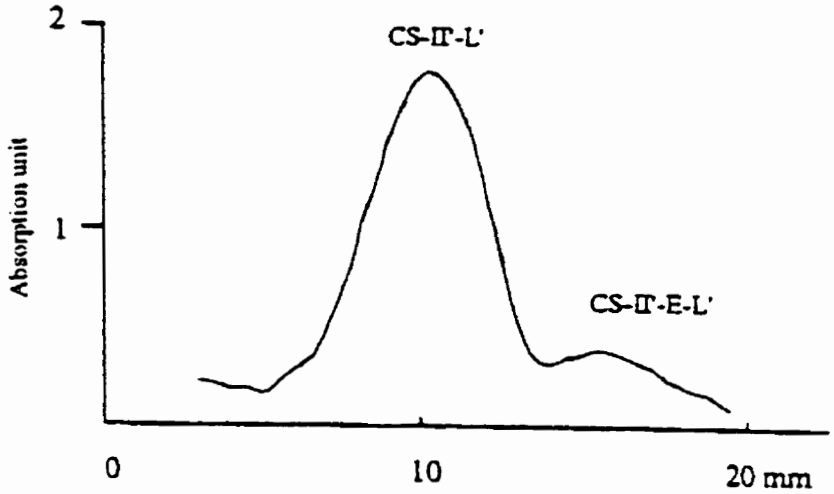


Figure 2.5 RT-PCR of total RNA from adult articular cartilage.

(a) Agarose gel (2%) electrophoresis of the RT-PCR products using the L2/R2 primers.

(b) Scanning of the Polaroid film of the agarose gel of the OA sample lane. The ratio of L': EL' is 5:1.

Note: The band below the 236 bp band in Figure 2.5a was a primer dimmer.

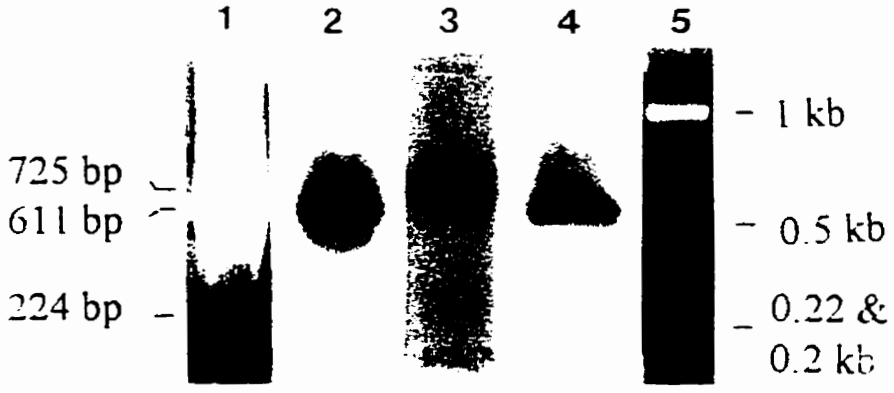


Figure 2.6 Identification of the LC (611 bp) and ELC (725 bp) isoforms by Southern hybridizations.

Lane 1 = The RT-PCR products (L1/R1 primers) fractionated in a 1% agarose gel stained with EthBr.

Lane 2 = Southern hybridization with the P1 riboprobe (exposure for 2 hours).

Lane 3 = Southern hybridization with the 21E riboprobe (exposure for 16 hours).

Lane 4 = Southern hybridization with the C riboprobe (exposure for 24 hours).

Lane 5 = The 1 kb DNA ladder.

2.3.3 Verification of the G3 isoforms cloned from RT-PCR by Northern hybridization, ribonuclease protection assay, or screening the cDNA library

The P1 cDNA clone (contained the LC isoform, see Figure 2.2) has previously been used in the laboratory (Matyas *et al* 1995, and also see below) for Northern blot hybridization to detect aggrecan mRNA in canine articular cartilage at the hypertrophic stage of osteoarthritis. However, it is likely that the P1 probe would not discriminate any of the isoforms of aggrecan (LC, ELC, and a putative "C-like module alone" isoforms) in Northern hybridization because of the small difference in their sizes (range from 0.114 kb to 0.388 kb) relative to the aggrecan mRNA (8 kb). Therefore, Northern hybridization with the 21E riboprobe and an oligonucleotide probe (RCSIIC) as well as ribonuclease (RNase) protection assays with 21E and ELC riboprobes were performed in order to verify the existence of the ELC and "C-like module alone" isoforms of aggrecan in canine articular cartilage.

Northern hybridization with the 21E riboprobe detected the ELC isoform-containing aggrecan mRNA (8 kb) in the total RNA of canine articular cartilage (middle panel of Figure 2.7). As a positive control, the P1 riboprobe also hybridized to the aggrecan mRNA in the same blot (bottom panel of Figure 2.7). Only the LC isoform (570 nt), but neither the ELC nor the "C-like module alone", was detected when the ELC riboprobe was used in the RNase protection assay (Figure 2.8a). In the RNase protection assays, the 21E riboprobe (152 nt) did not protect the E2-like module in the samples tested (Figure 2.8b).

These data suggested that the ELC isoform are expressed at a much lower level than that of the LC isoform in the samples tested. In theory, RNase protection assay would be a more sensitive method than the Northern hybridization for detecting a mRNA. The fact that we did not protect the ELC mRNA in the RNase protection assay may have resulted from the experimental condition of the RNase protection assay not having been optimized.

Since it is not possible to misprime the ELC with other G3 isoforms in the RT-PCR using primers CSIIE/CT, and this isoform had been reported in human aggrecan (Baldwin

et al. 1989), we concluded that the ELC cloned from the RT-PCR product was a true native G3 isoform, even though it was not able to be detected in the RNase protection assays in the samples tested.

To explore further the existence of the putative new isoform with "C-like module alone" cloned from RT-PCR, Northern hybridization was performed with the oligonucleotide probe RCSIIC (Table 2.1), which spans the putative exon boundary of "CSIIC" (for the putative "C-like module alone" isoform). However, the isoform with "C-like module alone" was not detected in the total RNA samples from canine articular cartilage by Northern hybridization with the RCSIIC oligoprobe (data not shown). The RCSIIC oligonucleotide probe was used to re-screen the cDNA library and no clone of the "C-like module alone" isoform was isolated (data not shown).

Finally, to test the hypothesis that the "C-like module alone" was a PCR artifact due to mispriming (because 11 nt out of 16 nt of the CSIIC primer are identical to the LC exon boundaries), clone of pure LC/pCR™II plasmid DNA (used as template) and the CSIIC and R1 primers (the pair of primers that had generated the "C-like module alone" cDNA in RT-PCR, Figure 2.1d and Table 2.2) were used in a PCR reaction. This generated a cDNA identical to "C-like module alone" (Figure 2.9). This PCR result suggested that the cDNA of the "C-like module alone" generated by RT-PCR using CSIIC/R1 primers is a PCR artifact (by mispriming to LC template) and does not represent a native G3 isoform.

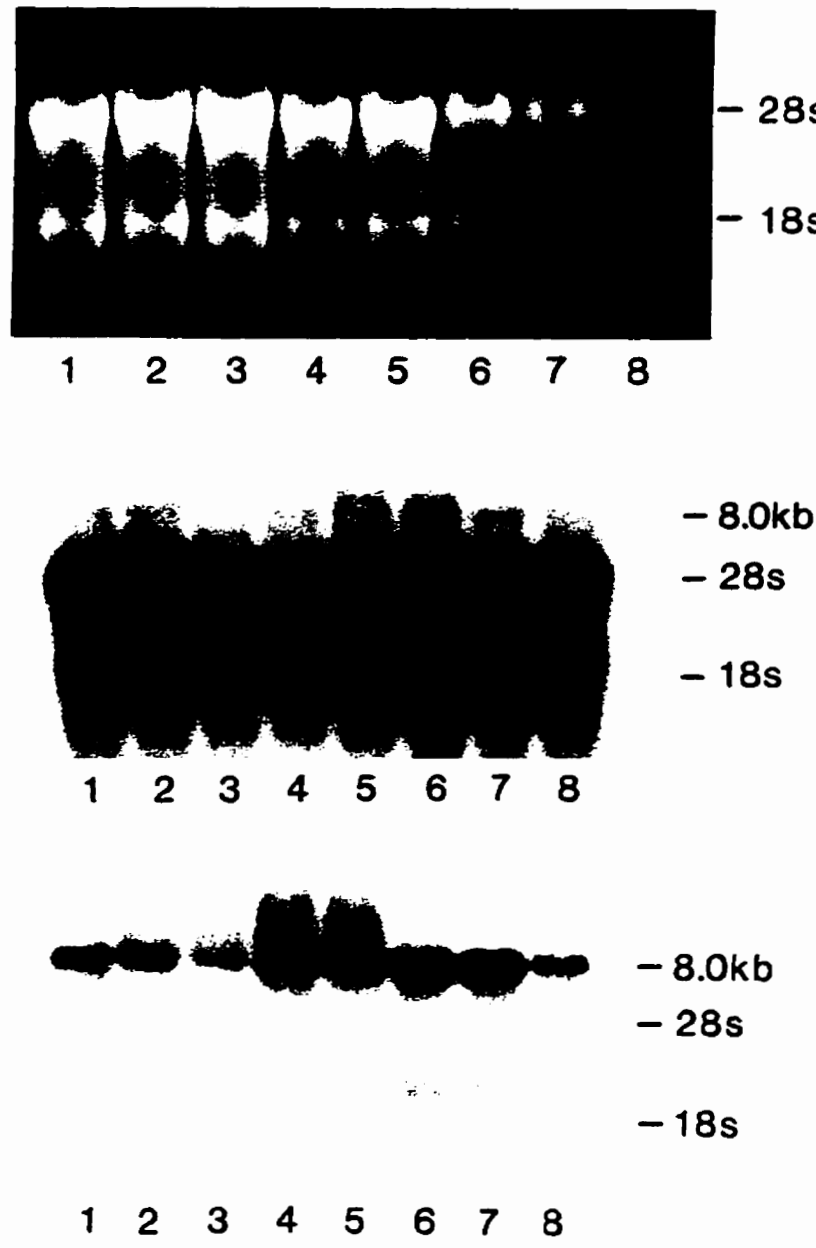


Figure 2.7 Northern hybridizations for canine aggrecan (8 kb).

Top panel: Polaroid film of the 0.8% agarose/formaldehyde gel electrophoresis of the total RNA from canine articular cartilage. Lanes 1 to 6 are OA samples of dogs 1, 2 ,3, 4, 5 and 8, lanes 7 is a normal adult sample and lane 8 is a normal puppy sample.

Middle panel: Northern hybridization using 21E riboprobe.

Bottom panel: Northern hybridization using the P1 riboprobe.

Figure 2.8 RNase protection assays for the G3 isoforms.

(a) RNase protection assay using the ELC riboprobe: Lane 1 = Negative control (5 μg of tRNA alone); Lane 2 = Total RNA (5 μg) from the articular cartilage of a normal puppy; Lane 3 = Total RNA (5 μg) from the articular cartilage of a normal adult dog.

(b) RNase protection assay using the 21E riboprobe: Lane 4 = Negative control (5 μg of tRNA alone); Lane 5 = Positive control (the 21E riboprobe alone); Lane 6 = Total RNA (5 μg) from the articular cartilage of a normal puppy.

Note: The size of the LC mRNA is 570 nt. The size of the 21E riboprobe is 152 nt.

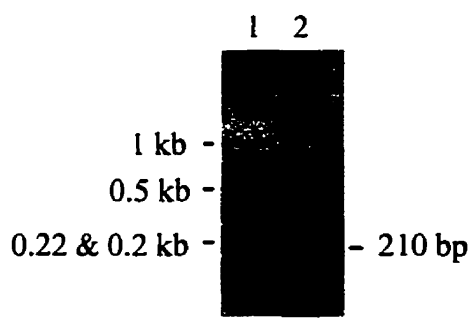


Figure 2.9 PCR using the LC/pCR™II plasmid as template and CSIIC/R1 as primers.

CSIIC/R1 misprimed to the LC/pCR™II plasmid template and generated an artifact cDNA with "C-like module alone" sequence (the 210 bp in the lane 2). Lane 1 = 1 kb DNA ladder.

2.4 DISCUSSION

The exon boundaries of the protein domains and modules of aggrecan were chosen from the genomic DNA sequence of chicken aggrecan (Tanaka *et al.* 1988), the cDNA sequences of the human aggrecan (Baldwin *et al.* 1989; Doege *et al.* 1991), and the G3 domain of canine aggrecan. The sequences at the intron-exon boundaries of human aggrecan are now known (Valhmu *et al.* 1995). Although the exon sequence of the canine G3 domain of aggrecan is less homologous with chicken than human G3, the intron-exon boundaries are highly conserved throughout aggrecan when comparing between the chicken and the human (Valhmu *et al.* 1995; Tanaka *et al.* 1988). Thus, we have assumed that the exon splicing sites of the G3 domain of canine aggrecan are the same as those in chicken and human aggrecan.

Although the exon for an E2-like module in the genomic DNA sequence of chicken aggrecan was not described by the authors (Tanaka *et al.* 1988), a putative exon for the E2-like module with appropriate intron/exon splicing sites can be found at numbers 389 to 502 of the sequence. Therefore, the E2-like module has the potential to be expressed in chicken aggrecan.

In this study, the alternatively spliced mRNA isoforms of the G3 domain of canine aggrecan core protein were studied extensively by RT-PCR and confirmed by Northern hybridization and RNase protection assays. The most important observations and conclusions are summarized below:

(1) Only two of the previously reported isoforms, LC and ELC, were found to be expressed in the canine articular cartilage. Although we have results for a putative new G3 isoform with the "C-like module alone" from RT-PCR (using CSIIC/R1 primers but not L1/R1 primers), the natural existence of this isoform is not completely defined by methods other than RT-PCR. This "C-like module alone" cDNA may be a true G3 isoform expressed at a very low level, or an RT-PCR artifact.

(2) It is interesting that an isoform of the G3 domain containing only the lectin-like module was reported in human fetal and juvenile cartilage (Doege *et al.* 1991). However, using primer pairs such as CSIIL/LT, CSIIL/R1 and other combinations, we were unable

to amplify this isoform from total RNA of articular cartilage in the normal puppies, normal adult dogs, or adult dogs in the hypertrophic stage of experimental osteoarthritis. This suggests that the isoform with the L-like module alone may be a specific isoform of aggrecan expressed at distinct phases of development, or alternatively it may be a species-specific isoform. If the hypothesis is true, it may suggest that the different isoforms of the G3 domain may have different biological significance in the cartilage.

(3) The high degree of conservation of the cysteine residues in the G3 domain suggests that formation of the correct disulfide bonds is critical to the proper structure and function of the G3 domain.

(4) The high degree of conservation across species of the "aggrecanase" cleavage site in the C-terminal portion of aggrecan suggests an important role for this cleavage. Thus, the fragment of the G3 domain that would be released from the aggrecan molecule by this cleavage may be of pathophysiological significance in aging cartilage and in osteoarthritis.

(5) Although RT-PCR was used extensively to detect G3 isoforms, different pairs of primers may not work for amplifying the same isoform (for example, CSIIE/R1 primers did not work but CSIIE/CT primers worked on amplifying the ELC isoform; L3/R3 primers did not worked but L2/R2 primers worked on amplifying the EL' and L'). Therefore, the existence of other mRNA isoforms with a very low abundance cannot be absolutely excluded. Nevertheless, this study is the first investigation of the alternatively spliced mRNA isoforms of the G3 domain of canine aggrecan in normal young, normal adult, and adult osteoarthritic cartilage. The data from this study will facilitate future studies of the structure and function of the G3 domain of aggrecan in normal and osteoarthritic articular cartilage.

CHAPTER 3.

3. RT-PCR QUANTIFICATION OF AGGRECAN G3 ISOFORMS IN ARTICULAR CARTILAGE IN THE HYPERTROPHIC STAGE OF EXPERIMENTAL CANINE OSTEOARTHRITIS

3.1 INTRODUCTION

Aggrecan, a large proteoglycan that aggregates with hyauronan, is a major macromolecular component of cartilage and plays an important role in maintaining cartilage integrity (Heinegård & Oldberg, 1989). It contains multiple domains, including three globular domains (G1, G2 and G3), a short interglobular domain (IGD) and a large glycosaminoglycan attachment domains which is sub-divided into a keratan sulfate-rich region (KSRR) and two chondroitin sulfate-rich regions (CS-I, CS-II) (Heinegård & Oldberg, 1989). The G3 domain of aggrecan contains four different components, one or two epidermal growth factor-like modules (E1 or E2), a lectin-like module (L), a complement regulatory protein-like module (C) and a C-terminal peptide. The G3 domain of aggrecan is subject to alternative mRNA splicing (Baldwin *et al.* 1989). Although two types of E-like module (E1 and E2) can be present in human aggrecan, only the E2-like module is detected in canine cartilage (Fülöp *et al.* 1993).

In a previous study we defined, using RT-PCR and Northern hybridization, that two isoforms, the LC and ELC (with the E2-like module), were expressed in the articular cartilage of normal puppies, normal adult dogs, and adult dogs in the hypertrophic stage of experimental osteoarthritis (OA) (see Chapter 2 for detail). Although a putative isoform of G3 with the "C-like module alone" sequence was identified by RT-PCR (with primers CSIIC/R1), its natural existence had not been further verified by Northern hybridization, ribonuclease protection assay, and rescreening of a cDNA library derived from normal canine articular cartilage (see Chapter 2 for detail).

The expression of aggrecan mRNA was found to be increased in the cartilage from the hypertrophic stage of experimental OA as measured by Northern hybridization with probes to the KSRR and to the LC region of the G3 domain (Matyas *et al.* 1995). However, the LC probe that was used would not be expected to define the differential expression of the ELC and the LC isoforms in the Northern hybridization because of the small difference in size (114 nt) between these two isoforms relative to the size of aggrecan mRNA (8.0 kb) (Adams *et al.* 1992). An E2-specific riboprobe, which only hybridizes to the ELC isoform of aggrecan in a Northern hybridization, detected the ELC containing aggrecan mRNA in canine articular cartilage (see Chapter 2 for detail). The relative amounts of the expression of the LC and the ELC isoforms of canine aggrecan in experimental OA has not been quantified before this work.

The exact function of the G3 domain of aggrecan is not known. We have formulated the hypothesis that these two isoforms of the G3 domain may have different physiological and pathological functions in normal and osteoarthritic cartilages. Before directly testing this hypothesis, it is important to determine whether the expression of these isoforms is changed in osteoarthritic articular cartilage. In this study, we quantified the expression of the two major G3 mRNA isoforms of canine aggrecan, LC and ELC, in articular cartilages from the hypertrophic stage of experimental OA and from the contralateral non-operated (NOP) stifles. A method for competitive RT-PCR with an internal standard was used to measure the mRNA of the LC isoform (Gilliland *et al.* 1990). For quantifying the ELC mRNA isoform, we used a method of quantitative RT-PCR with an external standard (Arcellana-Panlilio & Schultz, 1993).

3.2 EXPERIMENTAL

3.2.1 Materials

Proteinase K, Hoechst 33258 dye was from Boehringer-Mannheim, Laval, PQ. Nitrocellulose membrane (0.45 μm pore size, S&S NC) was from Schleicher & Schuell, Keene, NH. The α-[^{32}P]-dCTP (10 μCi/μl, S.A.=3000 Ci/mmole), γ-[^{32}P]-ATP (10 μCi/μl,

3000 Ci/mmole), X-ray films (Hyperfilm-MP, #RPN1675) and a Senstitize Pre-flash unit (#RPN 2051) were from Amersham, Oakville, ON. T4 polynucleotide kinase (PNK) and a 1 kb DNA ladder were from GIBCO/BRL, Burlington, ON. Sephadex G-25, restriction enzymes and ultra pure free deoxynucleotides (dATP, dCTP, dGTA, dTTP, 100 mM each) were from Pharmacia, Baie D'Urfé, PQ. Taq DNA polymerase was from Perkin-Elmer Cetus, Mississauga, ON. Calf thymus DNA, D-19 developer, general purpose hardening fixer and all other Molecular Biology grade chemical reagents used in this study were from Sigma, St. Loius, MO. The Pfu DNA polymerase and 10×Pfu buffer were from Stratagene, La Jolla, CA. The cDNA Cycle™ kit and TA Cloning™ kit were from Invitrogen, San Diego, CA. Plasmid vector (pSP64.polyA), Wizard™ Minipreps DNA Purification System for plasmid and Riboprobe™ Gemini System II (#P2020) were from Promega, Madison, WI. The Taq Dydeoxy™ Terminator Cycle Sequencing kit was from Applied Biosystems Canada Inc., Mississauga, ON. The Oligotex™ mRNA kit was from Qiagen Inc., Chatsworth, CA. Scintillation cocktail (Ready Safe™) was from Beckman, Mississauga, ON.

The 20×SSC, 50×Denhardt's solution, TE buffer, 6×DNA loading buffer, 50×TAE buffer, 5×formaldehyde running buffer and RNA loading buffer were prepared as described in "Molecule Cloning: A Laboratory Manual" (Sambrook *et al.* 1989). The stock Hoechst 33258 was prepared at 1.5×10^{-4} M in distilled deionized water (ddH_2O) and aliquots were stored at -20°C. The working solution was freshly prepared from the stock solution of Hoechst 33258 by diluting it 100-fold in ddH_2O. Calf thymus DNA was prepared at 1 mg/ml in 0.01 M Tris·HCl (pH 8.0) and aliquots were stored at -20°C. Proteinase K was prepared at 5 mg/ml in 0.01 M Tris·HCl (pH 8.0) and 0.001 M $CaCl_2$ and aliquots were stored at -20°C. X-ray films were pre-flashed using the Sensitize Pre-flash unit (Amersham), as described in the manufacturer's manual. All the PCR reactions were carried out in GeneAmp PCR System 9600 (Perkin-Elmer Cetus).

3.2.2 Source of cartilage

Experimental OA was induced in six skeletally mature male dogs (husky × shepherd) by transection of the ACL of one stifle, leaving the contralateral stifle as a non-operated control (NOP) (Adams & Pelletier, 1988; Adams, 1994). Cartilage was taken from the contralateral NOP control stifles and the operated stifles at 10 weeks after surgery at which time was the cartilage in the hypertrophic stage of the experimental OA. These dogs were numbered dog 1 to 6. However, for a dog of its size, dog 6 had an abnormal total cartilage mass in the contralateral NOP stifle, based on our previous data with this model, and, thus, it was not included in the results. Fresh cartilage was harvested from the stifles as thoroughly as possible using scalpel blades and was immediately frozen in liquid nitrogen and transferred to a -70°C freezer.

3.2.3 Extraction of total RNA from cartilage

The cartilage was pulverized while submerged in liquid nitrogen in a Spex freezer mill (Spex Industries, Inc., Edison, NJ), as described in Chapter 2. Cartilage powder from each joint was mixed well and a few milligrams (approximately 1% of the cartilage powder) was saved for measurement of total DNA concentration content. The remainder of the samples were used to extract and purify total RNA as previously described (Adams *et al.* 1992). The yield and the purity of the total RNA were measured by optical density at 260 nm and 280 nm (OD_{260nm} and OD_{280nm}) using an Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia).

3.2.4 Measurement of total DNA concentration in the cartilage

Total DNA in the cartilage was measured using a fluorescent method derived from Lipman (Lipman, 1989). DNA standards (0.5 to 5 μg/ml) were prepared by diluting stock calf thymus DNA (1 mg/ml) in 0.01 M Tris·HCl (pH 8.0). Each cartilage sample or standard was mixed with 0.2 ml of 1% SDS, boiled for 10 min, and was then cooled to room temperature. After addition of 0.7 ml of 0.01 M Tris·HCl (pH 8.0) and 0.1 ml of proteinase

K (5 mg/ml), the mixture was incubated at 60°C for 18 to 20 hours and then cooled to room temperature. Twenty-five μl of each sample or standard was mixed with 2 ml of 0.154 M NaCl / 0.015 M sodium citrate, 0.075 ml of 0.01 M Tris·HCl (pH 8.0) and 1 ml of the 100-fold ddH_2O diluted stock Hoechst 33258, and was allowed to stand at room temperature in the dark for at least 10 min. The fluorescence was measured in a luminescence spectrophotometer (Model LS-5, Perkin-Elmer Cetus), with the excitation wavelength at 350 nm, emission wavelength at 450 nm, and slit width at 10.

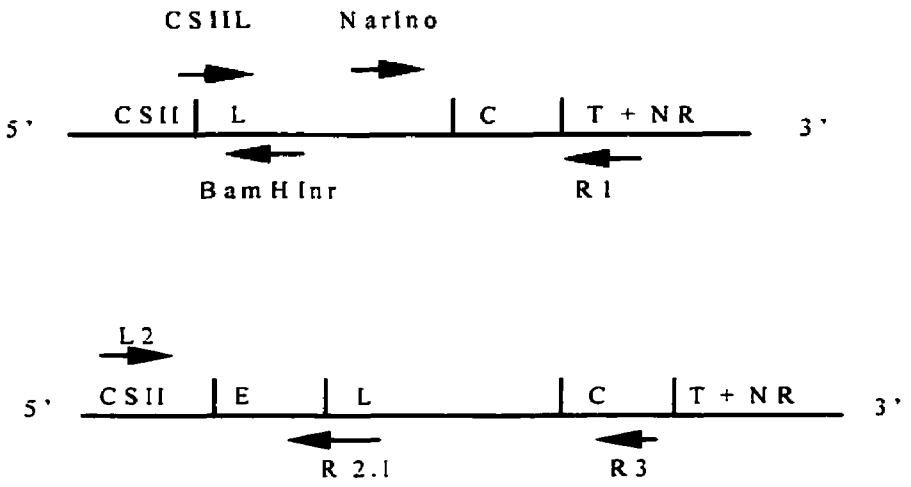


Figure 3.1 Schematic diagram of the locations of the oligonucleotide primers in the G3 domain of canine aggrecan.

The sizes of the oligonucleotides, the cDNA region, and the modules are not proportional. (T=C-terminus, NR=3' non-translation region).

Table 3.1 Canine aggrecan specific oligonucleotides.

Name	DNA Sequence		Tm[e]	Location[f]
BamHInr[a]	5'-dACCGGCTCTCCGG[1]ATCCA-3'	(A[d])	77	947-964
NarIno[a]	5'-dATTTGAGAACTGA[2]CGCCCC-3'	(S[d])	75	1113-1131
*CSIIL	5'-dACCAGCCATTCCAG|[c]ACCAGG-3'	(S)	75	741-754/869-874
*L2[b]	5'-dGAGGTGTCCGCACCAGCCATT-3'	(S)	73	730-750
*R1	5'-dCTGCGCTTGTAAGTGGAGG|-3'	(A)	70	1439-1457
*R2.1	5'-dCTCACACAGCTCCTGGT|CGAT-3'	(A)	73	865-868/869-885
*R3	5'-dTCGTAGCGGTCCTTCTTCTG-3'	(A)	71	1303-1322

Notes (letters correspond to the superscripts):

(a) The point mutations in the canine aggrecan specific primers by design were: G^1 (the original aggrecan cDNA is C) and A^2 (the original aggrecan cDNA is G);

(b) Three errors in synthesis (underlined) were found in the L2 primer. However, it was able to prime the canine aggrecan mRNA or cDNA at the correct location and so was still used in the experiments;

(c) The vertical lines indicate the exon boundaries;

(d) S = sense strand, A = antisense strand;

(e) Tm = melting temperature (°C);

(f) The locations of the oligonucleotides refer to the numbering of canine aggrecan as in Figure 2.3. The slashes separate the putative exon boundaries.

* Asterisks indicate primers that are also listed in Table 2.1.

3.2.5 Oligonucleotides

An oligo (dT) 30-mer was used for slot-blot hybridization to measure the amount of mRNA in the total RNA samples. This and the other oligonucleotides, which were used as primers in RT-PCR or in PCR site-directed mutagenesis, were synthesized by the Calgary Regional DNA Synthesis Laboratory (Calgary, AB). A summary of these oligonucleotides is listed in Table 3.1 and schematic diagrams of their locations in aggrecan are presented in Figure 3.1.

3.2.6 Slot-blot analysis of the total RNA with an oligo dT probe

Total RNA (0.5 μg, based on the OD_{260nm}) from each sample or a negative control (0.5 μg of tRNA alone) was prepared and blotted onto a nitrocellulose membrane using a Bio-Dot™ (SF) manifold (Bio-Rad) as described in "Molecular Cloning: A Laboratory Manual" (Sambrook *et al.* 1989). After blotting, the samples and control were fixed onto the membrane by baking at 80°C for 2 hours. The membrane was incubated with 20 ml of prehybridization solution containing 0.02 M NaH_2PO_4 / 0.7% sodium dodecyl sulfate (SDS) / 5×SSC / 10×Denhardt's solution / 50 μg/ml freshly denatured salmon sperm DNA (ssDNA) at 46°C for 4 hours.

To prepare an oligoprobe, 40 pmoles of the oligo (dT) 30-mer was labeled with γ-[^{32}P]-ATP at the 5' end using T4 PNK kinase (Sambrook *et al.* 1989). It was then extracted with one volume of Tris-buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1). The supernatant was loaded onto a Sephadex G-25 column which had been freshly packed into a baked Pasteur pipette and equilibrated with TE buffer. The sample was eluted with autoclaved ddH_2O. Fifteen fractions of the eluate were collected (0.1 ml each). Aliquots of the eluate (1μl/fraction) were used for liquid scintillation counting (LKB Wallac 1214 Rackbeta, Stockholm, Sweden). The highest peaks (in fractions No. 6 to 10) were pooled together and saved for hybridization.

About 14 ml of the pre-hybridization solution was removed from the pre-hybridization bag. The oligo (dT) probe (about 6.5×10^5 cpm total radioactivity) was added

into the bag and hybridization was continued at 46°C overnight. The post-hybridization conditions are listed in Table 3.2. An autoradiograph was made by exposing the membrane to a preflashed X-ray film at -70°C for 2 hours. The density of the autoradiographic signal was quantified by scanning the X-ray film with a CamScan (1993 Scanalytics, CSP, Inc., MA).

Table 3.2 Post-hybridization conditions for the slot-blot hybridization with the oligo (dT) probe.

Wash Solution	Temp (°C)	Wash time (min)
6×SSC and 0.01% SDS	25	30
2×SSC and 0.01% SDS	25	10
2×SSC and 0.01% SDS	27 to 30	60
2×SSC and 0.01% SDS	37	30
0.2×SSC and 0.01% SDS	35	30
0.2×SSC and 0.01% SDS	40	5

3.2.7 Creation of an internal standard for the LC isoform by site-directed mutagenesis

The native LC cDNA (clone LC/pCR™II) was derived from RT-PCR using primers CSIIL and R1 and cloned into the pCR™II vector in the TA Cloning™ kit. Using this clone as a template, PCR site-directed mutagenesis was carried out to create an internal standard for the LC isoform (Landt *et al.* 1990). In the first round of PCR, 1.0 μg of the LC/pCR™II plasmid and 60 pmole each of primers (CSIIL and BamHInr) were mixed with 0.8 unit of Taq DNA polymerase and the other PCR reagents as described in the cDNA Cycle™ kit. The PCR conditions were: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min (called program No.4). Four replicate PCR samples were pooled and fractionated in a 3% low melting point (LMP) agarose gel. A 94 bp cDNA called "L'BamHInew" (with a newly created *BamH*I restriction site) was purified from the LMP agarose gel by combination of heating to 68°C and phenol extraction (Sambrook *et*

al. 1989). For the second round of PCR, 1 μg of the LC/pCR™II plasmid, about 0.4 μg of the gel purified "L'BamHInew" cDNA, 60 pmole of primer R1 and the other PCR reagents were used for PCR reactions as above. The PCR product was fractionated in a 2% LMP agarose gel. cDNA at 603 bp, called "LCmut1", which corresponded to LC with a point mutation creating a new *BamH*I restriction site, was purified from the gel and cloned into the pCR™II vector, as described above.

Similarly, a LCmut2 cDNA, with a second point mutation that obliterated the native *Nar*I restriction site in the LCmut1 cDNA, was created by PCR site-directed mutagenesis using oligonucleotides NarIno/R1 as the primers and LCmut1/pCR™II plasmid as the template for the first round of PCR. The second round of PCR used oligonucleotide CSIIL and the cDNA fragment ("L'CNarIno"), that had been purified from the first round of PCR (with NarIno/R1 primers), as the primers and LCmut1/pCR™II plasmid as the template. The cDNA from the second round of PCR was purified and cloned into the pCR™II vector, as described above. LCmut2 was subcloned from pCR™II vector into the pSP64.polyA vector at restriction sites of *Xba*I and *Sac*I (called "LCmut2/pSP64.polyA").

Minipreps of the plasmid DNA for the clones of LCmut1/pCR™II, LCmut2/pCR™II, and LCmut2/pSP64.polyA were prepared using the Wizard™ Minipreps DNA Purification System. The plasmids were analyzed by restriction enzyme mapping and then by DNA sequencing using the automatic DNA sequencing method described in the Taq DyeDeoxy™ Terminator Cycle Sequencing kit. The purpose of creating two restriction sites by PCR site-directed mutagenesis in the LCmut2 clone was to compare the completeness of the restriction enzyme digestion after competitive RT-PCR.

3.2.8 Synthesis of the LCmut2 mRNA by in vitro transcription

The LCmut2/pSP64.polyA plasmid was linearized by restriction enzyme *Pvu*II and further purified by phenol/chloroform extraction. It was transcribed *in vitro* with SP6 RNA polymerase, as described in the Riboprobe™ Gemini System, except that only non-radioactive rNTPs were used in the reaction. This transcript contained 603 nucleotides (nt)

of LCmut2 mRNA, a 30 nt polyA tail and 306 nt derived from the pCR™II and pSP64.polyA plasmids (total 939 nt in length). The *in vitro*-transcribed mRNA of the LCmut2 was further purified with an Oligotex™ purification kit, as described by the manufacturer's manual. The LCmut2 mRNA was quantified by OD_{260nm} and OD_{280nm}; and its size and purity were confirmed by electrophoresis in a 1% agarose gel containing 6.6% formaldehyde (data not shown). Aliquots were stored at -80°C.

3.2.9 Competitive RT-PCR for quantifying the LC isoform

To determine the optimal cycle number for amplifying the LC isoform, 1 μg of total RNA was reverse transcribed (RT) twice, using an oligo (dT) primer, as described in the manual of the cDNA Cycle™ kit. The RT sample (20 μl) was then diluted 2.5-fold by adding 30 μl of autoclaved ddH_2O to the original RT sample. A master mix was made for seven identical PCR reactions, each of which contained 4 μl of the diluted RT sample, 5 μl of 10×Pfu buffer, 1 μl of dNTPs (25 mM each), 6 μl of the CSIIL primer (10 pmole/μl), 6 μl of the R1 primer (10 pmole/μl), 0.5 μl Pfu DNA polymerase (2.5 unit/μl) and 27.5 μl of ddH_2O. PCR was performed with program No.4 as described in section 3.2.7, but one tube was removed from the PCR machine at each of cycle numbers 20, 22, 24, 25, 26, 28, and 30. Samples (20 μl) of the PCR products were fractionated in a 2% agarose gel and photographed with type 55 Polaroid film. The film was analyzed by 2D scanning densitometry (CamScan System). It was thus determined that the PCR cycle number 25 was within the linear range for LC isoform under these conditions (data not shown).

The amount of the internal standard (mRNA of the LCmut2) that needed to be added to the added to the total RNA at the reverse transcription step was estimated based on preliminary trials (data not shown). To quantify the LC isoform, a method of competitive RT-PCR with internal standard was used (Gilliland *et al.* 1990). Basically, LCmut2 mRNA from 0.016 to 1.291 fmole (as had been defined in preliminary experiments) was added to 1 μg of the total RNA. The mixtures were reverse transcribed and each RT sample (20 μl) was diluted 2.5-fold using autoclaved ddH_2O as described above in the determination of the optimal cycle number for amplifying the LC isoform.

Four μl of each of these diluted RT samples (corresponding to 0.08 μg of original total RNA and 0.0013 to 0.103 fmole of internal standard added in the RT-PCR) was mixed with 46 μl of the master mix containing 0.1 μl of α-[^{32}P]-dCTP (10 μCi/μl) and other reagents as described above in the determination of the optimal cycle numbers for the LC isoform. The PCR conditions were: 94°C for 3 min, followed by 25 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, and a final 72°C for 7 min. Samples (20 μl) of the PCR products were digested with 50 units of *BamH*I at 37°C for 1.5 hours, and then fractionated in 2% agarose gels stained with EthBr. The gels were photographed under UV illumination using Polaroid type 55 film. The native LC band (603 bp), one of the *BamH*I digested LCmut2 cDNA (509 bp) and a blank area were sliced out of the gel, each mixed with 3 ml of the Ready Safe™ scintillation fluid and the radioactivity of each was measured in a liquid scintillation counter. The negatives of the photographs of the agarose gels for the quantitative RT-PCR were analyzed by 2D scanning densitometry (CamScan System). The radioactivity or the scan units of the intact LCmut2 were calculated by multiplying the CPM or scan unit of the 509 bp *BamH*I fragment by 603/509.

3.2.10 Quantifying the E2-like module by an external standard method

To prepare an external standard, plasmid DNA from clone 21E/pCR™II, which contains the native E2-like module of canine aggrecan (see Chapter 2) was linearized by restriction enzyme digestion with *BamH*I and then purified by phenol and chloroform extraction (Sambrook *et al.* 1989). The concentration of the linearized plasmid DNA was first estimated by OD_{260nm} measured by spectrophotometry, and then measured more precisely by spotting it and a range of known amount of standard (1 kb DNA ladder) stained with EthBr on saran wrap. The spots were then photographed under UV light using Polaroid type 55 film and the densities of the sample and standards in the Polaroid type 55 negative were analyzed by the CamScan (data not shown).

The RT-PCR conditions, the sample and external standard concentrations, and the optimal cycle number for quantifying the ELC isoform were determined in the similar way as described above for the internal standard method (data not shown). PCR program No.4

(see section 3.2.7) was found to be optimal at 25 cycles using 0.5 μg of total RNA and a range of external standard of 0.0025 fmole to 0.025 fmole.

To quantify the E2 mRNA, which represents the ELC isoform, 5 μg of the total RNA from each sample was reverse transcribed twice as described in the cDNA Cycle™ kit, except that 10 pmole of the specific primer R3 was used instead of oligo (dT). Two μl of the undiluted RT sample (corresponding to 0.5 μg of original total RNA added), or 2 μl of the external standard (*BamHI* linearized 21E/pCR™II) from 0.0025 to 0.025 fmole, or 2 μl blank (ddH_2O alone) was mixed with an aliquot of the master PCR mix which contained 29.1 μl of ddH_2O, 1 μl of dNTPs (25 mM each), 5 μl of 10×PCR buffer, 0.1 μl of α-[^{32}P]-dCTP (10 μCi/μl), 6 μl of L2 (10 pmole/μl), 6 μl of R2.1 (10 pmole/μl) and 0.8 μl of the Taq DNA polymerase (5 units/μl). PCR was performed with program No. 4 as described in section 3.2.7. All the samples were partially dried in a SpeedVac (model SVC100, Savant, State Line, PA) to reduce the volume to about 15 μl, and 5 μl of 6×DNA loading buffer was added to each sample. Then, the entire amount of the PCR reaction was fractionated in a 2% agarose gel. The cDNA at 152 bp (corresponding to the E2-like module containing cDNA) in each sample, standards, and a blank area were sliced out of the gel for scintillation counting as described above.

3.2.11 Data analysis

The amount of total RNA (based on the value of OD_{260nm}) was normalized to total mRNA using the data from the slot-blot hybridization of total RNA with the oligo (dT) probe. The results of the OD_{260nm} (for the total RNA) and the slot blots (for the total mRNA in the total RNA samples) were analyzed by paired Student t-test. Linear regression analyses were performed for the DNA assay on the standard and for the log plots of the quantitative RT-PCR data, using the computer software Microsoft Excel (version 5). Using Microsoft Excel (version 5), paired Student's t-tests were performed on the sample data of the total cartilage mass (g per stifle), DNA concentration (mg per gram of cartilage), total DNA concentration (mg per stifle), total RNA concentration (μg per gram cartilage), DNA-

normalized amount of total RNA (μg per mg DNA) in the cartilage, total mRNA amount (scan unit per gram cartilage), DNA-normalized amount of mRNA (scan units per mg DNA), the concentration of LC mRNA (fmole per gram of cartilage), the DNA-normalized amount of LC mRNA (fmole per mg DNA), the concentration of ELC mRNA (fmole per gram of cartilage), and the DNA-normalized amount of the ELC (fmole per mg DNA). The significant difference was accepted at $p \leq 0.05$ (one tail).

3.3 RESULTS

3.3.1 Assessment of experimental OA

Visual examination of the opened stifles at 10-week post-ACL transection showed that dogs 1 and 2 had developed more osteophytoses at the margins of the articular cartilage than dogs 3, 4 and 5. Photos of the opened stifles for dog 1 are shown in Figure 3.2. There was no focal cartilage depletion in any of the stifles (the usual finding at this stage of experimental OA). All the NOP control stifles and the shoulder joints appeared normal. Total cartilage mass per stifle was significantly ($p \leq 0.0004$) increased 1.57±0.18 fold (mean ± SD, n=5) when compared between the OA and the contralateral NOP stifles (Table 3.3).

The total DNA concentration was measured for each cartilage sample and the DNA concentration was used to estimate the cellularity in the cartilage samples. A standard curve for the DNA assay was constructed by linear regression of the calf thymus DNA standards (Figure 3.3). The DNA concentrations for all the samples were calculated from this standard curve (Table 3.4). The total DNA concentration (mg per gram cartilage or mg per stifle) was not significantly different between the OA sample and NOP controls (Table 3.4). This suggested that the number of cells in the cartilage tissue was not significantly changed in the hypertrophic stage of the experimental OA. This lack of increase in the DNA concentration per stifle also suggested that an increased rate of matrix production by chondrocytes in the tissue is an important factors contributing to the increase cartilage mass in the osteoarthritic joints, rather than a proliferation of the cells.

Table 3.3 Total cartilage mass from stifles with experimental OA and the contralateral NOP controls.

Dog	Lab ID	Body wt. (kg)	Cartilage mass (g)/joint OA	NOP	OA/NOP
1	B	30	1.71	0.95	1.80
2	C5	25	1.55	0.98	1.58
3	L138	24	1.99	1.34	1.49
4	L127	25	1.43	1.07	1.34
5	L146	23	1.56	0.94	1.66
Mean		25	1.65	1.06	1.57
SD		2.7	0.22	0.17	0.18

A paired Student's t-test showed that there was a significant increase ($P \leq 0.0004$) in the cartilage mass in the samples with OA when compared with the contralateral NOP controls.

Figure 3.2 Comparison of the cartilage surface in the 10 weeks post-ACL transected and the contralateral NOP stifles.

(a) The contralateral NOP stifle in dog 1.

(b) The 10 weeks post-ACL transected stifle in dog 1. The arrow indicates the osteophytes formed at the margin of the joint.

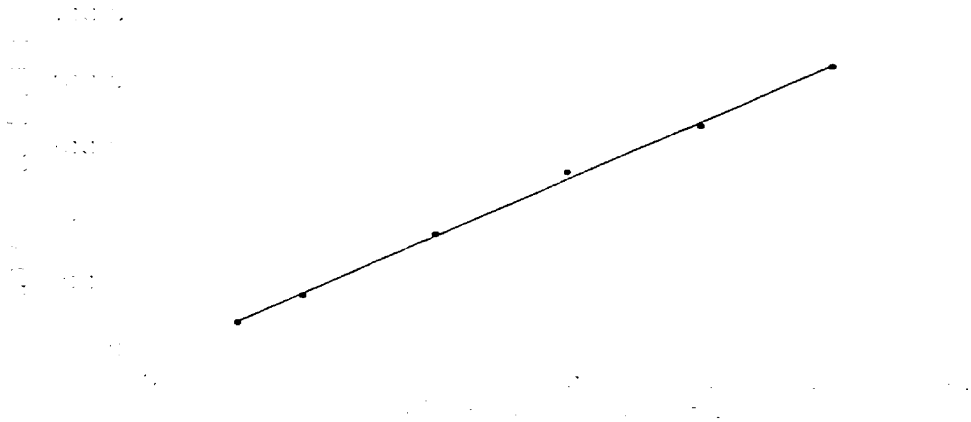


Figure 3.3 Linear regression analysis of the DNA standard.

(The regression coefficient was 0.999).

Table 3.4 Fluorescence assay and DNA concentration in the cartilage in the experimental OA and the contralateral NOP controls.

Dog	F./mg tissue		[DNA] (mg)/g cartilage			[DNA] (mg)/ joint		
	OA	NOP	OA	NOP	OA/NOP	OA	NOP	OA/NOP
1	32.8	27.4	0.40	0.30	1.30	0.70	0.30	2.30
2	20.8	30.3	0.20	0.30	0.70	0.30	0.30	1.00
3	24.5	29.6	0.30	0.30	1.00	0.60	0.40	1.50
4	61.2	31.4	0.70	0.40	1.70	1.10	0.40	2.70
5	29.7	37.4	0.30	0.40	0.70	0.40	0.40	1.00
Mean	33.8	31.2	0.38	0.34	1.08	0.62	0.36	1.70
SD	14.4	4.0	0.19	0.05	0.43	0.31	0.05	0.80

The fluorescence unit (F.) is an arbitrary unit of the luminescence spectrophotometer. The raw data of the sample fluorescence units were within the range of the standard curve. Paired Student's t-tests showed that there was no significant difference between the samples with OA and the NOP controls whether expressed as the total concentration of DNA (mg per gram cartilage, $P \leq 0.2$), or as the total amount of DNA (mg per joint, $p \leq 0.06$).

The total RNA concentration in each sample was estimated by spectrophotometry (OD260nm) (Table 3.5). The amount of total RNA used in the quantitative RT-PCR was based on the OD260nm value. Because any contaminants in total RNA samples might have influenced the optical density at 260nm, the total RNA concentrations from the value of OD260nm were then normalized with the total mRNA value, which had been measured by a slot-blot hybridization using a radiolabelled oligo (dT) probe (Figure 3.4 and Table 3.6). There were similar trends in the changes of total RNA and total mRNA in all of the five pairs of samples: *i.e.*, both the concentration of total RNA (μg per mg DNA or μg per gram cartilage) and the concentration of mRNA (scan unit per mg DNA or scan unit per gram cartilage) were significantly increased in the OA compared with the NOP controls (Table 3.5 and 3.6).

The positions of the samples in the slot-blot are:

Rows	Column A	Column B	Column C
1	dog 1 OA	dog 3 OA	tRNA
2	dog 1 NOP	dog 3 NOP	Blank
3	dog 6* OA	dog 4 OA	Blank
4	dog 6* NOP	dog 4 NOP	Blank
5	dog 2 NOP	dog 5 OA	Blank
6	dog 2 OA	dog 5 NOP	Blank

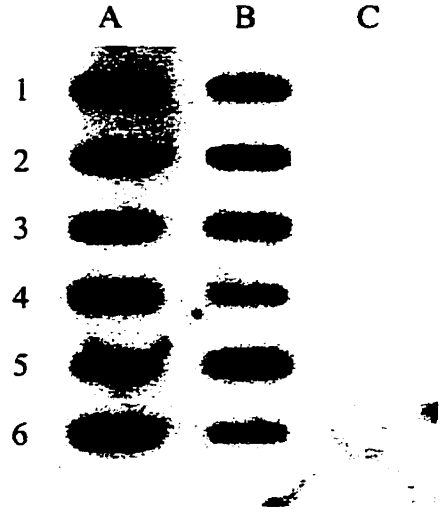


Figure 3.4 Slot-blot hybridization of the total RNA with an oligo (dT) 30-mer probe.

The tRNA did not hybridize with the probe. The slots of C2 to C6 were blanks.

*The data of dog 6 was not included in the final results in this Chapter (see section 5.2 in Chapter 5 for details).

Table 3.5 Total RNA of experimental OA and the contralateral NOP controls.

Dog	Total RNA (μg)/g cartilage			Total RNA (μg)/mg DNA		
	OA	NOP	OA/NOP	OA	NOP	OA/NOP
1	144	58	2.5	359	195	1.8
2	125	77	1.6	625	257	2.4
3	112	46	2.4	375	154	2.4
4	96	55	1.7	137	137	1.0
5	104	48	2.2	348	120	2.9
Mean	116	57	2.1	369	172	2.1
SD	18.7	12	0.4	173	55	0.7

A paired Student's t-test showed that there was significant difference between the OA and NOP samples whether expressed as a total RNA (μg) per gram cartilage ($p \leq 0.0008$) or as total RNA normalized per mg DNA ($p \leq 0.015$).

Table 3.6 Summary of the measurement of the mRNA by slot-blot hybridization with an oligo (dT) probe.

Dog	mRNA*/μg of total RNA			mRNA*/g cartilage			mRNA*/mg DNA		
	OA	NOP	OA/NOP	OA	NOP	OA/NOP	OA	NOP	OA/NOP
1	1.34	1.17	1.15	192	68	2.8	481	227	2.1
2	1.42	1.17	1.21	177	90	2.0	886	300	2.9
3	0.92	0.92	0.99	103	43	2.4	344	142	2.4
4	0.94	0.69	1.37	90	38	2.4	129	94	1.4
5	1.21	0.71	1.69	126	34	3.7	420	75	5.6
Mean	1.16	0.91	1.28	138	54	2.7	452	168	2.9
SD	0.23	0.26	0.26	45	24	0.7	277	94	1.6

Paired Student's t-tests showed that there were significant differences between the OA and NOP samples whether expressed as mRNA (scan units) per gram cartilage ($p \leq 0.0014$) or as mRNA (scan units) per mg DNA ($p \leq 0.017$).

*The mRNA was measured as "scan units" which are arbitrary units of the densitometer.

3.3.2 Quantification of the LC mRNA isoform in the osteoarthritic canine articular cartilage by competitive RT-PCR

Photographs of the EthBr-stained 2% agarose gels of the products of the competitive RT-PCR for the LC isoform and the LCmut2 internal standard are presented in the top panel of Figures 3.5 to 3.9. Linear plots of the logarithm of fmole of the LCmut2 added at RT-PCR vs the logarithm of the ratio of the scan units of the LCmut2 to the scan units of the native LC after RT-PCR are presented in the bottom panel of Figures 3.5 to 3.9. The amount of the PCR products loaded in the agarose gel for the native LC cDNA and the internal standard (LCmut2) were measured as scan units (arbitrary unit of the densitometer) (Table 3.7) or CPM (data not shown) from the slices of the agarose gel containing the samples or standards.

The amounts of the PCR products in the agarose gel for the native LC isoform expressed per 0.080 μg of total RNA in the five osteoarthritic dogs were calculated from the corresponding linear regression curves as the anti-log of the fmole of the LCmut2 at the 1:1 ratio of the logarithmic ratio of the scan units of the LCmut2/native LC (Methods & Application Book 3, by Clontech Laboratories, Inc). This calculation was based on the assumption that when the original amounts of the internal standard and native LC (in the 0.08 μg of total RNA) added in the RT-PCR were at 1:1 ratio, the amounts of the PCR products of both the internal standard and the native LC would be equal. An example of the detail calculation is presented in Appendix 7.2.1 of the thesis.

Except in dogs 1 and 2, in which errors were made in preparing the samples for scintillation counting, the results of the calculated LC isoform expressed were similar when using either the scan units derived from the Polaroid films of the gels of the RT-PCR products or the CPM of the LC and LCmut2 cDNA bands. Therefore, only results from the scan units of the films are presented.

The expression level of the LC isoform (fmole per μg total RNA) in the five dogs were normalized to total RNA in 1 gram of cartilage tissue or in 1 mg DNA. The mean amount of the LC isoform (fmole/g cartilage or fmole/mg DNA) expressed in the five OA

dogs was significantly increased ($p \leq 0.0016$ or $p \leq 0.0078$, respectively) when compared between the OA to the NOP samples were made (Table 3.8). The amount of the LC isoform expressed was $1.8 \times 10^{10} \pm 0.66 \times 10^{10}$ (copies/mg DNA; n=5) in the NOP and $5.7 \times 10^{10} \pm 2.6 \times 10^{10}$ (copies/mg DNA; n=5) in the OA.

Table 3.7 Data of competitive RT-PCR for the LC isoform in the cartilage of experimental OA and contralateral NOP controls.

Dog	LCmut2	LCmut2/LC[b]		Log LCmut2	Log LCmut2/LC[b]		Calculated native LC[c]	
	(fmole)[a]	OA	NOP	(fmole)[a]	OA	NOP	OA	NOP
1							0.025	0.017
	0.0065	0.10	—[d]	-2.187	-1.002	—		
	0.013	0.074	0.54	-1.886	-1.128	-0.264		
	0.019	0.32	1.1	-1.721	-0.490	0.046		
	0.026	8.69	2.9	-1.585	0.940	0.469		
	0.032	—	2.8	-1.495	—	0.453		
2							0.020	0.012
	0.013	0.64	1.1	-1.886	-0.197	0.030		
	0.026	1.3	2.6	-1.582	0.121	0.417		
	0.052	2.3	1.6	-1.284	0.360	0.200		
	0.103	1.5	1.8	-0.987	0.190	0.270		
3							0.014	0.014
	0.0013	0.023	—	-2.886	-1.640	—		
	0.0026	0.15	—	-2.585	-0.834	—		
	0.0065	0.31	0.40	-2.187	-0.502	-0.393		
	0.013	0.62	0.64	-1.886	-0.205	-0.195		
	0.019	—	1.3	-1.721	—	0.130		
	0.026	—	3.1	-1.585	—	0.496		

(Table 3.7 Continued)

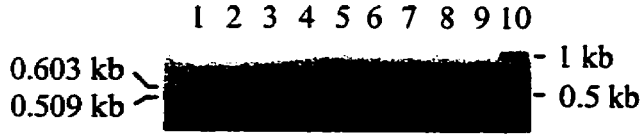
Dog	LCmut2	LCmut2/LC[b]		Log LCmut2	Log LCmut2/LC[b]		Calculated native LC[c]	
	(fmole)[a]	OA	NOP	(fmole)[a]	OA	NOP	OA	NOP
4							0.027	0.014
	0.0065	0.16	__[d]	-2.187	-0.803	__		
	0.013	0.35	0.95	-1.886	-0.455	-0.023		
	0.019	0.64	1.3	-1.721	-0.193	0.126		
	0.026	0.94	1.6	-1.585	-0.025	0.216		
	0.039	__	2.7	-1.409	__	0.431		
5							0.022	0.010
	0.0065	0.32	__	-2.187	-0.492			
	0.013	0.46	1.2	-1.886	-0.338	0.070		
	0.019	0.87	1.7	-1.721	-0.062	0.229		
	0.026	1.2	2.1	-1.585	0.098	0.316		
	0.039	__	2.6	-1.409		0.424		

(a) These are the amounts of LCmut2 mRNA added to the total RNA samples (80 ng per PCR) for RT-PCR;

(b) These are the ratios of LCmut2 to native LC. The LCmut2 and the LC after RT-PCR were measured as scan units. For calculation of this ratio, the LCmut2 was normalized (to account for its smaller size) by multiplying it by 603/509 (Note: The size of the LC cDNA is 603 bp, and the LCmut2 cDNA is 509 bp);

(c) These are the calculated level of the LC isoform expressed in canine stifles (fmole/80 ng total RNA). The method of calculation was explained in section 3.3.2 and in Appendix 7.2.1 of this thesis;

(d) The " __ " are those points that were not assayed because they were expected not to fall into the linear range as estimated from the results of preliminary experiments (data not shown).



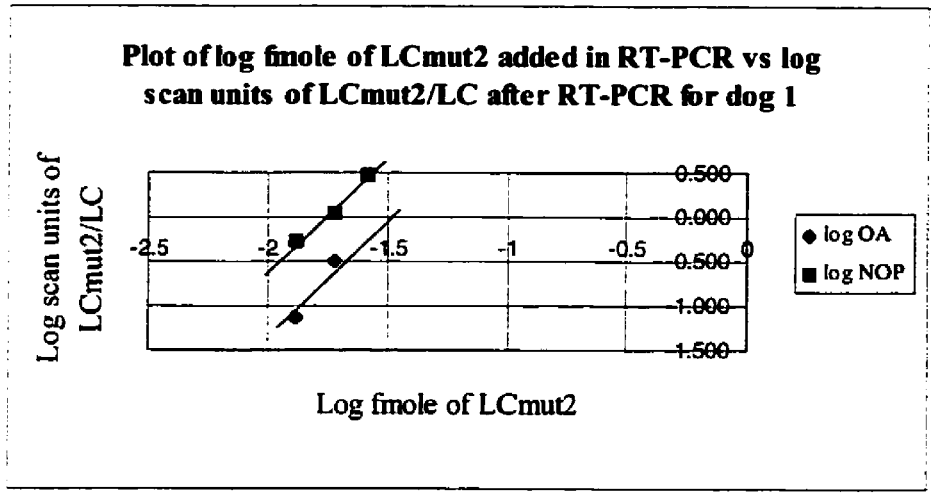
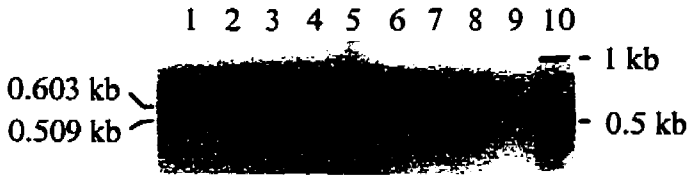


Figure 3.5 Competitive RT-PCR for the LC isoform for dog 1.

Top: Agarose gel electrophoresis of the native LC and LCmut2 after digestion of the PCR products with *BamHI* restriction enzyme: Lanes 1 to 4 are the dog 1 OA samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.0065, 0.013, 0.019, and 0.026 fmole of LCmut2 (original LCmut2 mRNA in RT step), respectively. Lanes 5 to 8 are the dog 1 NOP samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.013, 0.019, 0.026, and 0.032 fmole of LCmut2 (original LCmut2 mRNA in the RT step), respectively. Lane 9 is a blank and lane 10 is a 1 kb DNA ladder.

Bottom: Linear regression plots of the quantitative RT-PCR for the LC isoform from dog 1. Log fmole of the LCmut2 added (original LCmut2 mRNA in the RT step) and Log scan units ratio of LCmut2/native LC after RT-PCR are plotted on the abscissa and ordinate, respectively. There were errors in preparation of the sample points with 0.0065 fmole and 0.026 fmole of LCmut2 in the OA samples (in lanes 1 and 4 of the gel) and 0.032 fmole of LCmut2 in the NOP sample (in lane 8 of the gel). Therefore, they were not included in the plots. The regression coefficient for each line is 0.99.



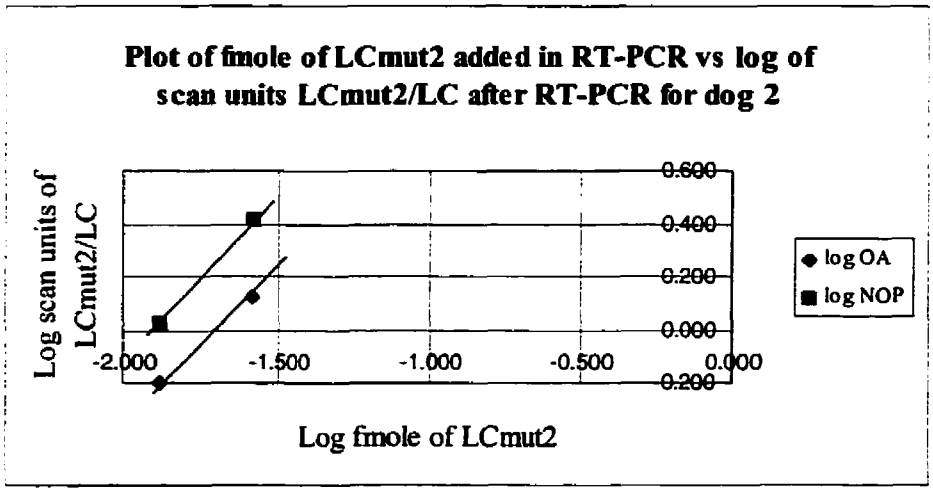


Figure 3.6 Competitive RT-PCR for the LC isoform for dog 2.

Top: Agarose gel electrophoresis of the native LC and LCmut2 after digestion of the PCR products with *BamH*I restriction enzyme: Lanes 1 to 4 are the dog 2 NOP samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.013, 0.026, 0.052, and 0.103 fmole of LCmut2 (original LCmut2 mRNA in the RT step), respectively. Lanes 5 to 8 are the dog 2 OA samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.013, 0.026, 0.052, and 0.103 fmole of LCmut2 (original LCmut2 mRNA in the RT step), respectively. Lane 9 is a blank and lane 10 is a 1 kb DNA ladder.

Bottom: Linear regression plots of the quantitative RT-PCR for the LC isoform from dog 2. Log fmole of the LCmut2 added (original LCmut2 mRNA in the RT step) and Log scan units ratio of LCmut2/native LC after RT-PCR are plotted on the abscissa and ordinate, respectively. There were errors in preparation of the sample points with 0.052 fmole and 0.103 fmole of LCmut2 in the OA sample (in lanes 7 and 8 of the gel) and NOP samples (in lanes 3 and 4 of the gel). Therefore, they were not included in the plots. The regression coefficient for each line is 0.99.

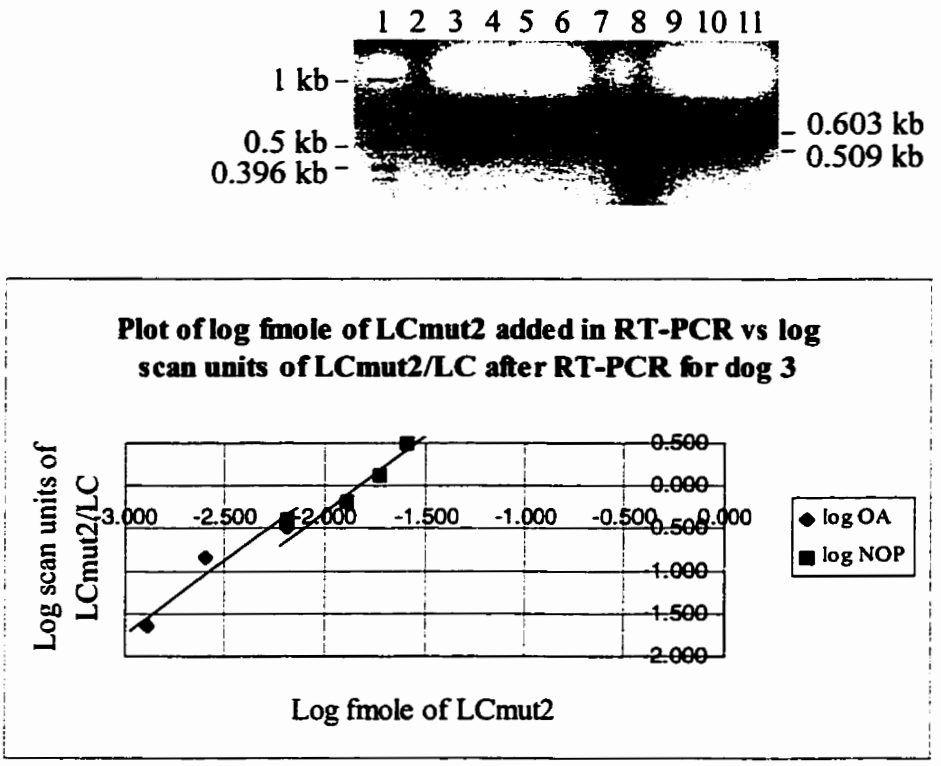


Figure 3.7 Competitive RT-PCR for the LC isoform for dog 3.

Top: Agarose gel electrophoresis of the native LC and LCmut2 after digestion of the PCR products with *BamH*I restriction enzyme. Lanes 3 to 6 are the dog 3 OA samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.013, 0.0065, 0.0026, and 0.0013 fmole of LCmut2 (original LCmut2 mRNA in the RT step), respectively. Lanes 8 to 11 are the dog 3 NOP samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.026, 0.019, 0.013, and 0.0065 fmole of LCmut2 (original LCmut2 mRNA in the RT step), respectively. Lanes 2 and 7 are blanks and lane 1 is a 1 kb DNA ladder.

Bottom: Linear regression plots of the quantitative RT-PCR for the LC isoform from dog 3. Log fmole of the LCmut2 added (original LCmut2 mRNA in the RT step) and Log scan units ratio of LCmut2/native LC after RT-PCR are plotted on the abscissa and ordinate, respectively. The regression coefficient for each line is 0.99.

(Please note that the order of the lanes of agarose gels in this Figure and in Figures 3.8 and 3.9 is different from the order of the lanes of agarose gels in Figure 3.5 and 3.6).

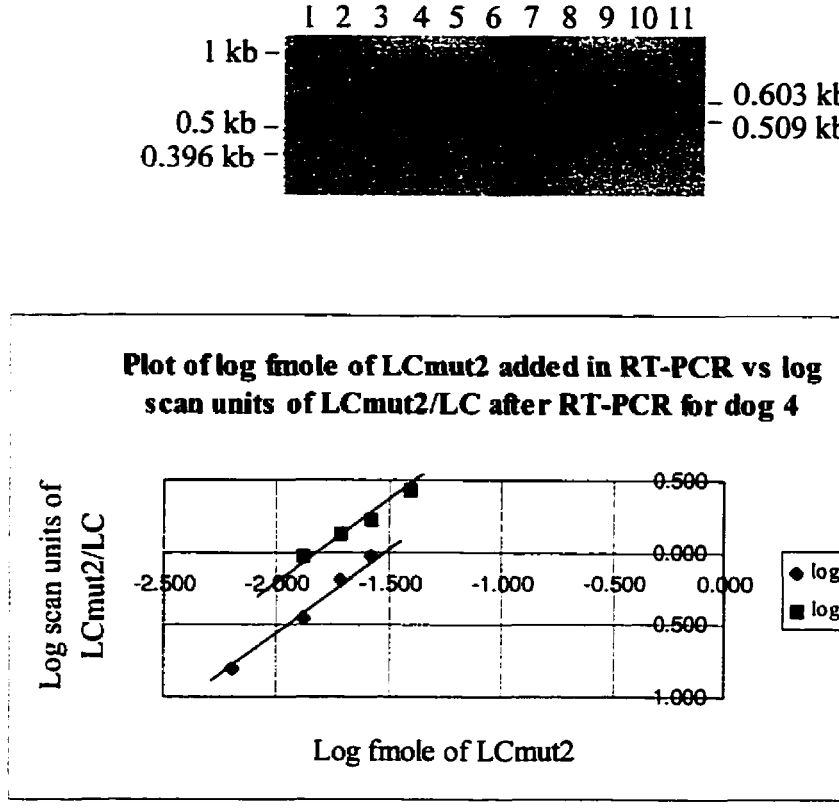
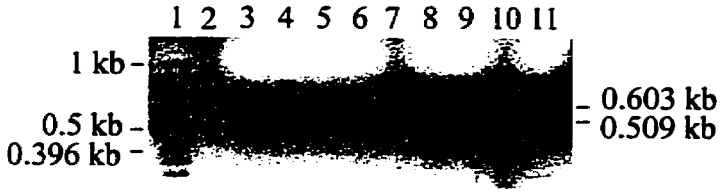


Figure 3.8 Competitive RT-PCR for the LC isoform for dog 4.

Top: Agarose gel electrophoresis of the native LC and LCmut2 after digestion of the PCR products with *BamHI* restriction enzyme: Lanes 3 to 6 are the dog 4 OA samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.026, 0.019, 0.013, and 0.0065 fmole of LCmut2 (original LCmut2 mRNA in the RT step), respectively. Lanes 8 to 11 are the dog 4 NOP samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.039, 0.026, 0.019, and 0.013 fmole of LCmut2 (original LCmut2 mRNA in the RT step), respectively. Lanes 2 and 7 are blanks and lane 1 is a 1 kb DNA ladder.

Bottom: Linear regression plots of the quantitative RT-PCR for the LC isoform from dog 4. Log fmole of the LCmut2 added (original LCmut2 mRNA in the RT step) and Log scan units ratio of LCmut2/native LC after RT-PCR are plotted on the abscissa and ordinate, respectively. The regression coefficient for each line is 0.99.



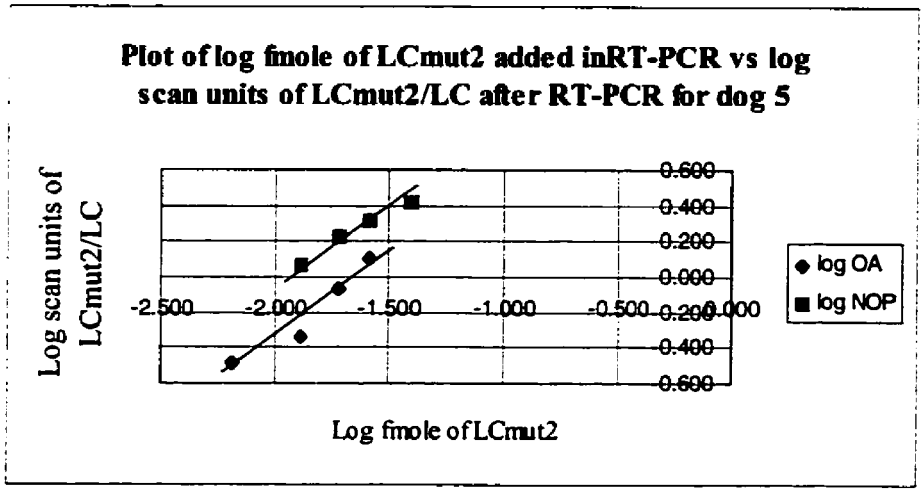


Figure 3.9 Competitive RT-PCR for the LC isoform for dog 5.

Top: Agarose gel electrophoresis of the native LC and LCmut2 after digestion of the PCR products with *BamH*I restriction enzyme: Lanes 3 to 6 are the dog 5 OA samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.026, 0.019, 0.013, and 0.0065 fmole of LCmut2 (original LCmut2 mRNA in the RT step), respectively. Lanes 8 to 11 are the dog 5 NOP samples, each of which contained 80 ng total RNA (equivalent to the original amount of total RNA in the RT step) and 0.039, 0.026, 0.019, and 0.013 fmole of LCmut2 (original LCmut2 mRNA in the RT step), respectively. Lanes 2 and 7 are blanks and lane 1 is a 1 kb DNA ladder.

Bottom: Linear regression plots of the quantitative RT-PCR for the LC isoform from dog 5. Log fmole of the LCmut2 added (original LCmut2 mRNA in the RT step) and Log scan units ratio of LCmut2/native LC after RT-PCR are plotted on the abscissa and ordinate, respectively. The regression coefficient for each line is 0.99.

Table 3.8 Level of expression of the LC mRNA isoform in the cartilage of experimental OA and the contralateral NOP controls.

Dog	fmole / μg total RNA		fmole / g cartilage			fmole / mg DNA		
	OA	NOP	OA	NOP	OA/NOP	OA	NOP	OA/NOP
1	0.32	0.21	46	13	3.6	114	42	2.7
2	0.25	0.15	31	12	2.6	157	39	4.0
3	0.18	0.18	20	8	2.5	67	27	2.5
4	0.34	0.18	33	10	3.4	47	24	1.9
5	0.27	0.12	29	6	4.8	96	15	6.4
Mean	0.27	0.17	32	10	3.4	95	30	3.5
SD	0.06	0.03	9	3	0.9	43	11	1.8

Paired Student's t-tests showed that there was a significant difference between the OA and the NOP samples whether expressed as LC fmole/g cartilage ($p \leq 0.0016$) or as the LC fmole/mg DNA ($p \leq 0.0078$).

3.3.3 Quantification of the ELC isoform in the osteoarthritic canine articular cartilage by RT-PCR with an external standard method

Due to technical reasons (see discussion), a method of quantitative RT-PCR with an external standard was used to quantify the mRNA of the E2-like module (Arcellana-Panlilio & Schultz, 1993). The mRNA of the E2-like module is an appropriate measure of the ELC isoform because there are no other detectable isoforms with the E2-like module in canine aggrecan (see Chapter 2).

The CPM data for the PCR of all the samples and the calculated fmole of E2-like module (representing the ELC isoform) expressed per 0.5 μg of total RNA are listed in Table 3.9. Figure 3.10 shows an agarose gel of the first batch of PCR products of samples and external standard after separation by electrophoresis. Two standard curves of the E2-like module are presented in Figure 3.11. Curve No.1 is the standard curve that was done with the first batch of samples, which included dogs 1 and 2. Curve No. 2 is the standard curve that was done with the second batch of samples, which included dogs 3, 4 and 5. Both standard curves were constructed as linear plots of the log CPM of the 152 bp cDNA (the E2-like module) of the PCR products for the external standard vs the log fmole of the external standard (21E/pCR™II plasmid DNA) added into the PCR reaction. The amounts of the E2-like module (fmole per 0.5 μg of total RNA) in the PCR product in OA and NOP samples for each dog were calculated from the corresponding standard curve. The amount of the E2-like module (fmole/μg total RNA) was normalized to the amount of total RNA in 1 gram cartilage or to 1 mg of DNA (Table 3.10).

The expression of the ELC isoform was increased in the OA samples of four out of the five dogs studied when compared to the respected NOP samples. Despite a large degree of variability among the individual dogs, the mean value for the expression of the ELC isoform for the five dogs (whether calculated as fmole/g cartilage or fmole/mg DNA) was significantly increased ($p \leq 0.027$ or $p \leq 0.048$, respectively) in the OA sample when compared to the NOP controls (Table 3.10). The amount of the ELC isoform expressed was $0.24 \times 10^{10} \pm 0.12 \times 10^{10}$ (copies/mg DNA; n=5) in the NOP and $0.54 \times 10^{10} \pm 0.36 \times 10^{10}$

(copies/mg DNA; n=5) in the OA. In the OA samples, the mean of the expression of the ELC isoform was 9% of the total G3 isoform (the LC and the ELC) mRNA; in the NOP samples, it was 12% (Table 3.11).

Table 3.9 Quantitative RT-PCR for the E2-like module.

Dog	CPM / 0.5 µg total RNA		Log CPM / 0.5 µg total RNA		fmole / 0.5 µg total RNA	
	OA	NOP	OA	NOP	OA	NOP
1	9818	7618	3.99	3.88	0.004	0.001
2	12704	13266	4.10	4.12	0.009	0.010
3	4054	3096	3.61	3.49	0.023	0.017
4	2890	4000	3.46	3.60	0.016	0.023
5	2238	1555	3.35	3.19	0.012	0.008

Table 3.10 The level of expression of the E2-like module in the cartilage of experimental OA and the contralateral NOP controls.

Dog	fmole/µg total RNA		fmole/g cartilage			fmole/mg DNA		
	OA	NOP	OA	NOP	OA/NOP	OA	NOP	OA/NOP
1	0.008	0.002	1.0	0.1	9.8	3.0	0.4	7.5
2	0.018	0.020	2.0	1.0	2.0	11	5.0	2.2
3	0.046	0.034	5.0	2.0	2.5	17	5.0	3.4
4	0.032	0.046	3.0	2.0	1.2	4.0	6.0	0.7
5	0.024	0.016	2.0	0.8	3.3	8.0	2.0	4.0
Mean	0.026	0.024	3.0	1.0	3.8	9.0	4.0	3.6
SD	0.014	0.017	1.0	1.0	3.5	6.0	2.0	2.5

Paired Student's t-tests showed that there was significant difference between the OA and NOP samples for the ELC (fmole)/g cartilage ($p \leq 0.027$) or ELC (fmole)/mg DNA ($p \leq 0.048$).

Table 3.11 Percentage of the ELC and LC isoforms in the total G3 domain of aggrecan per mg of DNA in canine articular cartilage.

Dog	Percentage of the ELC isoform		Percentage of the LC isoform	
	OA	NOP	OA	NOP
1	2.5	0.9	97	99
2	6.7	11	93	89
3	20	16	80	84
4	8.5	20	91	80
5	8.0	10	92	90
Mean	9.2	12	89	88
SD	6.7	7.3	8.0	7.0

This is assumed that only two isoforms (ELC and LC) of aggrecan are expressed in canine articular cartilage.

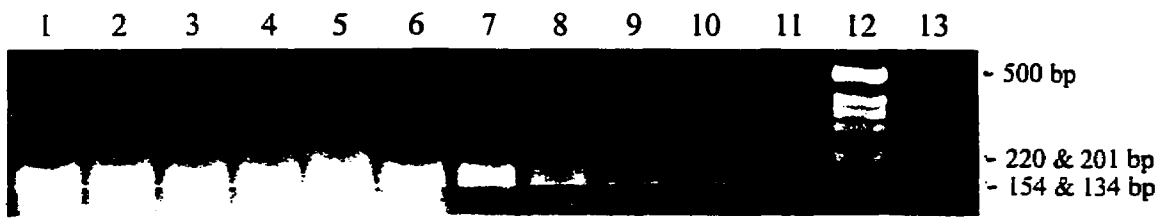


Figure 3.10 Quantitative RT-PCR for the E2-like module.

An example of the 2% agarose gel after electrophoresis of the first batch of PCR products (the E2-like module) for the samples and the external standard (for standard curve No. 1). Lane 13 is a blank, lane 12 is a 1 kb DNA standard; Lane 1=dog 1 OA, lane 2=dog 1 NOP, lane 3 =dog 6 OA, lane 4=dog 6 NOP, lane 5=dog 2 NOP, lane 6=dog 2 OA; lanes 7 to 11 are the external standard (0.025, 0.01, 0.0075, 0.005, and 0.0025 fmole) added into the PCR reactions.

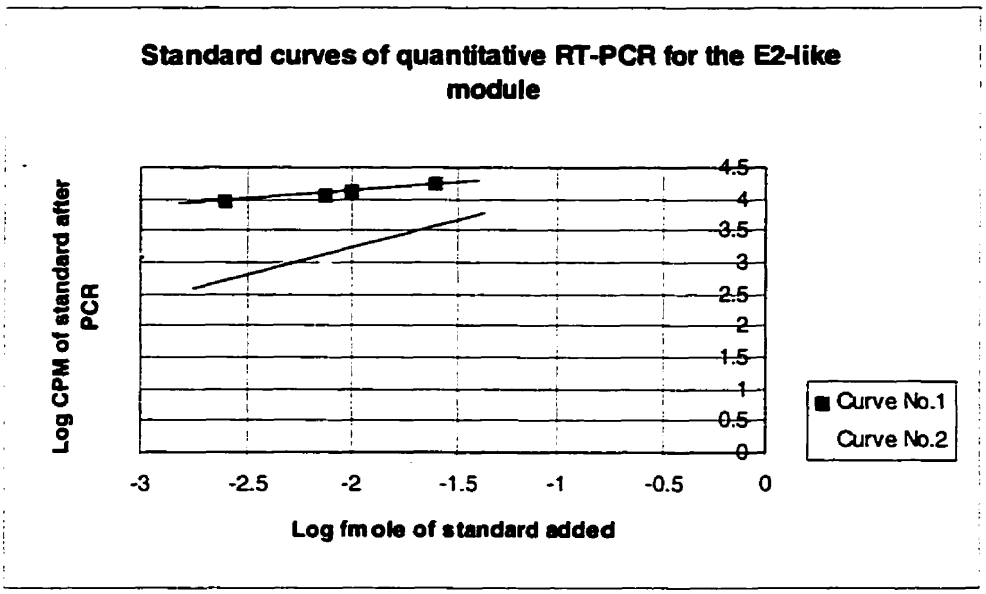


Figure 3.11 Standard curves of the external standard for the E2-like module.

These are linear regression plots of the external standard 21E/pCR™II for quantitative RT-PCR for the E2-like module. The regression coefficient for each curve is >0.99. Logarithmic data for the CPM of the external standard after PCR and the amount of the external standard (fmole) added to the PCR, respectively, was used for the plots. For the standard curve No. 1, the data point for the 0.005 fmole was ignored in the graph because there was an error during its preparation for scintillation counting.

3.4 DISCUSSION

In this study, despite the relative absence of osteophytosis in dogs 3, 4 and 5 (which is very rare at 10 weeks post ACL-transected stifles), the anatomical appearance of the articular cartilage, the changes in the cartilage mass, and the amounts and concentrations of total RNA were consistent with the previous data from this animal model at the hypertrophic stage of experimental OA (Matyas *et al.* 1995; Adams, 1994).

Traditional methods such as Northern hybridization and ribonuclease protection assay are not as sensitive as RT-PCR for quantifying a mRNA. Therefore, we used RT-PCR to quantify the mRNA isoforms of the G3 domain of canine aggrecan of adult articular cartilages from experimental OA and the contralateral NOP controls. There are many acceptable ways to do quantitative RT-PCR (Wiesner, 1992; Yu *et al.* 1991; Arcellana-Panlilio & Schultz, 1993; Wang *et al.* 1989; Gilliland *et al.* 1990), though none of them is 100% ideal. However, when care is taken to minimize the potential error during experiments, all these methods can yield reliable results.

The level of expression of the ELC mRNA isoform is lower than that of the LC mRNA isoform in the adult canine articular cartilage. One of the difficulties in quantifying the ELC isoform by PCR came from the interference with the LC isoform. For example, many primers that could be used for the ELC in PCR also primed or misprimed to the LC isoform. The L2 and R2.1 primers which amplified the E2-like module in the ELC isoform did not have detectable mispriming bands in the PCR products when using specific G3 primer (R3) for the reverse transcription. Thus, the E2-like module could be modified to an internal standard for the competitive RT-PCR to quantify the E2-like module of aggrecan. However, it was estimated that competitive RT-PCR with the internal standard method would require 12.5-fold more total RNA to quantify the ELC isoform than would have been required for the LC isoform. Quantitative RT-PCR with an external standard is a much simpler experimental procedure and uses much less sample than do methods with internal standards. For these reasons, quantitative RT-PCR with an external standard was chosen to quantify the ELC isoform.

One of the major disadvantages of using a method with external standard is that it is difficult to control the sample-to-sample variation. In this study, in order to reduce the sample-to-sample variability, we used a master mix for all the reagents in all the PCR reactions, and the amount of the standard and total RNA of the samples used in the PCR was carefully calibrated.

Although competitive RT-PCR with an internal standard is a better approach to control for PCR variability than the external standard method, it also has its own

limitation. The accuracy of the internal standard method used in this study depends upon the completeness of the restriction enzyme digestion of the internal standard (in this particular study, we used *BamH*I) in order to distinguish the internal standard from the native cDNA after PCR amplification. Even though a large excess of *BamH*I was used, the digestion was not complete. However, we found a much higher percentage of uncut native LC cDNA remained after *Nar*I restriction enzyme digestion than after *BamH*I digestion (> 30% after *Nar*I digestion vs ≤10% after *BamH*I digestion of the highest amount of LCmut2 alone used in the RT-PCR) (data not shown). Thus, only the *BamH*I was used for the digestion of the competitive RT-PCR samples for quantifying the LC isoform.

Possible factors that could contribute to incomplete restriction enzyme digestion include: formation of heterodimers between the internal standard and the native cDNA, which was not determined in this study and interference of proteins in the reaction mixture during restriction enzyme digestion. The PCR cycle number was optimized to be within the linear range for this study so that the reagents would be sufficient for the PCR reaction. This also should minimize the formation of the heterodimers. We did not extract the PCR samples with phenol and chloroform, which could have removed most of the proteins in the PCR sample and thus might have improved efficiency of the restriction enzyme digestion. However, new errors could have been introduced if phenol chloroform extraction were carried out, and this would potentially increase the variation in the samples.

One of the advantages of using the internal standard LCmut2 in this study was that this internal standard is almost identical to the native LC cDNA except that two nucleotides were changed by PCR site-directed mutagenesis. Therefore, it should have a similar efficiency in RT-PCR as the native LC when the amount of the internal standard added and the gene being tested were close to a 1:1 ratio.

From this study, it was found that there was a large variability in the expression both of the G3 isoforms of canine aggrecan. The level of expression of the ELC isoform was less than that of the LC isoform in adult canine articular cartilage. The expression of both ELC and LC mRNA isoforms of canine aggrecan was significantly increased in the

hypertrophic stage of the experimental OA. This may reflect that the overall rate of synthesis of aggrecan was increased in the experimental OA.

The biological function of the G3 domain is unknown. It has been reported that G3 domain containing the LC isoform which was expressed as a recombinant peptide could bind to galactose and fucose (Halberg *et al.* 1988). There are reports that the E-like module in proteins other than aggrecan binds to calcium ions and that influences its function (Handford *et al.* 1995). There is also evidence that the G3 domain may help in transport of aggrecan to the extracellular millieu and, thus, may be crucial for aggrecan secretion (Luo *et al.* 1996; Vertel *et al.* 1993). Irrespective of its function, the G3 domain of aggrecan could be released from aggrecan in the tissue because a putative "aggrecanase" cleavage site exists in the C-terminal portion of the CS-II domain (see Chapter 2). Released fragments of aggrecan containing the G3 domain may have physiological and pathological roles in normal and diseased cartilage. In future studies, it will be important to define both the specific binding of the G3 isoforms of aggrecan to cartilage molecules and the physiological and pathological functions of the G3 isoforms.

CHAPTER 4.

4. PRODUCTION OF RECOMBINANT PEPTIDES OF THE ISOFORMS OF THE G3 DOMAIN OF CANINE AGGRECAN IN THE PMAL-C2 EXPRESSION SYSTEM

4.1 INTRODUCTION

The third globular domain (G3) of canine aggrecan contains three different modules: an epidermal growth factor 2-like module (E2), a lectin-like module (L), a complement regulatory protein-like module (C), and a module containing the C-terminal 25 amino acids. The exact function of the G3 domain of aggrecan is unknown. The G3 domain of aggrecan is subject to mRNA alternative splicing (Baldwin *et al.* 1989; Fülöp *et al.* 1993). Two mRNA isoforms (LC and ELC) were positively identified as expressed in articular cartilage of normal young dogs, normal adult dogs, and experimentally osteoarthritic adult dogs (see Chapter 2 for details). Expression of the ELC and LC isoforms is increased in the hypertrophic stage of experimentally induced osteoarthritis (OA), although it varied among dogs (see Chapter 3 for detail).

The pattern of the disulfide bonds were mapped by analysis of the disulfide-bonded peptides from three globular domains of aggrecan (Sandy *et al.* 1990). The secondary structure of the LC isoform of the G3 domain of aggrecan was predicted by the authors (Figure 4.1) (Sandy *et al.* 1990). The disulfide bonding and the secondary structure of the E2-like module has been predicted in another report (Fülöp *et al.* 1993). Comparison of the secondary structures of human EGF and the E2-like module of aggrecan is presented in Figure 4.2.

The exact function of the G3 domain is not clear. The G3 domain of rat aggrecan has been expressed using an *in vitro* transcription and translation system and a lectin-like activity (similar to C-type animal lectin) was found in the G3 domain with galactose and

fucose as the preferred ligands (Halberg *et al.* 1988). Recombinant peptides of the G3 domain were also produced in an *Escherischia coli* (*E. coli*) system (plasmid vector: pINIIIompA2, host cell: JA221) and their binding to a variety of monosaccharides was studied (Saleque *et al.* 1993). The CD spectrum of the recombinant peptides of the G3 domain was similar to that of the domain found in a serum mannose-binding protein (Saleque *et al.* 1993). Thus, the L-like module is also labelled a carbohydrate recognition domain (CRD). There is a great deal of similarity between the solution structure of link protein and C-type lectins (Kohda *et al.* 1996), and the L-module contains BX_7B (B = basic amino acids; X = other amino acids) sequences, which has been reported to be a hyaluronan binding motif (Yang *et al.* 1994), suggesting the C-type lectin module may bind hyaluronan.

In this study, in order to generate reagents to study the function of the G3 domain, we used the pMAL-c2 expression system to produce recombinant proteins of canine aggrecan fused to maltose binding protein (MBP-ELC and MBP-LC) (Figure 4.3). The recombinant peptides for the isoforms of the G3 domain will be used for raising polyclonal antibodies, and could be used as competitive blocking reagents. These recombinant peptides will facilitate future studies of the structure and function of the G3 domain of aggrecan both *in vitro* and *in vivo*.

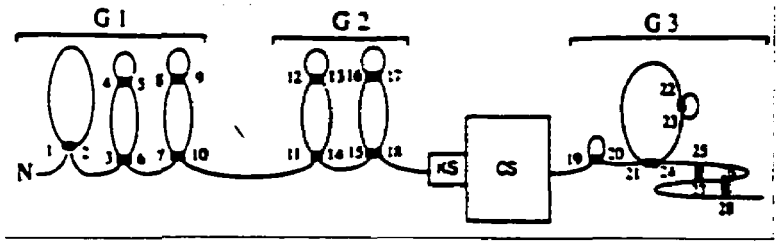


Figure 4.1 Assignments of disulfide bonds for three globular domains of aggrecan.

The numbers represent the cysteine residues numbered sequentially from the N-terminal to the C-terminal end of aggrecan (Sandy *et al.* 1990). Note: The EGF-like module is not shown in this G3 domain.

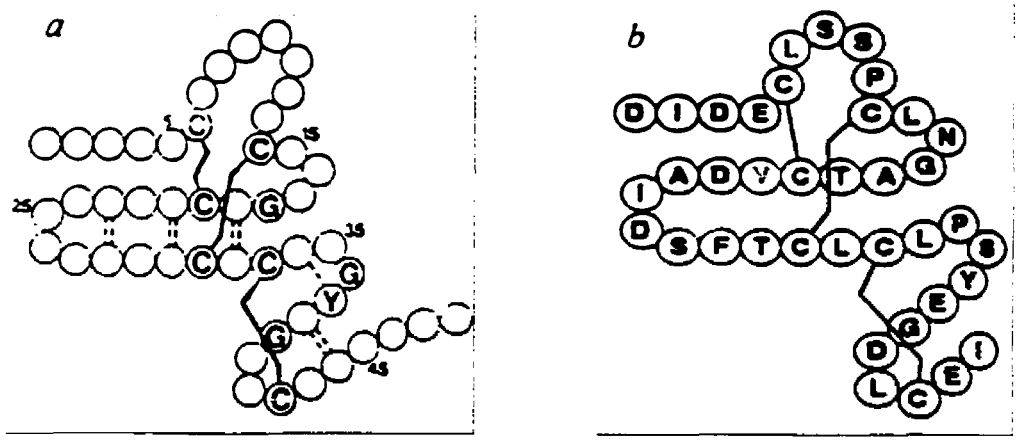


Figure 4.2 Comparison of the secondary structure of human EGF with the predicted structure of the E2-like module of aggrecan.

a. Human EGF with aa numbers starting at the N-terminus (Cooke *et al*, 1987). The marked amino acids are those highly conserved in EGF and EGF-like modules.

b. The E2-like module of aggrecan (Fülöp *et al.* 1993). The first D residues in this figure is at the N-terminal side of the module.

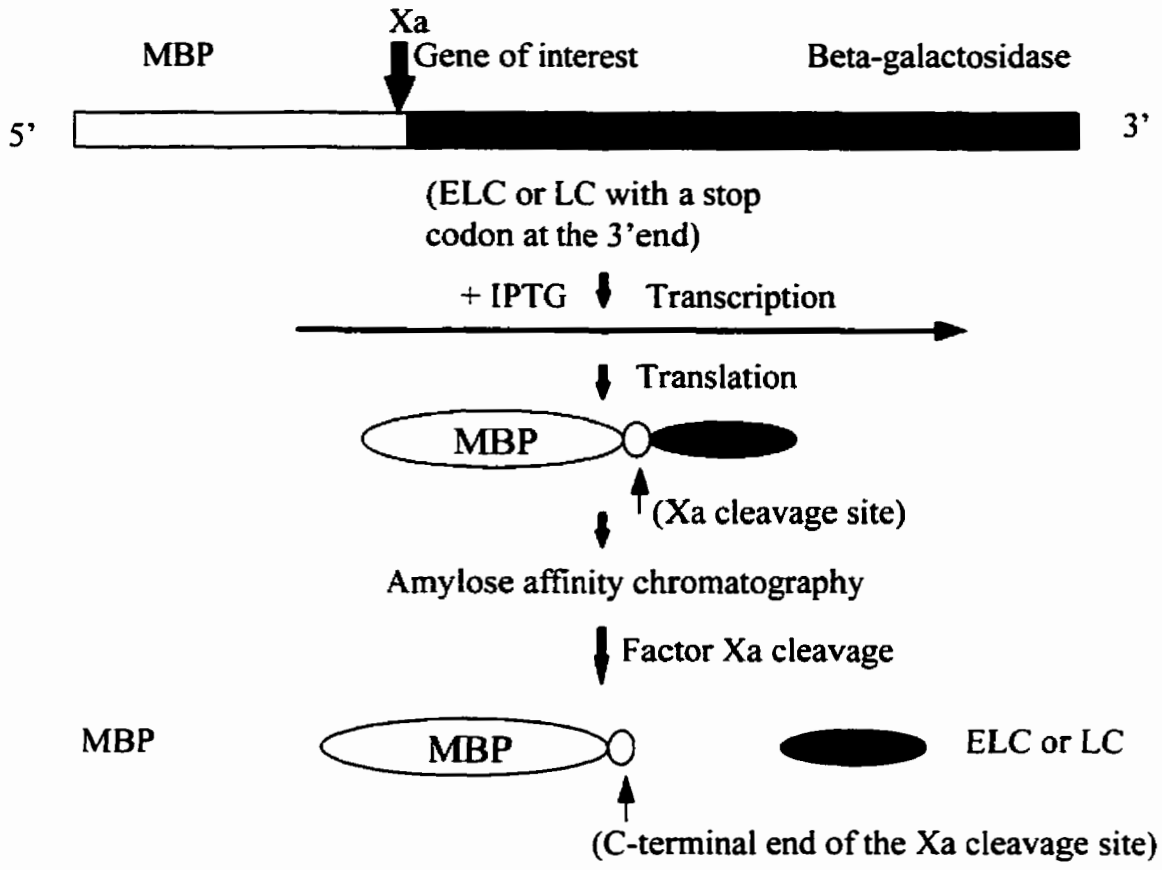


Figure 4.3 Schematic diagram of the production of recombinant fusion proteins in the pMAL-c2 expression system.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The cDNA Cycle™ kit was from Invitrogen (San Diego, CA). T4 DNA ligase and its 10×reaction buffer, the pMAL-c2 expression system, pre-swollen amylose resin, and coagulation factor Xa were from New England Biolabs (Mississauga, ON). *E. coli* host cells JM109, the Pfu DNA polymerase, and 10×Pfu buffer were from Stratagene (La Jolla, CA). Agarose, a 1 kb DNA ladder, calf intestine alkaline phosphatase, restriction enzymes and their stock buffers, as well as isopropyl β-D-thiogalacto-pyranoside (IPTG) were from GIBCO/BRL (Burlington, ON). The Wizard™ Plus Miniprep DNA Purification kit was from Promega (Madison, WI). A Bradford Protein Detection kit, low M.W. protein standards, polyvinylidene difluoride (PVDF) membranes, mini polyacrylamide gel apparatus, all the reagents for polyacrylamide gel, and the Bio-Gel® P-60 resin were from Bio-Rad (Mississauga, ON). Ampicillin, 2-mercaptoethanol, phenyl methyl-sulfonyl fluoride (PMSF), and maltose were from Sigma (St. Louis, MO). Methanol (HPLC grade), isopropanol, glacial acetic acid (analytical grade), and Tween 20 were from Fisher Scientific (Ottawa, ON). Three canine aggrecan specific oligonucleotides for use as primers were synthesized by the Calgary Regional DNA Synthesis Laboratory, University of Calgary, AB and they are listed in Table 4.1. The Taq DyeDeoxy™ Terminator Cycle Sequencing kit was from Applied Biosystems Canada Inc. (Mississauga, ON).

Standard methods (Sambrook *et al.* 1989) were followed for preparations of LB broth, LB-ampicillin agar plates, competent *E. coli* cells, TE buffer, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and 1× and 2×SDS-PAGE sample buffer. Stock IPTG (0.1 M) was prepared in autoclaved double distilled water (ddH_2O) and stored at -20°C. Stock PMSF (100 mM) was prepared in isopropanol and stored at -20°C.

Table 4.1 Oligonucleotides used for subcloning of the G3 isoforms.

Primers	DNA sequence	Tm[d]	Location[e]
Fegf2	5'-d*GGGGAATTC*[a]-ATTCCAGACATTGACGAGTGC-3' (S[c])	73	748-763
FLEcoRI	5'-d*CGCGAATTC*-CAGGAGCTGTGTGAGGAGGG-3' (S)	70	871-890
RCNBamHI	5'-d*CGCGGATCCTTA*[b]-GGAGGGGTCTGTGCAGGT-3' (A[c])	70	1426-1443

(a) The 5' extensions (additional sequences added to the aggrecan specific primers) are in italic font and separated from the canine aggrecan specific sequence by a hyphen. Restriction enzyme sites are added to the beginning (*EcoR*I) and the end (*BamH*I) of the LC and ELC constructs (underlined);
(b) There is a stop codon (TTA) in the RCNBamHI primer. This stop codon is added to the end of the LC and the ELC constructs;
(c) S = sense strand, and A = antisense strand;
(d) Tm = melting temperature at °C;
(e) The locations of the sequence for the oligonucleotides specific to canine aggrecan are referred to Figure 2.3.

4.2.2 Subcloning of the isoforms of the G3 domain of canine aggrecan from pCR™II to the pMAL-c2 plasmid vectors

Two cDNA clones derived from alternatively spliced G3 isoforms of canine aggrecan were subcloned into pMAL-c2 vector by polymerase chain reaction (PCR) manipulation (from the LC and ELC in pCR™II vector of Invitrogen, both without the last 25 amino acid terminus) (Figure 4.4). Two separate PCR reaction mixtures were prepared:

	Mixture (1)	Mixture (2)
LC/pCR™II plasmid (0.19 μg/μl)	2.6 μl	—
ELC/pCR™II plasmid (0.24 μg/μl)	—	2.1 μl
ddH_2O	28.9 μl	29.4 μl
dNTPs (25 mM each)	1 μl	1 μl
10×Pfu buffer	5 μl	5 μl
Fegf2 (10 pmole/μl)	—	6 μl
RCNBamHI (10 pmole/μl)	6 μl	6 μl
FLEcoRI (10 pmole/μl)	6 μl	—
Pfu (2.5 unit/μl)	0.5 μl	0.5 μl

The PCR conditions were: 95°C for 3 min, followed by 30 cycles of 94°C 1 min, 58°C for 1 min and 72°C for 1.5 min, then a last cycle of 72°C for 10 min. The PCR products were fractionated in a 2% low melting point (LMP) agarose gel. The cDNAs with correct sizes were purified from the gel by a combination of heating at 68°C and phenol extraction (see Chapter 2 for details). The gel-purified PCR products were digested with *EcoR*I and *BamH*I restriction enzymes, fractionated in a 2% LMP agarose gel, and then the cDNA was purified from the agarose gel, as described above.

To prepare the plasmid for ligation, about 22 μg of the pMAL-c2 plasmid was digested with the *EcoR*I and *BamH*I restriction enzymes. The plasmid was dephosphorylated by the addition of 2.3 units of calf intestinal alkaline phosphatase and incubated at 37°C for 1 hour. The reaction was stopped by the addition of 8 μl of 0.5 M EDTA, pH 8.0 and followed by extraction with one volume of Tris-buffer-saturated phenol:chloroform/isoamyl alcohol (25:24:1). The linearized and dephosphorylated plasmid was purified by electrophoresis in LMP agarose gel as described above. The purified pMAL-c2 plasmid prepared above (*i.e.*, linearized by *EcoR*I and *BamH*I restriction enzymes and dephosphrylated) was aliquoted into two microfuge tubes.

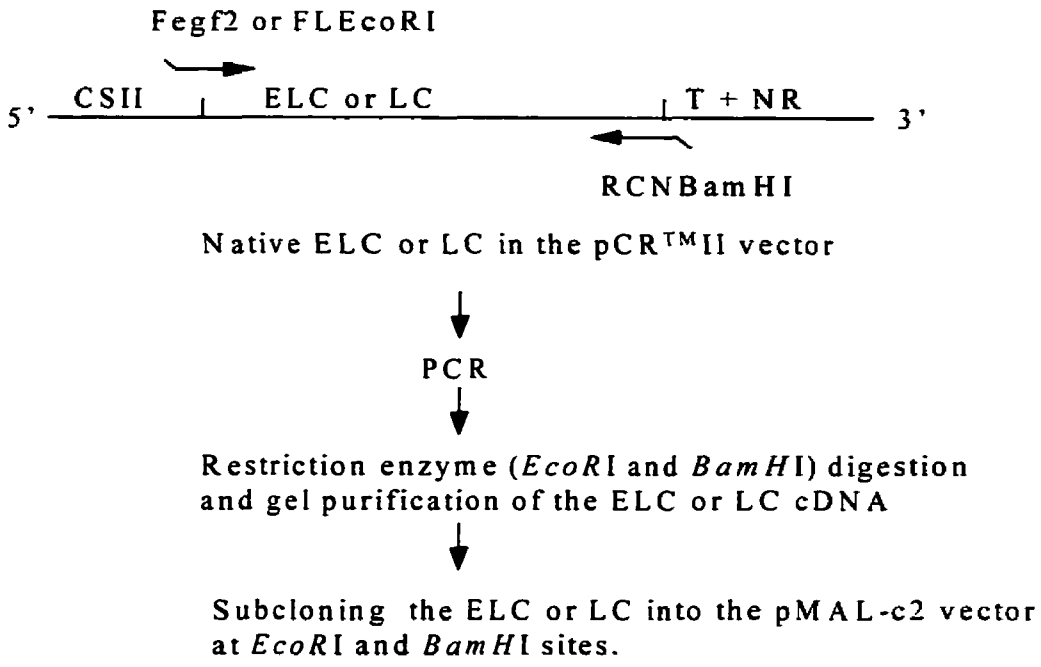


Figure 4.4 Schematic diagram of the PCR manipulation and subcloning of the LC and ELC from the pCR™II vector into the pMAL-c2 vector.

T= the last 25 amino acid of the C-terminus; NR=3' non-translated region.

For the ligation, each sample of pMAL-c2 that had been digested with *EcoR*I and *BamH*I and dephosphorylated as described above, and all of the gel-purified PCR product of the ELC or LC that had been digested with *EcoR*I and *BamH*I, respectively, were dissolved in 16 µl of ddH_2O (total volume). They were incubated at 65°C for 5 min, then cooled on ice for 5 min. To each reaction tube, 2 µl of 10×T4 DNA ligation buffer and 2 µl of T4 DNA ligase was added. They were incubated at 16°C overnight, then at 65°C for 5 min and finally chilled on ice for 5 min.

For the transformation reaction, each ligation sample, prepared as described above, was mixed with 25 µl of competent cells (JM109). They were incubated on ice for 5 min, followed by incubation at 42°C for 2 min, then on ice for 30 seconds. To each tube, 0.1 ml of LB broth was added and incubated at 37°C with shaking at 225 rpm for 20 min. They were briefly microfuged and resuspended in 0.1 ml fresh LB broth. Aliquots (0.05

ml/plate) of the samples were spread on LB-ampicillin (100 μg/ml) plates. They were incubated at 37°C overnight.

Several random clones were chosen for preparing plasmid DNA using the Wizard™ Plus Plasmid DNA Purification System. They were mapped by restriction enzyme digestion (*EcoR*I and *BamH*I). Positive clones of the LC/pMAL-c2 and ELC/pMAL-c2 were prepared for DNA sequencing using the Taq DyeDeoxy™ Terminator Cycle Sequencing kit and sequenced in the Calgary Regional DNA Sequencing Laboratory.

4.2.3 Production and partial purification of the recombinant peptides of the G3 isoforms as fusion proteins to maltose binding protein

4.2.3.1 Small scale preparation of recombinant fusion proteins

Protocols for producing the recombinant fusion proteins (MBP-LC and MBP-ELC) were derived from the manufacturer's manual (New England Biolabs). First, small scale production of the recombinant fusion protein was performed. *E. coli* (JM109) cells containing the cloned ELC or LC in pMAL-c2 plasmid (from section 4.2.2) were grown in a the LB/ampicillin plate at 37°C overnight and a single colony of LC or ELC clone was inoculated in 2 ml LB broth containing 100 μg/ml ampicillin and incubated at 37°C overnight with shaking at 300 rpm. A sample (0.8 ml) of the overnight culture was transferred into 80 ml of fresh LB/ampicillin (100 μg/ml) medium and incubation was continued as above until the cell density reached 2×10^8 cells/ml (OD_{600nm} about 0.5). One ml of the cells was transferred into a microfuge tube, centrifuged 2 min, and the cell pellet was dissolved in 0.1 ml of 2×SDS-PAGE sample buffer (labelled as "uninduced cells"). IPTG (final concentration at 0.3 mM) was added into the remaining culture and incubation was continued for a further 2 hours. One ml of the sample was transferred into a microfuge tube, centrifuged 2 min, and the cell pellet was resuspended in 0.1 ml 2×SDS-PAGE sample buffer (labelled as "induced cells").

The remaining sample was dispensed into 2 centrifuge tubes (labelled as No. 1 and 2) and the cells were harvested by centrifugation at 4000***g*** for 10 min. The cell pellet in

sample No. 1 was resuspended in 5 ml of lysis buffer (10 mM phosphate, pH 7.2 / 30 mM NaCl / 0.25% Tween 20) and frozen at -20°C overnight. The sample was thawed on ice, the cells were broken by sonicating 2 min with a microtip (Branson Sonifier Cell Disruptor 200, Bronson Ultrasonics, Markham, ON) and 0.6 ml 5 M NaCl was added into the sample. The sample was centrifuged at 9000*g* for 20 min at 4°C; the supernatant was saved and labelled as "soluble crude extract". The cell pellet was dissolved in 0.1 ml of 2×SDS-PAGE buffer and labelled as "insoluble sample".

The "soluble crude extract" sample was further analyzed for its ability to bind to amylose resin. First, 0.2 ml of amylose resin was washed twice in 1.5 ml of column buffer (10 mM phosphate, pH 7.2 / 0.5 M NaCl / 1 mM sodium azide). The resin was resuspended in 0.2 ml of column buffer to make a slurry of about 50% amylose resin. Fifty µl of this amylose resin was mixed with 50 µl of the "soluble crude extract", incubated on ice for 15 min, centrifuged 1 min 10,000*g* in a microfuge, and the supernatant was discarded. The resin was washed with 1 ml of column buffer, centrifuged 1 min as described above, and the supernatant was discarded. The resin was resuspended in 50 µl of 2xSDS-PAGE buffer and labelled as "amylose bound soluble crude extract". All the samples prepared above, including the "uninduced cell, induced cell, insoluble sample, soluble crude extract and amylose bound soluble extract", were boiled for 5 min. They were then analyzed in 12% or 15 % SDS-PAGEs (8 µl / lane).

Sample No. 2 was stored at -20°C. Using the above method, it was possible to detect the MBP-LC and MBP-ELC fusion recombinant proteins in the No. 1 sample. Therefore, it was not necessary to analyze the No. 2 sample using an alternate method.

4.2.3.2 Large scale preparation of recombinant fusion proteins

For the large scale preparation of the recombinant fusion proteins, a single colony of the LC/pMAL-c2 or the ELC/pMAL-c2 clone was inoculated in 30 ml LB/ampicillin (100 µg/ml) medium and cultured overnight at 37°C with shaking at 300 rpm. Ten ml of the overnight culture was added to 1 liter of fresh LB/ampicillin (100 µg/ml) medium and cultured as described above until the OD_{600nm} was about 0.5. Three ml of stock IPTG (0.1

M) was added to the cell suspension and the incubation was continued for 2 hours. Samples (1 ml) of the cell suspension before and after IPTG induction were collected for analyzing the production of the recombinant fusion protein by SDS-PAGE as described in section 4.2.3.1.

All the remaining cells were harvested by centrifugation at 4000*g* for 20 min, the cell pellet was resuspended in 50 ml lysis buffer with a fresh addition of 0.5 ml of stock PMSF (100 mM), and the sample was frozen at -20°C overnight. The sample was thawed on ice and the cells were broken by sonication until the release of the protein from the cells reached a maximum (monitored by the Bradford assay). NaCl (final concentration of 0.5 M) was added to the sample and it was centrifuged at 9000*g*, at 4°C for 30 min. The cell pellet (insoluble sample) was stored at -20°C. The supernatant (soluble crude extract) was saved for amylose affinity chromatography (see below). The protein concentration in the supernatant was measured by the Bradford assay. The protein concentration in the crude extract was kept below 2.5 mg/ml, otherwise, the sample would have to be diluted with column buffer.

To prepare the amylose resin for affinity chromatography, 20 ml of pre-swollen amylose resin was de-gassed at room temperature with an aspirator for 20 min and poured into a 3×23 cm (inner diameter×length) Econo column (Bio-Rad). The column was washed with 3 bed volumes of the column buffer containing 0.25% (v/v) Tween 20 and then washed with 5 bed volumes of column buffer without Tween 20. The recombinant fusion protein (MBP-LC or MBP-ELC) was eluted with column buffer containing 10 mM maltose. The eluate was collected into 20 tubes at 3 ml/tube. The flow rate of the column was 1 ml/min. The Bradford assay was performed on the eluate and the protein containing fractions were pooled.

Small samples of the recombinant fusion proteins that had been purified by amylose affinity chromatography were analyzed by SDS-PAGE and the remaining samples were dialyzed against 2 liters of 0.5 M ammonium acetate, pH 6.0 three times, and then lyophilized.

To purify further the recombinant fusion proteins, a Bio-Gel® P-60 gel filtration column (0.75×30 cm, inner diameter×length) was prepared and equilibrated with 5 bed volumes of 0.1 M ammonium bicarbonate. The MBP-LC or MBP-ELC recombinant proteins prepared by amylose affinity chromatography was redissolved in 0.1 M ammonium bicarbonate, loaded onto a Bio-Gel® P-60 column, and eluted with 0.1 M ammonium bicarbonate (2 ml/tube, a total of 30 tubes) with flow rate 0.33 ml/min. The protein containing fractions (in the void volume) were assayed by the Bradford assay and were pooled. Small samples of the pooled fraction were analyzed by SDS-PAGE and the remainder was lyophilized.

4.2.4 Analytical procedures

4.2.4.1 General method for analysis of protein

Protein concentrations were measured by the method of Bradford, using Bio-Rad's Bradford Protein Detection kit. SDS-PAGE (12% or 15% gels) was performed by the method of Laemmli (Laemmli, 1970). Proteins in the SDS-PAGE gels were detected by staining with Coomassie brilliant blue (0.1% Coomassie® Blue R-250, w/v in 40% methanol, v/v, and 10% acetic acid, v/v) and destaining with 40% methanol (v/v) / 10% acetic acid (v/v).

4.2.4.2 Preparation of samples for amino acid analysis

For transferring proteins onto PVDF membrane, the manufacturer's (Bio-Rad) protocol was followed. The PVDF membrane was pre-wetted with 100% methanol for a few seconds, then equilibrated with Towbin buffer (25 mM Tris base / 192 M glycine / 20% methanol) for a few minutes. The SDS-PAGE gels of the MBP-ELC and MBP-LC were transferred onto PVDF membranes in pre-chilled Towbin buffer at 250 mA (constant current) at 4°C for 90 min. The membranes were stained with Coomassie brilliant blue (0.025%, w/v, Coomassie® Blue R-250 in 40% methanol, v/v), and destained with 50% methanol (v/v). The membrane was air dried, sealed in a vacuumed plastic bag, and stored

at -20°C. This was used for amino acid composition analysis by the Protein Core Laboratory, University of Calgary, AB.

4.2.4.3 Cleavage of MBP from LC or ELC by coagulation factor Xa

A sample of each recombinant fusion protein (MBP-LC or MBP-ELC) was dissolved in 20 µl of factor Xa buffer (20 mM Tris·HCl, pH 8.0 / 100 mM NaCl / 2 mM $CaCl_2$ / 1 mM sodium azide) at 1 mg/ml. One µl (0.2 mg/ml) of factor Xa was added to each sample and the samples were incubated at room temperature. At 2, 4, 8 and 24 hours, 5 µl of each sample was removed from the reaction, mixed with 5 µl of the 2×SDS-PAGE sample buffer and stored at -20°C. The negative control contained only 5 µl of the MBP-LC or MBP-ELC alone. All the samples were analyzed by SDS-PAGE.

It was known from the manufacturer (New England Biolabs) that if the disulfide bonds of the factor Xa were disrupted, it would dissociate into two smaller peptides which would co-migrated with LC or ELC, respectively, in a SDS-polyacrylamide gel. This problem could be solved by dissolving the sample in SDS-PAGE sample buffer containing neither dithiothreitol nor 2-mercaptoethanol and incubating it at 65°C instead of boiling.

4.3 RESULTS

4.3.1 Production and partial purification of the recombinant fusion proteins

After subcloning of LC or ELC cDNA from pCR™II vector into the pMAL-c2 vector by PCR manipulation, the new clones (LC/pMAL-c2 and ELC/pMAL-c2) were verified by DNA sequencing. Analysis of the cells before and after IPTG induction by SDS-PAGE showed that there was an enhanced protein band with the expected M.W. of the recombinant fusion protein in the corresponding IPTG-induced samples (MBP-ELC = 71.7 kD and MBP-LC = 67.5 kD) (Figure 4.5). The cells containing the vector plasmid alone produced MBP-β-gal-α fusion protein alone with a MW of 52 kD (data not shown). The amylose-bound soluble fraction of the recombinant fusion proteins from small scale preparation of the samples are shown in Figure 4.6.

A large proportion of the recombinant fusion proteins remained in the insoluble fraction of the samples, as estimated from the SDS-polyacrylamide gels (data not shown). The yield of the soluble fusion proteins for both MBP-LC and MBP-ELC after amylose affinity chromatography was about 2 to 3 mg per liter of culture medium, with variable degree of contamination by impurities. Gel filtration on Bio-Gel® P-60 improved the purity of the samples that had been obtained from amylose chromatography, with good recovery; but the fusion proteins were not purified to homogeneity (data not shown).

4.3.2 Analysis of the G3 recombinant fusion proteins

From the cDNA-deduced amino acid sequences, it is known that there is no Xa cleavage site in the LC or ELC amino acid sequences. In a trial of cleavage of MBP from the LC or ELC in the fusion protein by factor Xa using the large scale preparation of the recombinant fusion proteins purified only by amylose affinity chromatography, the fusion proteins were only partially cleaved by the enzyme (data not shown). This might due to that the samples contained many impurities which might have interfered with the enzyme's function. However, the recombinant fusion proteins that were purified by combination of amylose affinity chromatography and Bio-Gel® P-60 gel filtration were more easily cleaved by factor Xa (Figure 4.7). The MBP after Xa cleavage had the expected MW (42 kD), which suggests that the MBP is stable during the Xa treatment. However, neither the LC nor ELC were clearly identified in the SDS-PAGE after Xa cleavage of the recombinant fusion proteins (Figure 4.7). The difficulty in identifying the LC or ELC band after Xa digestion may be due to partial degradation of the LC or ELC recombinant peptides during Xa treatment.

The manufacturer (New England Biolabs) reports the M.W. of the MBP to be 42 kD, yet the calculation of the M.W. deduced from the cDNA sequence of MBP yields 49 kD. The M.W.s deduced from cDNA of LC and ELC are 25.5 and 29.7 kD, respectively. Therefore, there are two theoretical M.W.s for each recombinant fusion protein, depending upon which M.W. for the MBP is used: (1) 67.5 kD and 71.7 kD for MB-LC and MBP-ELC, respectively, using 42 kD for MBP (the manufacturer reported M.W. of MBP); (2)

74.5 kD and 78.7 kD for MBP-LC and MBP-ELC, respectively, using 49 kD for MBP (the calculated M.W. of MBP).

The M.W.s of the MBP-LC and MBP-ELC determined by analysis of amino acid composition and SDS-PAGE for each sample was very close to first predicted values. Although the M.W.s of the MBP-LC and MBP-ELC determined by analysis of amino acid composition are 7 kD smaller than the second predicted values, the M.W.s for these two recombinant fusion proteins determined by the analysis of amino acid composition are still considered as very close to the second predicted values. The analysis of amino acid composition of the MBP-LC fusion proteins showed that the amino acid contents of the samples corresponded closely to the amino acid composition deduced from the cDNA sequences (Table 4.2). The analysis of amino acid composition of the MBP-ELC recombinant fusion proteins also corresponded closely to the value deduced from the cDNA sequence of the MBP-ELC except for the Ser and Gly residues which were 54% and 38% higher than the predicted values, respectively (Table 4.2). The difference in the data of analysis of amino acid composition and the deduced amino acid composition could result from (1) the method of the analysis of amino acid composition, which could have 10% to 15% error by itself; and (2) some impurity proteins might have co-migrated with the samples.

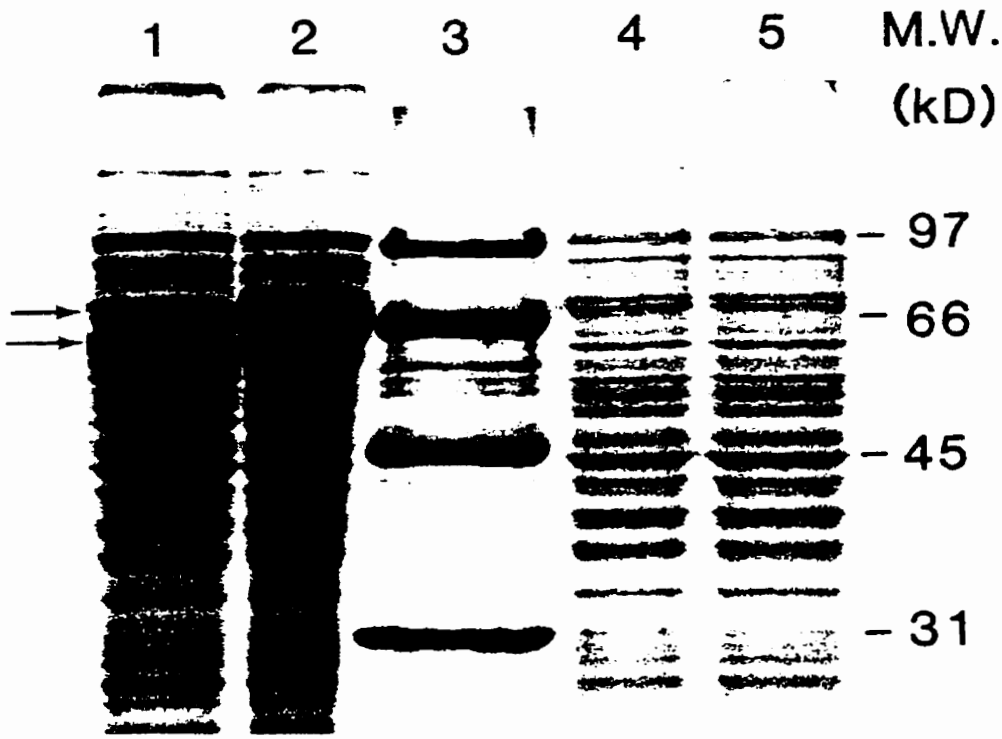


Figure 4.5 Analysis of recombinant fusion proteins before and after IPTG induction by a 12% SDS-PAGE gel stained with Coomassie brilliant blue.

The samples (cell lysis) of this gel were from cultures of cells that contained the MBP-LC or MBP-ELC plasmids before and after IPTG treatment as described in the method above.

Lane 1 = The MBP-LC clone: "induced cells". Arrow indicates the production of the recombinant fusion protein of MBP-LC after IPTG treatment.

Lane 2 = The MBP-ELC clone: "induced cells". Arrow indicates the production of the recombinant fusion protein of MBP-ELC after IPTG treatment.

Lane 3 = M.W. standard.

Lane 4 = The MBP-LC clone: before IPTG induction ("uninduced cell").

Lane 5 = For the MBP-ELC clone: before IPTG induction ("uninduced cell").

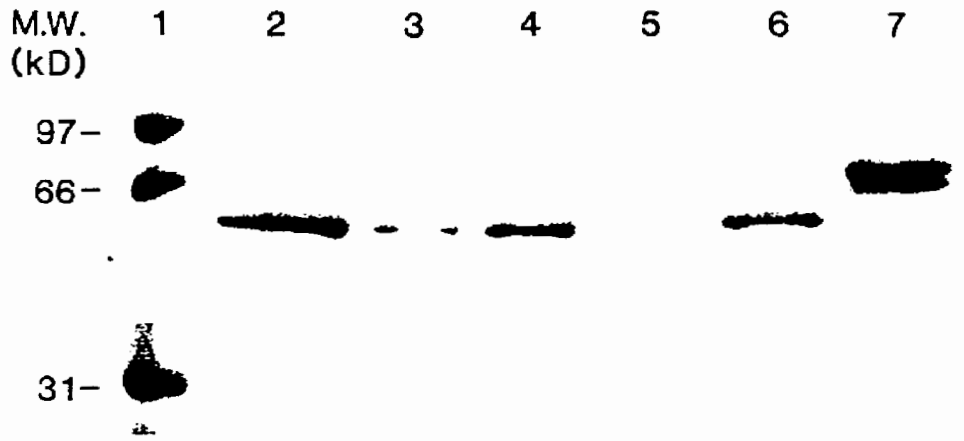


Figure 4.6 Analysis of the amylose-bound soluble fraction of the MBP-LC and the MBP-ELC by a 12% SDS-PAGE gel stained with Coomassie brilliant blue.

Lane 1 is the M.W. standard; lane 5 is a blank; lanes 2 to 4 and lane 6 are the MBP-LC; and lane 7 is the MBP-ELC.

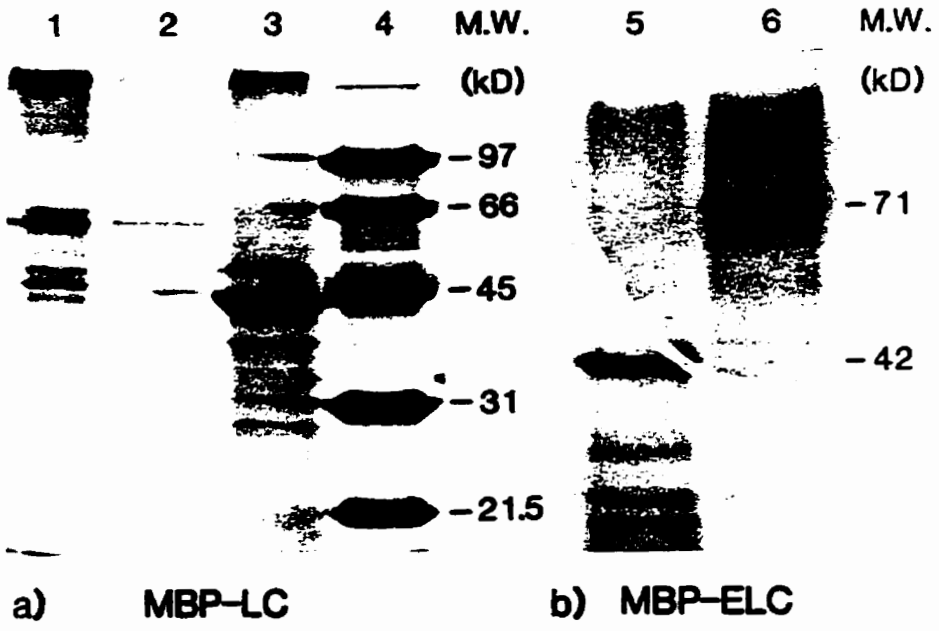


Figure 4.7 Factor Xa digestion of the recombinant fusion proteins in the soluble fraction after amylose affinity chromatography and Bio-Gel® P-60 gel filtration chromatography.

(a) Analysis of the MBP-LC with and without factor Xa treatment in a 12% SDS-PAGE gel stained with Coomassie brilliant blue. Lanes 1 and 2 are the MBP-LC before treatment with factor Xa. Lane 3 is the MBP-LC after treatment with factor Xa (6 hours). Lane 4 is a M.W. standard. Only the MBP (42 kD) alone (but not the LC, 25.5 kD) was clearly seen in the gel after factor Xa digestion of the sample.

(b) Analysis of MBP-ELC before and after treatment with factor Xa in a 15% SDS-PAGE gel stained with Coomassie brilliant blue. Lane 6 is the MBP-ELC before treatment with factor Xa; and lane 5 is the MBP-ELC after treatment with factor Xa (6 hours). The M.W. for this gel is marked on the right side of the Figure. Only the MBP (42 kD) alone (but not the ELC, 29.7 kD) was clearly seen in the gel after factor Xa digestion.

Table 4.2 Comparison of the analyses of the amino acid (aa) composition of the MBP-LC and the MBP-ELC recombinant fusion proteins with the amino acid composition deduced from their cDNA sequences.

Amino acid	MBP-LC (No. of aa / mole)		MBP-ELC (No. of aa / mole)	
residues	aa analysis	deduced from cDNA	aa analysis	deduced from cDNA
Asx[a]	82	85	83	91
Thr	32	30	34	32
Ser	34	26	67	31
Glx[b]	78	66	72	67
Pro	38	32	36	34
Gly	54	44	74	46
Ala	55	48	57	50
Val	34	33	32	34
Met	7	8	6	8
Ile	29	32	28	35
Leu	43	36	41	41
Tyr	23	22	22	23
Phe	27	27	24	28
His	14	10	20	10
Lys	40	43	52	43
Arg	21	19	18	20
Cys	N.D.[c]	10	N.D.	16
Trp	N.D.	16	N.D.	16
Total aa	612	587	666	625
M.W.	67 kD	74.5[d]	71 kD	78.7[d]

a. Both Asp and Asn.

b. Both Glu and Gln.

c. N.D.=not determined.

d. The M.W.s were determined by deduced from the cDNA sequences of the MBP-LC and MBP-ELC.

4.4 DISCUSSION

It was found from pilot studies that addition of glucose to the culture medium as recommended in the manufacturer's manual, reduced the yield of the G3 recombinant fusion proteins (S. Kwok, M.-P. Gastineau and M.E. Adams, unpublished observation). Therefore, only LB medium was used for the production of the G3 recombinant fusion proteins. The yield of the soluble recombinant fusion proteins of the G3 isoforms that was purified by amylose affinity chromatography was much lower than the average values (10 to 40 mg per liter culture) reported in the manufacturer's manual for the pMAL-c2 expression system. A large proportion of the G3 recombinant fusion proteins existed in an insoluble fraction (most likely in inclusion bodies), as estimated by SDS-PAGE gels. We tried reducing the culture temperature from 37°C to 25°C and adding a physiological concentration of Ca^{2+} into the LB medium, hoping to increase the amount of soluble fraction of the fusion recombinant proteins. However, these measures did not increase the yield of the soluble fraction of the G3 recombinant proteins.

Production of a recombinant protein as an MBP-fusion protein has the advantage of simplifying the purification of the recombinant protein, because the MBP can bound to an amylose affinity chromatography. However, large preparation of the G3 fusion proteins purified by one or two cycles of amylose affinity chromatography still contained a variable amount of impurities, even though a large excess of the amylose resin was used and the flow rate was properly controlled. The MBP-LC or the MBP-ELC fractionated from these preparations were only partially cleaved by factor Xa. In an effort to purify further the MBP-LC and MBP-ELC, the samples were passed through a Bio-Gel® P-60 gel filtration column (Note: Sephadex is not recommended for the gel filtration because fusion proteins can tightly bind to the Sephadex via the MBP). After gel filtration, the samples were less heterogeneous than those that had been purified by the amylose affinity chromatography alone. There were still impurities in the sample, as seen on SDS-PAGE gels. However, it is not known whether the extra bands detected in the SDS-PAGE gels were derived from non-specific degradation of the samples.

Although the samples that were purified by gel filtration were readily cleaved by factor Xa, there was another problem: the MBP remained intact (as estimated for the M.W. on SDS-PAGE gels), but the LC and ELC were not clearly identified on either the 12% or 15% SDS-polyacrylamide gels (Figure 4.7). This may be due to non-specific degradation of the LC and ELC recombinant peptides after Xa treatment.

Because the above problems became evident during the purification and factor Xa cleavage of the recombinant MBP-LC and MBP-ELC proteins, we were unable to perform N-terminal sequence analysis on the LC and ELC peptides to verify further the recombinant peptides. In order to obtain a peptide specific to the recombinant LC or ELC, cyanogen bromide digestion of the MBP-LC and MBP-ELC fusion proteins was tried. The numbers and sizes of the peptides thus yielded were predicted from the cDNA-deduced amino acid sequences for these two clones. However, the samples were not completely digested by cyanogen bromide and the expected peptides specific to the ELC and LC proteins were not well separated in 15% SDS-polyacrylamide gels (S. Kwok and M.E. Adams, unpublished observation). Thus, the MBP-LC and MBP-ELC were only partially characterized in this study by SDS-PAGE and analysis of amino acid composition.

In theory, foreign peptides and proteins produced in *E. coli* cells often have difficulty in forming disulfide bonds correctly (Goeddel *et al.*, 1991). We were unable to analyze the disulfide bonds due to the problems with purifying the fusion proteins.

In the hope of obtaining recombinant peptides with properly formed disulfide bonds, we initiated the subcloning and production of recombinant peptides of the G3 isoforms in a yeast expression systems (*Pichia*, from Invitrogen). In this system, the recombinant peptides are expected to be secreted extracellularly. In a trial production of the recombinant ELC peptides (without the last 25 amino acid of C-terminus, same construct as in the pMAL-c2 vector) and the E2-like module alone in the *Pichia* expression system, no recombinant peptides with the expected M.W.s were detected in the culture supernatant as assayed by Coomassie Blue staining of SDS-polyacrylamide gels (data not shown). This may be due to, among other reasons, (1) different properties of the eukaryotic cells and prokaryotic cells in processing the same foreign gene; (2) failure to optimize the

culture conditions of the yeast expression system for high yield of the recombinant peptides, or (3) intracellular or extracellular degradation of the foreign peptides. However, we did not have an antibody against the ELC or LC peptide. If the yeast expression system produced the recombinant ELC or LC peptide at a very low level, it might be detectable with the more sensitive method of Western blotting than the Coomassie brilliant blue staining.

It is unclear why the same constructs cloned into the pMAL-c2 vector (for intracellular production of the recombinant of the ELC and LC) and the pPIC9 vector (in the *Pichia* system, for secretion of the recombinant ELC and LC)) differed so profoundly in the expression of the G3 isoforms of canine aggrecan. Perhaps the last 25 amino acids of the C-terminus of aggrecan may be important in intracellular transportation of aggrecan. The reason that we did not include the C-terminal peptide from the constructs was based on one of our previous unpublished observations: when we subcloned the LC cDNA with this C-terminus and part of the 3' non-translated region into pGEX-3x (an *E. coli* expression system, with GST fusion protein system, from Pharmacia, Baie d'Urfé, PQ), no production of the recombinant peptides was detected. However, recombinant fusion proteins for both LC and ELC were produced with the constructs without the cDNA of the C-terminus and the 3' non-translation region in the pMAL-c2 vector. Furthermore, some evidence exists that the C-terminal peptide is cleaved from aggrecan after secretion (J. D. Sandy, personal communication).

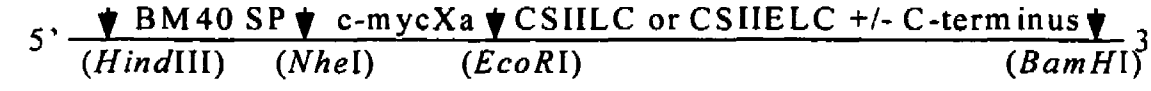


Figure 4.8 Schematic diagram of the new constructs designed for cloning into the pCEP/d4-7 vector.

Note: BM40 SP = The signal peptide of basement 40 protein in the pCEP/d4-7 vector (Mayer *et al.* 1993). The oligonucleotide for the c-mycXa (a cDNA codes for the c-myc epitope and the Xa cleavage site) was synthesized by the Calgary Regional DNA Synthesis Laboratory, University of Calgary, AB. cDNAs of the CSIILC, CSIIELC, CSIILC-C-terminus and CSIIELC-C-terminus would be subcloned from our previous clones of the G3 of canine aggrecan by PCR manipulations.

In order to determine whether the last 25 amino acid C-terminus of aggrecan would affect the expression the G3 isoforms in a mammalian system (The vector used was pCEP/d4-7 which was modified from the pCEP of Invitrogen) (Mayer *et al.* 1993), we have initiated cloning one of the four constructs for the two G3 isoforms of canine aggrecan (both with or without the last 25 amino acid C-terminus). These four constructs would contain a part of CS-II region up to most C-terminal cleavage site of the putative "aggrecanase", a human c-myc epitope tag for facilitating the isolation of the recombinant peptides using a c-myc monoclonal antibody (Oncogene Science, Inc., Hornby, ON), a factor Xa cleavage site and a human BM40 signal peptide (Mayer *et al.* 1993) at the 5' end of the G3 cDNA (Figure 4.8). Production of the recombinant peptides using the mammalian system will be performed by another researcher in our laboratory and thus, it is not included in this report.

Although we have initiated production of recombinant peptides of the G3 domain of canine aggrecan in different expression systems, more work is still needed to produce the recombinant peptides with proper secondary structure for future studies of the structure and function of the G3 domain. The recombinant peptides made in the pMAL-c2 expression system should be useful for producing polyclonal antibodies, which in turn will be useful tools for future studies of the G3 domain.

CHAPTER 5

5. GENERAL DISCUSSION

5.1 CONCLUSION AND SIGNIFICANCE OF THE STUDY

In this project, we accomplished all the aims proposed and tested our hypotheses. We defined qualitatively and quantitatively the gene expression of the G3 domain of aggrecan in the articular cartilage of adult dogs in the hypertrophic stage of experimental osteoarthritis (OA) and contralateral non-operated (NOP) controls. The results confirmed our first hypothesis, *i.e.*, the types of mRNA isoforms of the G3 domain of aggrecan core protein that are expressed are the same in the canine articular cartilage of normal young dogs, normal adult dogs and adult dogs in the hypertrophic stage of experimental osteoarthritis.

Previous studies have shown that only a portion of the aggrecan isolated from either bovine cartilage or cultured bovine chondrocytes contained marker peptides of the G3 domain (Flannery *et al.* 1992). It has also been shown that the content of the G3 domain of aggrecan in cartilage decreases with age, as measured by immunosorbant assay with an antiserum against the G3 domain (Dudhia *et al.* 1996). These biochemical and immunochemical studies, however, cannot define whether the aggrecan that is missing the G3 domain results from degradation of aggrecan or the expression of a mRNA isoform without the G3 domain in cartilage.

To test whether any aggrecan mRNA was missing the entire G3 domain, total RNA of canine articular cartilage was reverse transcribed with oligo (dT) and PCR performed with primers FCSII5 and R1 (Figure 2.1a in Chapter 1). All the RT-PCR products contained a complete G3 domain and CS-II region, *i.e.*, cDNA without a G3 domain was not seen in the PCR product (data not shown). This suggests that the G3 domain exists in all the original mRNA. Our findings support the hypothesis that the aggrecan without the G3 domain most likely is a product of extracellular enzymatic cleavage. This observation

is corroborated by a previous report which showed the same level of aggrecan mRNA in the osteoarthritic and control canine articular cartilages by Northern hybridization using probes specific for the KS-rich region and the G3 domain (with LC isoform) of canine aggrecan (Matyas *et al.* 1995).

The results of quantitative reverse transcription-polymerase chain reaction analyses demonstrated that the LC isoform is predominantly expressed in canine adult articular cartilage, both in the hypertrophic stage of OA and in the NOP controls. Although the expression of the LC and the ELC isoforms was significantly increased when comparing the OA samples with the NOP samples, the results did not support our second hypothesis, which was that "the isoform(s) with an E-like module is (are) expressed more abundantly in the osteoarthritic cartilage than the NOP controls". Nevertheless, our results suggest that aggrecan can undergo active metabolic changes in the hypertrophic stage of experimental OA, which agrees with the previous reports in this model of OA (Adams & Brandt, 1991; Adams, 1994; Matyas *et al.* 1995).

Alternative splicing endows a gene with the capacity to generate more than one related mRNA and the corresponding protein isoforms. Alternative splicing of pre-mRNA is now known to be of widespread importance for generating protein isoforms. For aggrecan, we provide evidence that differential splicing of pre-mRNA regulates the expression of the G3 domain of the aggrecan, that is, the ELC and LC. The ELC isoform contains an extra module, the E2-like module. The differential expression of the G3 domain of aggrecan raises the following intriguing but not understanding questions:

(1) How is the alternative splicing of aggrecan regulated *in vivo*?

(2) What is the function of the G3 domain of aggrecan?

(3) Is there a different function for the ELC and LC isoforms?

(4) What is the role of the G3 domain in the development of osteoarthritis?

There is very limited knowledge of the regulation of alternative splicing, however, growth factors and cytokines may be involved in the regulation of alternative splicing. For example, TGF-β was reported to regulate expression of tenascin alternative-spliced isoforms in fetal rat lung (Zhao & Young, 1995).

It is not clear what factors affect the alternative splicing of aggrecan. It is known, however, that many growth factors are involved in the maintenance of the homeostasis of differentiated cartilage and the metabolism of osteoarthritic cartilage (Malemud & Moskowitz, 1981; Rouslahti & Yamaguchi, 1991). Cytokines, on the other hand, are mainly responsible for inflammatory and destructive processes of cartilage by inhibiting extracellular matrix synthesis and regulating the neutral metalloproteinases production by chondrocytes and/or synovial fibroblasts (Larrick & Kunkel, 1988; Pratta *et al.* 1989; Pelletier *et al.* 1991). Insulin-like growth factor-I and II (IGF-I and IGF-II) can stimulate ^{35}S-sulfate incorporation into articular cartilage (an indication of synthesis of proteoglycan) in tissue culture (Zingg & Froesch, 1973). It was reported that transforming growth factor-β (TGF-β) enhanced the biosynthesis of both collagen and proteoglycans in culture articular cartilage (Redini *et al.* 1988; Redini *et al.* 1991). TGF-β was reported to counteract the effect of IL-1 in cultured articular cartilage chondrocyte (Pujol *et al.* 1991). Osborn *et al.* (1989) showed that epidermal growth factor (EGF) at 10 ng/ml had a maximal effect on stimulating incorporation of ^{35}S-sulfate into bovine adult cartilage. This effect of EGF can be synergistically enhanced by IGF-I, insulin and basic fibroblast growth factor (bFGF). EGF had no effect on DNA synthesis in chondrocyte when using alone in the culture system, but showed increase ^{3}H-thymidine incorporation into chondrocytes after co-culture with insulin (Osborn *et al.* 1989). Interleukin-1 (IL-1) plays a dual role in cartilage metabolism: it causes proteoglycan degradation by inducing the production of metalloproteinases such as stromelysin, but it also prevents cartilage repair by inhibiting proteoglycan synthesis (Ratcliffe *et al.* 1986). Induction of interleukin-6 (IL-6) by IL-1 was required for IL-1-induced inhibition of proteoglycan synthesis (Nietfeld *et al.* 1990). It would be interesting to test whether these growth factors and cytokines affect the alternative splicing of aggrecan *in vitro*.

The function of the G3 domain of aggrecan is not fully understood. It is very interesting that selectins, a group of lymphocyte homing receptors, possess E-like, L-like and C-like modules similar to those in the G3 domain of aggrecan (McEver, 1994). Selectins are involved in the cell-cell initial adhesive interaction between leukocyte-

endothelial cells and leukocyte-platelet at site of inflammation (Whelan, 1996). Although the molecular basis of selectin-ligand interaction has not been completely determined, individual selectins have been shown to bind specifically to a variety of natural and synthetic carbohydrates, including phosphated mono- and polysaccharides, sulfated polysaccharides, and oligosaccharides (Bevilacqua & Nelson, 1993; Rosen & Bertozzi, 1994). It would be important to investigate whether the recombinant peptides of the LC and the ELC isoform of aggrecan can bind specifically to synovial joint components, such as chondrocytes, cartilage extracellular matrix, or the synovium (as the free G3 domain may defuse into joint cavity and could then bind to synovium). Furthermore, it is also necessary to test whether the recombinant peptides of the LC and ELC isoform of aggrecan can affect chondrocyte metabolism. These studies would give some clues to the roles that the G3 isoform might play in normal cartilage and during the development of OA.

The ELC isoform may differ from the LC isoform either in binding specificity and biological function because the ELC isoform contains an E-like module. Stanescu *et al.* (1991) showed by Western blot that the EGF antibodies reacted to the deglycosylated human and baboon aggrecan which was extracted from different aged subjects of cartilage samples (both band I and band II of aggrecan that was fractionated in an agarose gel). This result suggested that the E-like module has the similar structure as the true EGF and may possess EGF-like activity. In the intact aggrecan, the epitope of the E-like module could be masked by the glycosaminoglycan side chains of the nearby CS-II domain and may not be able to exert any function to its target. However, when a G3 domain-containing fragment is released from the aggrecan core protein, as it may be *in vivo* (Ilic *et al.* 1992), the E-like module of the G3 domain of aggrecan might defuse to the target area in the joint and exert a biological function.

In summary, the knowledge of the alternative splicing patterns of the G3 domain of aggrecan in normal and osteoarthritic canine articular cartilage obtained from this study, and the results of other studies suggest that the G3 domain is a very important part of the aggrecan molecule, and that different isoforms of the G3 may play different roles in the

physiology and pathology of normal, aging, and osteoarthritic cartilage. It is important to define the function of the isoforms of the G3 domain.

5.2 SCOPE AND LIMITATIONS OF THE STUDY

In this study, we focused on the alternative splicing patterns of the G3 domain of canine aggrecan. Although we had hypothesized (see section 1.1.4 of Chapter 1) that the different isoforms of the G3 domain have different effects on the metabolism of chondrocytes in normal and experimental osteoarthritis, this hypothesis was not tested in this study, but it is worth testing in future studies.

As mentioned in the general introduction, there is much evidence that tissue repair may produce protein isoforms that resemble those that are present in juvenile tissue. In this study, we found that the same isoforms of the G3 domain are expressed in the articular cartilage of normal young dogs, normal adult dogs and adult dogs in the hypertrophic stages of experimental OA. However, the level of these isoforms expressed in articular cartilage of puppies (10 days old) and normal adult dogs vs the NOP contralaterals was not determined.

A survey by RT-PCR of the alternative splicing patterns and the expression level of the isoforms of the G3 domain of canine aggrecan provided an effective and efficient way to accomplish the aims of this study. However, this method had its limitations, and these were discussed in Chapters 2 and 3.

In Chapter 3, we excluded dog 6 from the study of quantifying the G3 isoforms. This is because after 10 weeks post-ACL transection, this dog has an abnormal cartilage mass (1.76 g/stifle) in its NOP control stifle (Table 5.1). According to our historical data on this model in dogs with matched age, sex and body weight, the average cartilage mass (g/stifle, mean±SD, n = 9) is 0.96±0.15 on the NOP control, 1.50±0.21 on the OA, and the cartilage mass ratio of OA to NOP is 1.58±0.08 (Table 5.2). Although total RNA and total mRNA level were raised in the OA stifle in dog 6, the level of expression of the LC isoform was decreased; and the level of expression of the ELC isoform was slightly increase (Table 5.1). The discoordinate changes in total RNA and specific G3 mRNA of

aggrecan is different from the other five dogs studied in Chapter 3. This may reflect that the NOP control has certain degree of hypertrophy as indicated by its cartilage mass, suggesting it may have been developing spontaneous OA in that joint, making it an invalid control.

Table 5.1 Summary of data for dog 6.

Variables	OA	NOP	OA/NOP
Cartilage mass (g/stifle)	1.95	1.76	1.1
DNA concentration (mg/g cartilage)	0.30	0.30	1.0
Total RNA (μg/mg DNA)	242	143	1.7
Total mRNA (scan unit/mg DNA)	319	211	1.5
LC mRNA (fmole/mg DNA)	91	129	0.7
ELC mRNA (fmole/mg DNA)	3.3	2.9	1.1

Note: The body weight of this dog was 26 kg.

Table 5.2 Historical data for cartilage mass (g/stifle) for 9 dogs 10 weeks post-ACL transection.

For 9 Dogs	OA	NOP	OA/NOP
Mean	1.50	0.96	1.58
SD	0.21	0.15	0.08

Assessment of the degree of OA in the abnormal cartilage (in the early stage of OA) depending mainly on cartilage mass is one of the limitations in this type of staging of this model of OA. Although the ACL-transected canine osteoarthritic model very closely resembles human OA, biological variation in the expression of the G3 isoforms was found in individual dogs. Therefore, larger samples other than the 5 that were used in this studies would be more appropriate for future studies.

The recombinant peptides of the G3 isoforms of canine aggrecan (ELC and LC, both without the last 25 amino acids of the C-terminus, as there are suggestions that this C-

terminus is cleaved from the mature secreted form of aggrecan) were successfully produced using the pMAL-c2 expression system. However, characterization of the secondary structure, large scale purification of the recombinant peptides and production of polyclonal antibodies for the G3 domain are beyond the scope of this project. The pMAL-c2 expression system provides a rapid way to express, on a large scale, recombinant proteins which are satisfactory for producing polyclonal antibodies specific for the G3 isoforms of aggrecan. Such antibodies are not readily available. Although it would be better to produce recombinant peptides which are structurally and functionally close, or ideally, identical to the native isoforms, the difficulty in forming correct disulfide bonds in prokaryotic expression systems could limit this. However, the antibodies that will be generated from these recombinant peptides should be useful for the future preparation of recombinant peptides of the G3 isoforms using eukaryotic expression systems.

5.3 FUTURE STUDIES OF THE G3 DOMAIN OF AGGRECAN

For the recombinant peptides produced by the pMal-c2 system to be most useful, it would be necessary to produce pure recombinant peptides derived from the maltose binding fusion proteins. It would also be necessary to characterize the structure of these recombinant peptides. These peptides could be used to produce polyclonal antibodies direct against the G3 domain, which in turn, could serve as useful tools to facilitate further studies of the structure and function of the G3 domain of aggrecan in normal and osteoarthritic cartilage.

Future studies of the G3 domain of aggrecan might focus on the ligand binding and other possible biological functions of the G3 domain. To define whether the G3 domain binds to any joint tissues or molecules, biotinylated recombinant G3 peptides could be used as ligands in ligand-based hitochemistry and in Western blots containing proteins of cultured chondrocytes or joint tissues fractionated by polyacrylamide gel electrophoresis. Alternatively, to test whether or not G3 binds to a peptide ligand, the labelled recombinant G3 peptides could be used for screening a chondrocyte expression cDNA library. To study whether the G3 peptides affect chondrocyte gene expression, recombinant G3 peptides could be added into chondrocyte culture medium, for example, with assessment of

expression of aggrecan, type II collagen, metalloproteinases and their tissue inhibitors, or other cartilage matrix proteins by Northern hybridization or PCR. The effects of the different alternatively spliced G3 isoforms (ELC and LC) could be compared in these assays.

To facilitate such future studies, a eukaryotic expression system (*Pichia*, a yeast expression system, for secretion of the recombinant G3 isoform) was tried for small scale production of recombinant peptides of two constructs: the ELC (without the last 25 amino acids of the C-terminus) and the E2-like module. However, successful production of these two recombinant peptides was not clearly demonstrated (S. Kwok, M.-P. Gastineau and M.E. Adams, unpublished observations). This might be due to a failure to optimize the culture conditions, and consequently the level of the production of the recombinant peptides might have been too low to have been detected by staining the polyacrylamide gels with Coomassie brilliant blue. Further analysis of these samples with Western blots might be appropriate if the antibodies were available. Another possible explanation for the low yield might be that the last 25 amino acids of the C-terminus could be the part of G3 involved in intracellular transportation and secretion of aggrecan. This could explain why the constructs with two G3 isoforms (ELC or LC) but without the C-terminus made in pPIC9 vector of the *Pichia* expression system did not have any detectable secretion of the recombinant G3. [Note: Some studies have suggested that the G3 domain is involved in translocation through the Golgi and secretion of aggrecan in chondrocytes (Luo *et al.* 1996; Vertel *et al.* 1993).]

Cloning of the cDNAs of the G3 isoforms (four constructs for CSIIELC and CSIILC, both with/without the last 25 amino acids of the C-terminus) into a pCEP/d4-7 vector for a mammalian expression system had been initiated in this project (see Chapter 4 for details). Production of the recombinant peptides for these constructs in a mammalian expression system will be performed in our laboratory (in the hope of obtaining recombinant peptides with correct disulfide bonding), but the information was not yet available for this thesis. By comparison of the expression of these G3 constructs, we can determine whether the C-terminus is responsible for intracellular translocation of the

aggrecan. These recombinant G3 isoforms would be useful for defining the biological function of the G3 domain of aggrecan.

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7. APPENDIX

7.1 BUFFERS AND REAGENTS

7.1.1 Buffers and reagents for general usage in molecular biology

The preparation of the following reagents was based on the recipes of Sambrook *et al.* (1989). (Note: The page numbers, e.g., A.6, B.15, B.24, *etc.*, refer to the page numbers in the reference, not the page numbers of this thesis.)

(1) Stock ampicillin (50 mg/ml) (p. A.6)

Dissolve of 500 mg of ampicillin powder in 10 ml autoclaved distilled deionized water (ddH_2O), filter through a 0.22 μm of sterile filter, aliquot into microfuge tubes and store the aliquots at -20°C until needed.

(2) 50×Denhardt's solution (p. B.15)

Dissolve the following chemicals in 400 ml ddH_2O:

5 g Ficoll (Type 400, Pharmacia),

5 g polyvinylpyrrolidone,

5 g bovine serum albumin (Fraction V, Sigma),

Q.S. with ddH_2O to 500 ml.

Filter the solution through a 0.22 μm of sterile filter and store aliquots at -20°C until needed.

(3) 6×DNA loading buffer (type III) (p. B.24)

Dissolve the following chemicals in 30 ml ddH_2O:

0.125 g bromophenol blue,

0.125 g xylene cyanol FF,

15 ml 99.9 % (v/v) glycerol,

Q.S. with ddH_2O to 50 ml.

(4) 5×formaldehyde RNA running (FRR) buffer (p. 7.43)

200 ml 0.5 M 3-(N-morpholino)propane sulfonic acid (MOPS) (pH 7.0),

80 ml 0.5M sodium citrate,

10 ml 0.5 M EDTA (pH 8.0),

Q.S. with DEPC-treated ddH_2O to 1 liter.

(5) LB (Luria-Bertani) broth (p. A.1)

Dissolve following chemicals in 950 ml ddH_2O:

10 g Bacto-tryptone,

5 g Bacto-yeast extract,

10 g NaCl.

Adjust the pH to 7.0 with 5 N NaOH (about 0.2 ml). Q.S. with deionized H_2O (dH_2O) to 1 liter. Sterilize by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle.

(6) LB-ampicillin agar plate (p. A.4)

Add 15 g of Bacto-agar to 1 liter of LB broth, autoclave as for the LB broth. When the temperature of the solution has cooled to about 50°C, add stock ampicillin (50 mg/ml) to the solution to achieve a concentration of 50 μg/ml, mix well and pour into Petri dishes (about 30 to 35 ml/plate). After the LB/agar has solidified at room temperature, the plates should be inverted and stored at 4°C until needed.

(7) RNA loading buffer (p. 7.44)

Dissolve following chemicals in 20 ml ddH_2O:

0.25% (w/v) bromophenol blue,

0.25% (w/v) xylene cyanol FF,

25 ml 99.9% (v/v) glycerol,

0.1 ml 0.5 M EDTA (pH 8.0),

Q.S. with ddH_2O to 50 ml.

(8) 1×SDS-PAGE sample buffer (p. 18.53)

5 ml 0.5 M Tris·HCl (pH 6.8),

5 ml 20% (w/v) SDS,

5 ml 1% (w/v) bromophenol blue,

5 ml 99.9% (v/v) glycerol,

Q.S. with ddH_2O to 45 ml.

Aliquots (450 μl) are stored at -20°C until needed. Add 50 μl of stock dithiolthreitol (1 M) to the 450 μl aliquot before using.

(9) 20×SSC (p. B.13)

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of ddH_2O, adjust pH to 7.0 with 1 M HCl, and then adjust the volume to 1 liter with ddH_2O. Sterilize by autoclave as above.

(10) 20×SSPE (p. B.13)

Dissolve 175.3 g of NaCl, 27.6 g of $NaH_2PO_4 \cdot H_2O$ and 7.4 g of EDTA in 800 ml of ddH_2O, adjust the pH to 7.4 with 10 N NaOH (about 6.5 ml). Adjust the volume to 1 liter with ddH_2O. Autoclave as above.

(11) 50×TAE buffer (p. B.23)

Dissolve 242 g Tris base in 700 ml ddH_2O. Add 57.1 ml glacial acetic acid and 200 ml 0.5 M EDTA (pH 8.0). Q.S. with ddH_2O to 1 liter.

(12) 5×TBE buffer (p. B.23)

Dissolve following chemicals in 900 ml ddH_2O:

54 g Tris base,

27.5 g boric acid,

20 ml 0.5 M EDTA (pH 8.0),

Q.S. with ddH_2O to 1 liter.

(13) TE buffer, pH 7.4 (p. B.20)

10 ml 1 M Tris·HCl (pH 7.4),

2 ml 0.5 M EDTA (pH 8.0),

Q.S. with ddH_2O to 1 liter.

7.1.2 Buffers and reagents specific for screening cDNA library and Northern hybridization

The preparation of the following buffers and reagents was based on the manufacturer's manuals for the Lamda Zap® II/*EcoR*I Cloning Kit and the *In Vitro* Transcription Kit (Stratagene, La Jolla, CA).

(1) NZY broth

Dissolve following chemicals in 900 ml ddH_2O:

5 g NaCl,

2 g $MgSO_4 \cdot 7H_2O$,

5 g yeast extract,

10 g NZ amine (casein hydrolysate),

Q.S. with ddH_2O to 1 liter. Autoclave as above.

(2) NZY plate

Add 15 g Difco agar per liter of NZY broth. Autoclave as above. Pour approximately 80 ml/ 150 mm plate.

(3) 5xPE buffer

250 ml 1 M Tris·HCl, pH 7.5,

10 ml 50% (w/v) sodium pyrophosphate,

250 ml 20% (w/v) SDS,

50 ml 20% (w/v) polyvinylpyrolidone (M.W. 40,000),

50 ml 20% (w/v) Ficoll (M.W. 400,000),

25 mM EDTA,

Q.S. with ddH_2O to 800 ml.

Autoclave as above, then add filter sterilized 200 ml 5% (w/v) bovine serum albumin (fraction V; Sigma) to it.

(4) SM buffer

Dissolve the following chemicals in 900 ml ddH_2O:

5.8 g NaCl,

2.0 g $MgSO_4$.

50 ml 1 M Tris·HCl (pH 7.5),

5 ml 2% gelatin,

Q.S. with ddH_2O to 1 liter.

Autoclave as above.

(5) 2×Y T medium

Dissolve following chemicals in 900 ml ddH_2O:

10 g NaCl,

10 g yeast extract,

16 g Bacto-tryptone,

Q.S. with ddH_2O to 1 liter.

Autoclave as above.

7.2 CALCULATION OF THE AMOUNTS OF THE G3 ISOFORMS OF CANINE AGGRECAN QUANTIFIED BY RT-PCR

Reference: Quantitative RT-PCR: Methods & Application Book 3, Clontech Laboratories, Inc. Mississauga, ON.

7.2.1 Calculation of the amount of LC mRNA quantified by competitive RT-PCR with an internal standard

In the method of competitive RT-PCR with an internal standard, the relative initial amounts of target mRNA (LC mRNA, see Chapter 3) and the internal standard (the LCmut2 mRNA, see Chapter 3) can be determined from Equation 1. The values for the efficiency of amplification, E_t or E_s for the target and the standard, respectively, can be calculated from the slope of a graph of Log N_t or Log N_s as a function of cycle number (n).

Equation 1:

$N_{0t}/N_{0s} = N_t\ (1 + E_s)^n/N_s\ (1+E_t)^n$

Where:

N_{0t} = The initial number of target molecules (the unknown)

N_{0s} = The initial number of standard molecules (known)

N_t = The number of amplified target molecules (measured directly after RT-PCR)

N_s = The number of amplified standard molecules (measured directly after RT-PCR)

E_t = Amplification efficiency of the target

E_s = Amplification efficxiency of the standard

n = The number of amplification cycles

When the amplification efficiencies of the two reactions (target and standard) are identical, *i.e.*, $E_t = E_s$, the analysis is greatly simplified (Equation 2).

Equation 2:

$N_{0t}/N_{0s} = N_t/N_s$

The equation 2 can be written as Equation 3:

$A_t/A_s = N_t/N_s$

Where:

A_t = The amount of amplified target (in cpm or scan units)

A_s = The amount of amplified standard (in cpm or scan units)

Thus, for any value of n (in the linear range of PCR amplification), the initial ratio of target to standard (N_{0t}/N_{0s}) is equal to the ratio of the amplification products (N_t/N_s or A_t/A_s). Therefore, if the standard and target mRNA amplify with the same efficiency, the absolute initial amount of target cDNA (and in turn target mRNA) can be determined by allowing known amounts of standard (mRNA or cDNA) molecules to compete with the target for primer binding during PCR amplification.

In the method of competitive RT-PCR that was used for quantifying the LC isoform, as described in Chapter 3, serial dilutions of the mRNA standard (LCmut2) were made, and these dilutions were added to RT-PCR reaction mixture containing a constant amount of sample (total RNA). Following RT-PCR, the amplification products were analyzed by agarose gel electrophoresis, and the amount of the PCR products generated by standard (A_s) and the target (A_t) were determined for each individual reaction as cpm or scan units. The logarithm of ratio of A_t/A_s was graphed as a function of the logarithm of the initial molar amount of standard (N_{0s}) (bottom panel of Figures 3.5 to 3.9). The initial amount of target mRNA (N_{0t}) in the reaction is extrapolated from the graph, assuming that N_{0t} is equal to the amount of the standard (N_{0s}) added, in the reaction that produce an equimolar ratio of the two types of products (*i.e.*, where the Log A_t/A_s = Log 1 = 0).

An example of the calculation of the LC isoform expressed in dog 4 is shown as follows:

The data for competitive RT-PCR are listed in Table 7.1. The graph of Log fmole of (N_{0s}) vs Log (scan units A_t/A_s) are presented in Figure 7.1. From Figure 7.1:

when $\text{Log}\,(A_t/A_s) = \text{Log}\,1 = 0,$

The Log fmole of LC is: Log A_t = Log A_s = -1.563 (for OA); or -1.849 (for NOP)

The LC mRNA contained in the 0.08 μg total RNA was:

Anti-log A_s = LC (fmole)/0.08 μg total RNA

i.e., For OA sample, LC = 0.027 fmole/0.08 μg total RNA

For NOP sample, LC = 0.014 fmole/0.08 μg total RNA

Therefore, the LC mRNA contained in the 1 μg total RNA was:

OA sample = 0.34 fmole/μg total RNA

NOP sample = 0.18 fmole/μg total RNA

The results of the LC (fmole)/μg total RNA were normalized to total RNA/g cartilage and total RNA/mg DNA:

It was known from Chapter 3 that:

the OA sample contained 96.6 μg total RNA/g cartilage

or 137 μg total RNA/mg DNA;

the NOP sample contained 55 μg total RNA/g cartilage

or 137 μg total RNA/mg DNA;

Therefore,

for the OA sample:

LC = (0.34 fmole/μg total RNA) × (96.6 μg total RNA/g cartilage)

=33 (fmole)/g cartilage

LC = (0.34 fmole/μg total RNA) × (137 μg total RNA/mg DNA)

= 47 (fmole)/mg DNA

for the NOP sample:

LC = (0.18 fmole/μg total RNA) × (55 μg total RNA/g cartilage)

=10 (fmole)/g cartilage

LC = (0.18 fmole/μg total RNA) × (137 μg total RNA/mg DNA)

= 24 (fmole)/mg DNA

Table 7.1 Competitive RT-PCR data for dog 4.

Sample added (µg total RNA)	N_{0s} (fmole)	Log (N_{0s}) (fmole)	Ratio of scan units A_s/A_t OA	NOP	Log A_s/A_t OA	NOP
0.08	0.0065	-2.187	0.16	—	-0.803	—
0.08	0.013	-1.886	0.35	0.95	-0.455	-0.023
0.08	0.019	-1.721	0.64	0.13	-0.193	0.126
0.08	0.026	-1.585	0.94	1.6	-0.025	0.216
0.08	0.039	-1.409	—	2.7	—	0.431

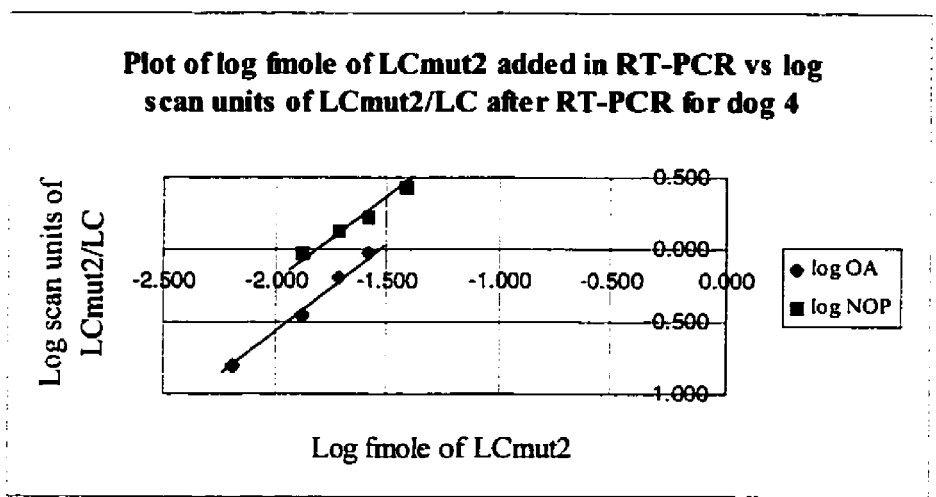
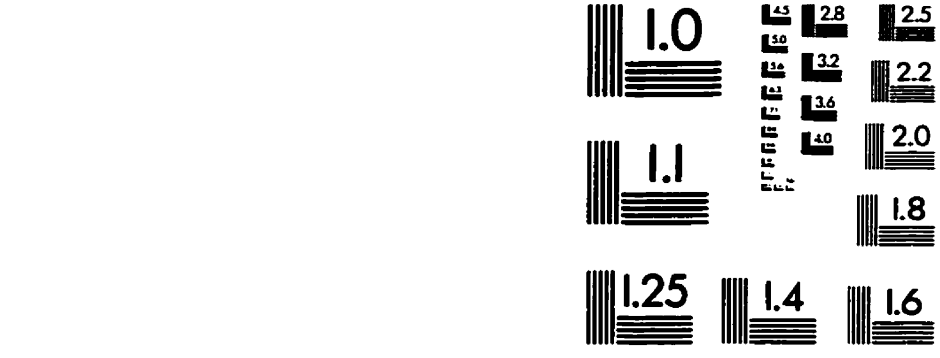
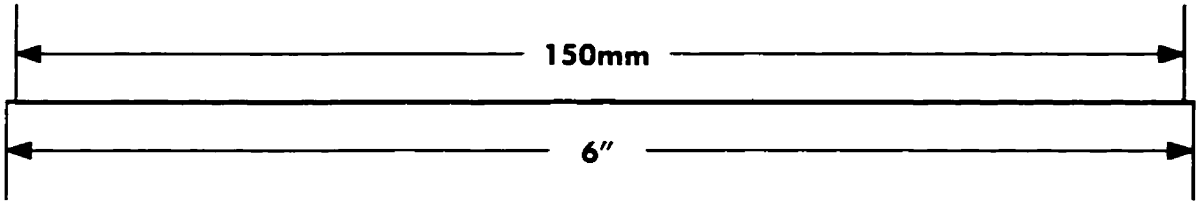


Figure 7.1 Linear plot of Log fmole of the standard added in RT-PCR vs Log scan units ratio of standard/sample after RT-PCR.

The mean LC or ELC isoforms of the five dogs measured as fmole/mg DNA were converted into copies of molecule by multiplying the fmole/mg DNA with the Avogadro's number (6.02×108 molecules/fmole).

IMAGE EVALUATION
TEST TARGET (QA-3)





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