Master's Theses   Theses and Dissertations

1991

Quantitation of Type I Collagen MRNA in Intact Human Fibroblasts

Edward Touma
Loyola University Chicago

QUANTITATION OF TYPE I COLLAGEN mRNA IN INTACT HUMAN FIBROBLASTS

EDWARD TOUMA

ABSTRACT

A number of human connective tissue disorders involve abnormalities in the synthesis of Type I collagen mRNAs. In order to screen large numbers of samples for such defects, a simple and rapid dot blot technique has been developed for the quantitation of collagen mRNA levels in small numbers of intact fibroblasts. Procedures devised for non-mammalian cells have been adapted for use with human fibroblasts. Cells growing in monolayer culture are harvested by trypsinization and fixed in phosphate buffered saline with 1% glutaraldehyde for 60 minutes at 4°C. The cells are then washed and processed through a series of dehydration steps and finally resuspended in 70% ethanol. The desired number of cells is loaded onto a poly-L-lysine coated glass fiber filter under 40 mm Hg of vacuum using a dot blot apparatus. After air drying the filter is incubated with proteinase K followed by triethanol amine and acetic anhydride treatments. The cells on the filter are then hybridized with RNA probes made from Type I collagen cDNAs or with nick translated probe for 28S ribosomal RNA. A strong signal is obtained with 5,000 cells per dot and the amount of hybridization to a dot can easily be quantitated by scintillation counting. The amount of cross hybridization between the probes utilized and structurally similar genes, and the specificity of hybridization of the probes to their target mRNAs was determined by southern blot

analysis and the use of the sense-strand as a negative control, respectively. The sensitivity of the procedure can be increased by increasing the size of the probe used. However, the effect of hyperpolymer formation between the probe molecules and their target mRNA, limits such uses to detection purposes only. When quantitation of the absolute amounts of a particular mRNA species is desired, small probes must be used.

The steady state levels of Type I collagen mRNA in two cell lines (GM0744 and GM02962) from individuals with the lethal form of osteogenesis imperfecta (OI) and one from a normal individual (GM03348B) were compared. The steady state level of total RNA, as measured by hybridization to the 28S rRNA probe, is elevated in both of the OI cell lines relative to the normal cell line. However, there was no proportional increase in the Type I collagen mRNAs. The levels of both collagen mRNAs are low in cell line GM0744 and only $\alpha1(I)$ is low in cell line GM02962. The ratio of $\alpha1(I)$ to $\alpha2(I)$ mRNA in GM0744 was the same as in GM03348B. However, the ratio of the two mRNAs was less than normal in GM02962.

Depending on the size of the probe used, this method could be used for the quantitation of the absolute or the relative amounts of a particular mRNA in a large number of samples, facilitating the systematic screening of affected cell lines for further studies.

QUANTITATION OF TYPE I COLLAGEN mRNA IN INTACT HUMAN
FIBROBLASTS

by

Edward Touma

Thesis Submitted to the Faculty of the Graduate School

of Loyola University of Chicago in Partial Fulfillment

of the Requirements for the Degree of

Master of Science

January

1991

ACKOWLEDGEMENTS

I would like to thank my committee members, Dr. Diane Suter and Dr. John Smarrelli for their time and effort. I extend a very special thanks to my advisor, Dr. Jeffrey L. Doering for his assistance and guidance. I also would like to thank the people in the department of biology especially those in Dr. Doering's laboratory for their help on many occasions during the past years. Most of all, I thank my family for their constant love, support, and encouragment. Without their help my education would not have been possible.

The author, Edward Touma, is the son of Emmanuel Touma and Shoshan Touma. He was born on August 9, 1962, in Baghdad, Iraq.

His high school education was completed in 1979 at Al-Gymhoria High School, Baghdad, Iraq. In October, 1980, he immigrated to the United States.

In June, 1987, he was awarded the degree of Bachelor of Science with honor in Biology from the University of Illinois at Chicago. In September, 1987, the author was granted an assistantship in biology at Loyola University of Chicago, allowing him to complete the requirements for the degree of Master of Science in 1991.

In 1989, Edward Touma presented a poster at the American Society for Cell Biology/The American Society for Biochemistry and Molecular Biology joint meeting in San Francisco. He also presented posters at the Illinios State Academy of Science 82nd Annual Meeting, 1989, and at the Graduate Research Forum sponsored by the Loyola University Chapter of Sigma Xi, where he was inducted into the society as an associate member.

PUBLICATIONS

P.M. Mathisen, E. Touma, A. Jurkowski, and L. Miller. 1987. The induction of keratin gene expresion during <u>Xenopus</u> development. <u>ICSU short reports</u>. Advances in Gene Technology: The Molecular Biology of Development. 7:89.

E. Touma and J. Doering. 1988. A rapid method for determining Type I collagen mRNA levels in human fibroblasts. <u>The Journal of Cell Biology</u>. 107:804a.

E. Touma and J. Doering. 1989. A rapid, improved method for determining Type I collagen mRNA levels in intact human fibroblasts. <u>Transactions of the Illinois State Academy of Science</u>. 82:81.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Osteogenesis imperfecta (OI) is a complex and heteroge-
nous human genetic disorder affecting the skeleton and other
connective tissues. It is characterized by brittle bones and,
to varying degrees, other abnormalities such as blue sclerae,
presenile hearing loss, dentinogenesis imperfecta, joint
hypermobility, and easily bruisable skin (Sillence et al.,
1979; Sillence et al., 1979b; Prockop and Kivirikko, 1984).
Type I collagen is the major component in the tissues involved
in OI. It accounts for about half of the dry weight of the
bone and over 80% of the dry weight of skin, tendons, and
ligaments (Prockop and Kivirikko, 1984). Defects in the
structure and/or synthesis of Type I collagen have been shown
to be the main cause of the OI phenotype (McKusick, 1972).
Mutations in one or the other of the two genes coding for Type
I collagen (COL1A1 and COL1A2) are common in OI, and include
DNA rearrangements, missense mutations, and splicing muta-
tions. Many OI cases are characterized by abnormal collagen
amounts reflected both at the mRNA and protein levels (Bateman
et al., 1988; Byers et al., 1988).

Due to the diversity of the mutations that could cause
OI symptoms and the difficulty in identifying those cases with

alterations in the levels of expression of Type I collagen genes, I opted to develop an _in_ _situ_ dot blot hybridization technique to screen for abnormalities in OI at the mRNA level. _In_ _situ_ dot blot method was chosen mainly for three reasons. The first is that this method requires no isolation and handling of RNA and thus is technically very simple to perform. Second, a large number of different samples can be analyzed at the same time, facilitating the rapid identification of any abnormalities in the synthesis of the Type I collagen mRNA, which can then be studied further. Third, unlike other quantitation methods, this procedure requires a very small sample size (about 25,000 cells) to perform, thus making it very appealing in cases where sample size is limiting.

This study consists of two parts, the first being the development and optimization of a simple method to quantitate the steady state levels of Type I collagen mRNAs in intact human fibroblast cells. The method is a modification of _in_ _situ_ hybridization techniques developed for non-mammalian systems (Yu and Gorovsky, 1987; Lawrence and Singer, 1985; Cox et al., 1984). Poly-L-lysine-coated glass fiber filters are used instead of glass slides or other immobilizing media. Fibroblast cell cultures are used instead of whole tissue and thus a predetermined number of cells can be used. Long ^{32}P-labeled RNA probes are employed instead of nick translated DNA probes to increase the sensitivity of the assay. One draw-

back to the use of large probes is the formation of a hyper-polymer of the probe on the target mRNA. Consequently, the absolute quantity of mRNA can not be determined using large probes. The signal is detected by exposure to X-ray film and quantitated by scintillation counting. The second part of the study tests whether the amounts of Type I collagen mRNAs in OI cell lines are different from that in normal fibroblasts by comparing the steady state levels of $\alpha 1(I)$ and $\alpha 2(I)$ collagen mRNAs in OI and normal fibroblasts. Using the 28S ribosomal RNA as a measure of the steady state of the total RNA, fibroblasts from OI patients appear to contain more RNA than fibroblasts from normal individuals. However, there is no proportional increase in the Type I collagen transcripts in these cells. Such screening may eventually characterize reproducible differences in collagen mRNA levels between the various classes of OI and quickly identify cases with collagen gene defects for further molecular genetic analysis.

LITERATURE REVIEW

Structure and Processing of Type I Collagen

Osteogenesis imperfecta, OI, is a highly heterogenous group of human diseases affecting bone and other connective tissues rich in Type I collagen (Sillence et al., 1979; Sillence et al., 1979b; Prockop, 1984). It is known that defects in Type I collagen are responsible for the majority of OI cases (for review see Byers et al., 1984; Cohn et al., 1988; Bateman et al., 1988). Type I collagen is a hetero-trimer made up of two $\alpha 1$ chains and one $\alpha 2$ chain ($[\alpha 1(I)]_2[\alpha 2(I)]$). Each of the polypeptide chains is encoded by a single gene, COL1A1 and COL1A2 respectively. COL1A1 is located on chromosome 17 (Huerre et al., 1982) and is 18 kilobases (kb) in length (Chu et al., 1985). COL1A2 is located on chromosome 7 (Sykes and Solomon, 1978; Junien et al., 1982) and is 40 kb in length (de Wet et al., 1987). Both genes are made up of 51 exons interrupted by introns of widely divergent sizes (Figure 1). Each exon is composed of codons for the characteristic repetitive triplet, Glycine-X-Y, where X and Y may be any amino acid except tryptophan, tyrosine, or cysteine, and are often proline and hydroxyproline respective-ly (for review see Prockop and Kivirikko, 1984; Byers et al.,

4

1984; Byers et al., 1987). Each exon begins with a glycine codon and ends with a Y codon and the majority of exons are multiples of nine nucleotides, the most common being 54 and 108 base pairs (bp). Thus each exon corresponds to 6 to 12 amino acid triplets (Wozney et al., 1981; Crombrugghe and Pastan, 1982). Based on these findings, it has been proposed that collagen genes evolved from an ancestral gene by multiple duplication or amplification of a single genetic unit containing a 54 bp exon (Yamada et al., 1980; Benveniste-Schrode et al., 1985).

The collagen genes are transcribed into precursor mRNAs that contain copies of both the exons and introns (Figure 1). The precursors are then processed by at least 50 splicing reactions to form the cytoplasmically translatable mRNAs. The transcripts from a single collagen gene are polymorphic in size due to the varying lengths of their 3' untranslated regions. In humans, two mRNAs of different sizes have been identified for pro-$\alpha 1$(I) (Chu et al., 1982), and five for pro-$\alpha 2$(I) (de Wet et al., 1987).

After transcription and processing, the mRNAs are translated by ribosomes on the rough endoplasmic reticulum (RER) (Prockop et al., 1979; Hollister and Holbrook, 1984). The peptides are translocated across the RER membrane into the cisterna. Both α chains are synthesized as procollagen molecules consisting of a central triple helix flanked by an amino and a carboxyl propeptide. The NH_2 and the COOH

terminals appear to aid in the "self assembly" process of the three chains into the characteristic triple helix. As the α chains pass through the membrane of the RER, certain prolyl and lysyl residues are hydroxylated (Figures 1 and 2), and some hydroxylysyl residues are glycosylated by enzymes within the cisterna (Kivirikko and Myllyla, 1982). After translation two $\alpha 1(I)$ and one $\alpha 2(I)$ propeptides come together at the COOH terminal and start to wind around each other into a right handed triple helix (Prockop and Kivirikko, 1984; Byers et al., 1984). The twisting of the three chains proceeds in a zipper-like fashion from the carboxyl to the amino terminus. The hydroxylation and glycosylation reactions continue until the completion of the triple helix prevents any further modification to the procollagen chains. Therefore, the extent of hydroxylation and glycosylation are dependent upon the rate of triple helix formation (i.e., decreased rate of triple helix assembly causes an overmodification of the procollagen molecules). This fact will be important later in understanding some of the mutations causing OI (Bateman et al., 1988).

After the intracellular processing of procollagen, the molecules are secreted into the extracellular matrix where the amino and carboxyl propeptides are cleaved by specific extracellular proteinases. The mature collagen molecules are arranged and crosslinked into stable, quarter-staggered fibrils (Figure 1). The crosslinking reaction occurs by catalyzing oxidative deamination of amino groups of specific

lysyl and hydroxylysyl residues between two adjacent collagen molecules (Prockop and Tuderman, 1982; Prockop and Kivirikko, 1984). Lysyl oxidase is the extracellular enzyme involved in the deamination step.

The two requirements which must be met for a stable collagen helix are a repeating Gly-X-Y sequence, and sufficient proline, hydroxyproline, lysine, and hydroxylysine in the X and Y positions. The presence of glycine in every third position in the chain is conformationally essential for the formation of a helical molecule. The hydroxylation of proline is essential for stabilizing the procollagen triple helix under physiological conditions. Non-hydroxylated pro-α chains cannot form triple helical molecules at 37°C (Kivirikko and Myllyla, 1982). In addition to its role in molecular stability, the hydroxyl group of hydroxylysine serves as the site of attachment of carbohydrate units (glyocsylation) and the location of intermolecular collagen crosslinks (Bornstein and Traub, 1979). The later covalent bonds enable collagen fibrils to serve as the major structural elements in the connective tissues. These linkages stabilize the fibrils and confer on them the tensile strength necessary to withstand the tensions exerted in different parts of the body.

Regulation of Type I Collagen Genes

The collagen family consists of some twenty different genes that give rise to at least thirteen distinct collagen

molecules (Mayne and Burgeson, 1987). Various tissues synthesize a family of different collagen peptides, and thus mechanisms must exist for the regulation of the various types and amounts of collagens expressed in each cell type. The type(s) and amount(s) of collagen(s) are regulated at the transcriptional levels, but quantitative control may also be exerted at the translational and post-translational levels (i.e., through the regulation of the modification enzymes, rate of degradation of collagen, and concentration of certain substrates).

The transcriptional regulation of the collagen genes is a complex process which involves both cis-acting and trans-acting elements (Schmidt et al., 1986; Khillan et al., 1986). Like other eukaryotic genes, the collagen genes contain several regulatory sequences upstream and downstream of the transcription initiation site. A TATA box and a CAAT box are located within a few hundred base pairs of the transcription start site (Chu et al., 1985). These sequences are essential for the proper initiation of transcription (Lewin, 1987). In addition to regulation of transcription initiation the mouse $\alpha2(I)$ promoter region has been shown to contain sufficient information for the proper developmental and tissue-specific expression of a chimeric gene in transgenic mice (Khillan et al., 1986). Several other cis-acting regulatory sequences in the 5' region of collagen genes are also known to play a role in the expression of the collagen genes. For example, the

human α1(I) gene contains two identical, GC rich, twelve bp direct repeats that increase the expression of a marker gene when studied in microinjected <u>Xenopus</u> <u>laevis</u> oocytes (Rossouw et al., 1987). These direct repeats have been shown to be highly conserved through evolution (Brenner et al., 1989). The mouse α2(I) collagen gene contains three upstream elements that serve as the binding sites for specific DNA-binding proteins. Mutations in these elements inhibit the binding of the trans-acting elements and decrease the activity of the mouse collagen promoter by eight to twelve fold (Karsenty et al., 1988).

In addition to these upstream elements Type I collagen genes contain enhancer core sequences within the first intron (Rossouw et al., 1987; Rossi and Crombrugghe, 1987). Enhancer elements exert a positive regulatory effect on the transcription of their respective genes in a distance- and orientation-independent manner (Lewin, 1987). In the case of the α1(I) gene, the enhancer, located between +700 and +1300, is flanked by a pair of inverted decanucleotide sequences on each side (Rossouw et al., 1987). These four consensus sequences serve as the sites for the binding of the RNA polymerase II constitutive transcription factor, Sp1 (Kadonaga et al., 1986; Rossouw et al., 1987). It has been postulated that intricate regulatory control of the α1(I) gene may be achieved by multiple interactions between a set of trans-acting factors that bind the promoter and 5' flanking sequence and a second

array of DNA-binding proteins that recognize intronic sequences (Bornstein and McKay, 1988). The mouse $\alpha2(I)$ collagen gene contains two enhancer sequences between +418 and +1524 of the first intron (Rossi and Crombrugghe, 1987). These enhancer elements are intermixed with several pentanucleotide inverted repeats. The $\alpha2(I)$ enhancers exert a cell-specific transcriptional activation of the gene (Rossi and Crombrugghe, 1987).

The quantity of collagen may also be regulated at stages beyond transcription. First, the amount of collagen produced is proportional to the amount of proline in the intracellular pool (Rojkind and Leon, 1970). A positive correlation between the concentration of free proline and the amount of collagen synthesized has been demonstrated in the liver fibrosis of rats and murine Schistosomiasis (Ehrinpreis et al., 1980; Dunn et al., 1977). The proline intracellular pool is affected by the catabolic activity of proline oxidase (the enzyme that converts proline into glutamate). Second, a highly conserved palindromic sequence around the translation initiation site (position +88 to position +135) of several collagen genes is capable of forming a hairpin structure which may regulate the translation (Chu et al., 1985). Third, the amino terminal peptide resulting from the cleavage of procollagen Type I and III molecules has been shown to exert a negative feedback effect specific for the synthesis of collagen (Wiestner et al., 1979; Paglia et al., 1979; Paglia et al., 1981). This biological activity resides in the NH_2 terminal globular

domain of the propeptide, and can be demonstrated by adding this fragment or the intact amino propeptide to fibroblast cell cultures or to a reticulocyte lysate cell-free translation system of procollagen mRNA. These findings suggest that the NH_2 terminal propeptide or a fragment of it may influence the efficiency with which the procollagen mRNA is translated. Although these peptides are present at a significant concentration in various tissues and biological fluids (e.g., serum, Rohde et al., 1979), whether the regulatory mechanism exists in vivo is not yet known since the peptide's concentration in the extracellular matrix is much lower than that required to cause a reduction in the rate of collagen synthesis in cultured cells. Further, the mechanism by which the peptide exerts its effect is not known.

Another level of control is post-translational. The amount of collagen secreted by cells is regulated through the degradation of a significant fraction of the newly synthesized procollagen within the cell (Bienkowski et al., 1978; Berg et al., 1980). This has been demonstrated by measuring the amount of low molecular weight hydroxyproline-containing peptides shortly after exposing the cells or tissue to radioactively labeled hydroxyproline. Approximately 30% of the newly synthesized collagen is degraded within the first 1/2 hour of labeling (Bienkowski et al., 1978). The rate of the intracellular degradation is in turn regulated by other cellular factors such as levels of cAMP (Baum et al., 1980).

It is apparent from this discussion that the biosynthesis of the collagen molecules is an intricate, multi-step process that requires precise biological control. Any error in this complex process may lead to deficiencies in the collagenous matrix, and in turn to some pathological conditions.

Osteogenesis Imperfecta (OI)

OI is a class of pathological conditions which are all characterized by bone fragility. The disorders result mainly from defects in the structural genes for Type I collagen, although abnormalities in other components of the extracellular matrix (Dickson et al., 1975) or other collagen-like genes (Doering et al., 1987) may also be involved. The mutations in the collagen genes can be divided into two broad classes: structural and synthetic. The structural class includes point mutations and insertions or deletions of various sizes. Synthetic mutations include splicing defects and quantitative abnormalities in the expression of procollagen.

OI has been classified into four different types based strictly on the clinical symptoms and the mode of inheritance (Sillence et al., 1979; Sillence et al., 1979b; Prockop, 1984). The mildest form of OI is Type I. It is a dominantly inherited disorder and is characterized by osseous fragility that decreases at puberty, short stature, blue sclerae, deafness, and sometimes dentinogenesis imperfecta (Sillence et

al., 1979; Sillence et al., 1979b; Prockop, 1984). In a number of studies the phenotype has been shown, by analysis of restriction fragment length polymorphisms, to segregate consistently with one or the other of the two Type I collagen genes, COL1A1 or COL1A2 (Tsipouras et al., 1984; Sykes et al., 1986; Sykes et al., 1990). Thus, defects in the structural genes for Type I collagen are clearly responsible. Biochemically, most cases in this class are characterized by a quantitative reduction in Type I collagen protein of the skin and bones (Cheah, 1985; Byers et al., 1988; Bateman et al., 1988; Barsh et al., 1982). Cultured skin fibroblasts from these patients synthesize about half the normal amounts of Type I collagen (Barsh et al., 1982; Rowe et al., 1985; Byers et al., 1988b). In some cases of this disorder the reduction in collagen propeptides is due to alteration in the absolute amounts of $\alpha1(I)$ collagen mRNA (Rowe et al., 1985). In other cases the $\alpha1(I)$ to $\alpha2(I)$ collagen mRNA ratio deviates from the normal 2:1 (Byers et al., 1988b; Bateman et al., 1988). This stoichiometric alteration in the levels of the two mRNAs ultimately results in a decrease in the amount of procollagen secreted by the fibroblasts. These results suggest that some regulatory mutation in the transcription of the collagen gene, altered stability of the collagen mRNA, and/or reduced transport of the mRNA from the nucleus to the cytoplasm may be the cause in these cases. In other cases, where no changes in mRNA levels are observed, translational defects and/or

increased post-translational degradation have been implicated (Byers et al., 1988b; Bateman et al., 1988). It is also possible in the latter cases, that although $\alpha 1(I)$ to $\alpha 2(I)$ mRNA ratios are unaltered, there may in fact be a coordinate down regulation of both genes (Bateman et al., 1988) which would not be detected by the methods used in these studies.

Although most cases of OI Type I are characterized by reduced levels of collagen, few cases with structural defects in collagen genes are known. A patient with OI type I studied by Sippola et al. (1984) and Kuivaniemi et al. (1988) was found to have a 19-bp deletion at the junction of the intervening sequence 10 and exon 11 of the $\alpha 2(I)$ gene that produced an RNA splicing defect. The abnormal pro-$\alpha 2$ mRNA was spliced from exon 10 to 12. There was no quantitative alteration in either the collagen Type I protein or mRNAs. The patient produced equal amounts of normal and shortened pro-$\alpha 2(I)$ chains. The presence of a mutant pro-$\alpha 2$ chain in procollagen trimers caused a decreased thermal stability of the molecule and made it resistant to cleavage by the extracellular N-protease. Another case studied by Steinmann et al. (1986), Cohn et al. (1988b), and Labhard et al. (1988) is the result of a point mutation in the $\alpha 1(I)$ collagen gene. The mutation results in the substitution of cysteine for glycine in the carboxyl terminus telopeptide of the mature molecule. The procollagen molecules had a normal denaturation temperature and normal translational modifications. Similar point

mutations in the triple helical region of α1(I) result in a more severe form of OI, Type II (see OI Type II for explanation). The reason for this is not known.

OI Type II is the severest form of the disease. It is characterized by neonatal dwarfism, severe bone deformity, and usually results in death during or soon after birth. In most cases it is the result of new dominant mutations in the genes for Type I collagen (Bateman et al., 1986; Byers et al., 1988b), but in about 5% of the cases it is inherited in an autosomal recessive fashion (Byers et al., 1988b). At the biochemical level, OI Type II is also characterized by decreased collagen production. In the few cases where collagen mRNAs have been studied, altered levels have been found (de Wet et al., 1983a; de Wet et al., 1983b). In two patients the steady state ratios of pro-α1(I) to pro-α2(I) mRNA were greater than 2:1 and the ratio of the two collagen proteins was increased proportionately (de Wet et al., 1983a). The presence of a non-functional α2(I) allele has been implicated in these altered mRNA ratios. However, most cases are caused by a point mutation in the triple helical coding region of Type I collagen genes. The point mutation is more frequently found in the α1(I) gene than in the α2(I) gene although a few such mutations have been found in the latter gene. The nature of the mutations is the replacement of glycine in the gly-X-Y repeat by another amino acid, commonly cysteine and arginine but also asparagine, valine, and serine

(Byers, 1989; Prockop et al., 1989). Since the presence of glycine, the smallest amino acid, in every third position is crucial for the formation of a functional triple helix, its substitution results in a decrease in the rate of the zipper-like winding of the procollagen chains from the site of the mutation to the end of the chain, the amino terminal. The slower helix formation leads to a cascade of events: over-modification of the polypeptide by co-translational and post-translational enzymes, a decrease in the thermal stability of the trimers, increased intracellular degradation, and a decreased secretion of procollagen polypeptide (Bateman et al., 1988; Prockop et al., 1989; Steinmann et al., 1984; Williams and Prockop, 1983).

In addition to point mutations, deletions and insertions in Type I collagen genes are known in OI Type II. In one patient a mutant α1(I) gene had undergone intragenic recombination between two non-homologous introns, which resulted in a 651-bp deletion, removing three exons coding for 84 amino acids in the triple helical domain (Chu et al., 1985b; Barsh et al., 1982). The deletion caused an increase in the modification of the procollagen molecules containing a mutant chain and their decreased extracellular secretion (Barsh and Byers, 1981; Williams and Prockop, 1983; Penttinen et al., 1975). As a result the ratio of Type I collagen to other extracellular matrix molecules was very low. Interestingly, the termini of the rearrangement were found to be located

within two short inverted repeats suggesting that the self-complementary nature of these DNA elements may have favored the formation of a DNA secondary structure intermediate which served as a substrate for the "recombination deletion" reaction (Chu et al., 1985b). Another case of deletion has been found in the α2(I) gene. In this patient a heterozygous 4.5 kb de novo deletion results in the removal of seven exons encoding 80 amino acids (residues 586-765) of the triple helical region (Willing et al., 1988). The intron-to-intron deletion preserved an integral number of the glycine-X-Y triplet repeats. The 5' junction of the deletion is located in the middle of a stretch of 23 AT base pairs but there is no evidence that this sequence is predisposed to recombination. In addition to deletion there is a case of insertion in one allele of the α1(I) collagen gene. The insertion, toward the amino terminal of triple helical domain, is 70-80 amino acids long (Byers et al., 1988b). The insertion appears to result from the duplication of a multi-exon 600 bp segment of the α1(I) gene. All of these cases of OI produce about equal amounts of mutant and normal Type I chains. The collagen molecules containing defective α chains become overmodified because of the delay in the triple helix formation of collagen molecules. These molecules are thermally unstable and are secreted either inefficiently or not at all. As a result, all of these cases are characterized by a reduced synthesis of collagen proteins.

OI Type III is a moderately severe form characterized by a progressive deformation of bones due to repeated multiple fractures. Both sporadic and autosomal recessive modes of inheritance have been demonstrated (Aitchison et al., 1988; Sillence et al., 1979; Sillence et al., 1979b). In the majority of cases examined the defect does not appear to be associated with the collagen Type I genes. Many of the patients in this class of OI show no collagen Type I abnormalities and the analysis of restriction fragment polymorphism markers indicated that the mutation is unlinked to either of the Type I collagen genes (Byers et al., 1987; Aitchison et al., 1988). Also, recent results in our lab indicate that in the majority of Type III OI patients, an $\alpha2(I)$ complementary DNA (cDNA) probe detects deletions at one or the other of two discrete sites in a Type I collagen-like gene other than COL1A1 or COL1A2 (Doering et al., 1987). While there is a significant reduction in Type I collagen production in some OI Type III fibroblast cells, this reduction does not correlate to altered $\alpha1(I)$ to $\alpha2(I)$ mRNA ratios (Bateman et al., 1988; Dickson et al., 1984; Bonaventure et al., 1986). Therefore, in most cases, mutations may be located not in Type I collagen genes themselves but in other genes which, by some unknown mechanism, produce OI with Type III phenotype. It has been suggested that some cases in this category may be due to abnormalities in the post-translational events (Bateman et al., 1988).

However, two Type III patients with structural mutations in Type I collagen genes are known. In the first case, the patient acquired two defective alleles of pro-α2(I) from his phenotypically normal, third cousin parents (Dickson et al., 1984; Pihlajaniemi et al., 1984). The recessive mutation had been stably inherited through at least five generations. The nature of the mutation is a four nucleotide deletion in the carboxyl propeptide (in exon number one) of the pro-α2(I) gene. The deletion shifts the reading frame of the last 33 amino acids of the propeptide and results in the utilization of a termination codon (TGA) four nucleotides 3' to the original termination site (TAA). Consequently, α2(I) collagen chains are not incorporated into the collagen molecule. Instead, thermally stable α1(I) trimers are formed and secreted extracellularly. Fibroblasts from the parents secrete α1(I) trimers in addition to normal Type I pro-collagen. The second case involves a substitution mutation in the α1(I) collagen gene (Pack et al., 1989). The glycine at position 844 is replaced by a serine. Although the phenotype in the proband was only moderate in severity, the nature of the mutation is very much like those found in lethal OI Type II. Why two similar mutations in the same general region result in two distinctly different phenotypes is not well understood. A possible explanation may lay in the fact that collagen chains are made up of thirteen "cooperative blocks" (Privalov, 1982). These blocks do not contribute equally to

the thermal stability of the protein (i.e., some blocks are more important than others to the over all stability of the molecule). Furthermore, glycyl residues in the same general region of the block are not equivalent in their stability factor. As a result, substitution of certain glycyl residues may have a more deleterious effect than the substitution of other glycyl residues within the same region.

The least understood class, OI Type IV, is characterized by moderate clinical severity, limited degree of skeletal deformity, and blue sclerae which lighten with age. Inheritance is usually dominant, although sporadic mutations are known to occur (Sillence et al., 1979; Sillence et al., 1979b; Prockop and Kivirikko, 1984). Biochemically, in addition to reduced collagen synthesis, most individuals with this form of OI produce two populations of Type I procollagen molecules, normal and overmodified (Bateman et al., 1988; Marini et al., 1989; Wenstrup et al., 1986; Wenstrup et al., 1988). The overmodified chains may be secreted extracellularly at a reduced rate. Unlike OI Type I, the reduction in collagen is not correlated with altered ratios of $\alpha1(I)$ to $\alpha2(I)$ mRNA (Bateman et al., 1988). At the molecular level only a single case of OI Type IV has been found to be caused by a point mutation in the $\alpha1(I)$ gene (Marini et al., 1989). The defect arose spontaneously in one of the $\alpha1$ alleles and resulted in the substitution of serine for a glycine at amino acid residue 832. The rest of the cases are due to defects in the $\alpha2(I)$

gene. Two such cases have been examined in detail. The first involves an arginine substitution for the last glycine of the triple helical domain (position 1012) of the $\alpha2(I)$ gene (Wenstrup et al., 1988). The second case involves a ten amino acid deletion from the middle of the $\alpha2(I)$ triple helical domain (Wenstrup et al., 1986). Because mutations in the $\alpha2(I)$ gene are less severe than similar mutations in the $\alpha1(I)$ gene (see below) it has been postulated that point mutations in $\alpha2(I)$ chains are likely to be a common feature of the Type IV OI phenotype (Byers et al., 1988b; Falk et al., 1986).

At first glance the innumerable mutations that give rise to the various clinical manifestations of OI render no consistent cause-and-effect relationship. Upon closer inspection of the nature of the defects, however, some general features emerge. First, the evidence indicates that mutations resulting in decreased levels of collagen (transcriptional or translational abnormalities) are phenotypically less severe than mutations which affect the primary structure of the proteins produced (point mutations, deletions, and insertion). The presence of a structurally abnormal pro-α chain in procollagen results in interference with the folding of the protein into the stable triple helical conformation, the processing of the NH_2 terminal propeptide, or the assembly of processed collagen into normal fibrils (Prockop et al., 1989). Second, mutations in the pro-$\alpha2(I)$ gene cause less severe symptoms than mutations in the pro-$\alpha1(I)$ gene. Stoichiometri-

cally, the incorporation of a mutant pro-α2(I) chain results in a 50% inactivation of the total procollagen trimers (Figure 3; Williams and Prockop, 1983). The incorporation of a mutant pro-α1(I) chain, on the other hand, causes the inactivation of up to 75% of the procollagen molecules. These abnormal molecules are rapidly degraded either before or after secretion from the cell. This phenomena of destruction of collagen harboring abnormal α chains is called "protein suicide" (Figure 3). Third, large rearrangements in either of the genes of Type I collagen cause more severe symptoms than point mutations. Small deletions vary in the severity of their effects depending on the chain and the location of the mutation in the chain. Fourth, mutations located toward the amino end of procollagen cause milder OI phenotypes than similar mutations located at the carboxyl terminal. This is because the winding of the three α chains around each other proceeds from the COOH toward the NH_2 terminal. As a result mutations closer to the carboxyl end cause a greater delay in the formation of the triple helix and consequently cause a greater degree of modification of the procollagen molecule.

In situ Hybridization

All forms of OI have reduction in collagen protein as a common biochemical feature, although the underlying defect may be very different from case to case. Mutations that give rise to structurally abnormal Type I collagen chains are more

accessible to analyses by current technologies than mutations that cause an alteration in the levels of expression of the genes. Therefore, a simple method for quantitating the level of collagen gene expression in a large number of OI patients would be very helpful in further understanding the disorder. Due to recent advances in _in situ_ hybridization techniques such quantitation at the mRNA level is possible. The _in situ_ hybridization technique was first described by Gall and Pardue (1969). It allows the visualization of cellular DNA or RNA in tissue sections, single cells or chromosome preparations (Yu and Gorovsky, 1987; Brahic and Haase, 1978; Capco and Jeffery, 1978). The technique has been used extensively in cell biology, gene mapping, prenatal and pathological diagnosis, and investigation of specific gene expression in tumor or normal cells. The technique has also been used successfully, but to a lesser extent, for quantitative purposes (Lawrence and Singer, 1985; Yu and Gorovsky, 1987). _In situ_ hybridization requires fixing of tissue or cells, immobilization, pretreatment, and hybridization of the sample with a radio-labeled probe of interest. The results of hybridization are studied by either autoradiography or microscopy (light or electron). Several different fixatives have been used but glutaraldehyde (Yu and Gorovsky, 1987) and paraformaldehyde (Lawrence and Singer, 1985) are the most reliable since they provide for the most RNA retention. Glutaraldehyde is a stronger fixative than paraformaldehyde and as a result cells

treated with glutaraldehyde require extensive digestion with proteinase K to optimize hybridization. Different immobilization media have also been utilized (e.g., cells embedded in paraffin or gelatin, cells fixed onto cover slip, or cells immobilized to poly-L-lysine coated glass fiber filters). For use with dissociated cells the latter immobilization method is the best for quantitation purposes because a predetermined number of cells can be loaded onto the filter (Yu and Gorovsky, 1987). The pretreatment and hybridization parameters vary markedly among different researchers and from one cell type to another.

Two in situ hybridization methods have been developed for the quantitation of mRNA. The first used paraformaldehyde to fix chicken embryonic muscle cells grown on coverslips treated with gelatin (Lawrence and Singer, 1985). The coverslips with the fixed cells were used directly in the hybridization reaction. The signal obtained was quantitated by scintillation counting of the coverslips. In this study paraformaldehyde was determined to be the fixative of choice because it provided for a good RNA retention, a good hybridization signal without a proteolytic treatment, the ability to use large probe fragments, and the advantage that cells treated with paraformaldehyde exhibit minimal autofluorescence (needed for use with fluorescent detection methods). The sensitivity reported was 20 copies of a 2 kb message per cell using nick-translated DNA probes. The second method was used
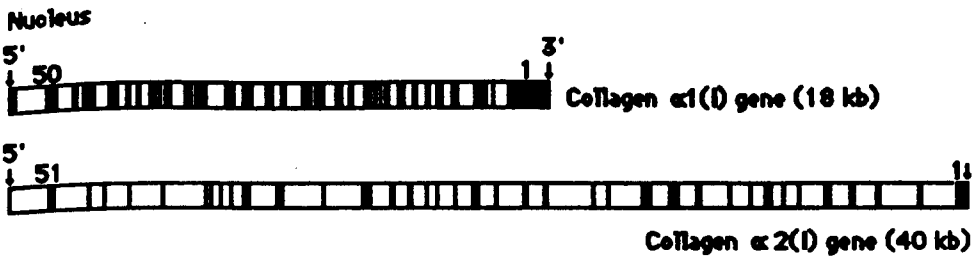
to quantitate histone mRNA in <u>Tetrahymena</u> <u>thermophile</u> (Yu and Gorovsky, 1987). It employed glutaraldehyde as a fixative and poly-L-lysine-coated glass fiber filters as the immobilizing medium. Complementary RNA probes with a mass average of 150 bases were used for the hybridization. The signal was quantitated by densitometry or scintillation counting. Unlike paraformaldehyde-fixed cells, glutaraldehyde-fixed cells required proteolytic treatment to allow for optimum probe penetrability into the cell. The sensitivity limit was 1 or a few messages per cell.

No quantitative <u>in</u> <u>situ</u> method has previously been devised for use with human cultured cells. Thus, such a technique had to be developed in the present study prior to examining collagen gene expression in fibroblasts of OI patients.

The usefulness of <u>in</u> <u>situ</u> hybridization as a quantitation method depends on the accuracy and sensitivity with which signals reflect the actual concentration of target mRNAs (Cox et al., 1984). Sensitivity has been increased by using high specific activity RNA probes corresponding to the coding strand and by decreasing non-specific binding of the probe to the immobilizing media by including ficoll, bovine serum albumin, and polyvinylpyrrolidone in the hybridization solution (Cox et al., 1984). The accuracy of <u>in</u> <u>situ</u> hybridization for RNA quantitation can be comparable to standard methods using purified RNA. The important advantage of <u>in</u>

situ techniques is that no isolation or handling of unstable mRNA is required, and as a result a measure of true mRNA concentration per cell can be established. This method also allows for the relatively easy processing of more samples at one time.

Figure 1. The biosynthesis of procollagen Type I. Schematic representation of the $\alpha 1(I)$ and $\alpha 2(I)$ genes. Gene structure: black box, exons; open box, introns. The two genes are transcribed and processed to yield the mature mRNAs which are translated on membrane-bound polysomes. The signal peptide cleavage, hydroxylation, glycosylation, disulfide bonding and helix formation occurs within the rough endoplasmic reticulum. The procollagen containing the amino and carboxyl propeptides are secreted into the extracellular matrix. Outside the cell the propeptides are cleaved by specific N- and C-proteinases. The mature collagen monomers are assembled into collagen fibrils by quarter staggered aggregation and cross-linking.
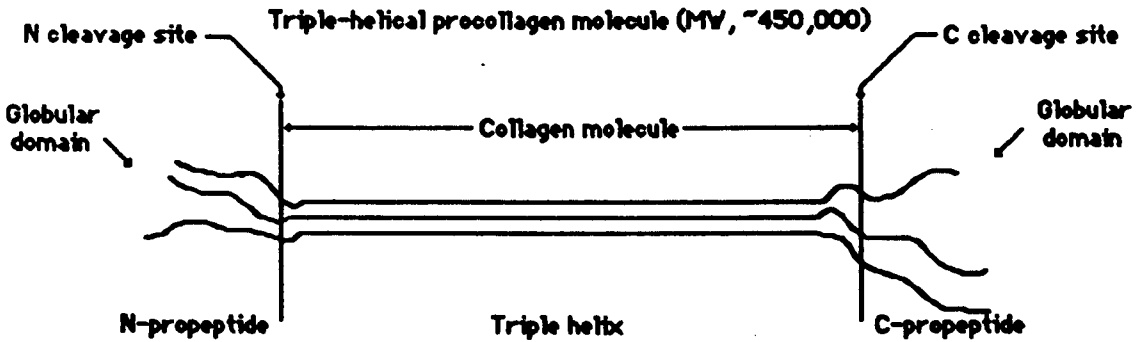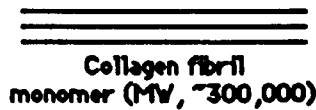
Figure 2. The translation and processing of procollagen Type I. Schematic representation of the events of collagen biosynthesis that occur in the rough endoplasmic reticulum and their secretion. After translocation across the RER the hydroxylation of proline and lysine residues and the glycosylation of certain hydroxylysine residues are initiated. The formation of the triple helix stops the modification process. The protein is secreted through the smooth vacuoles of the cells into the extracellular matrix. Abbreviations: Gal, galactose; Glc, glucose; GlcNac, N-acetylglucosamine; $(Man)_n$, mannose residues.
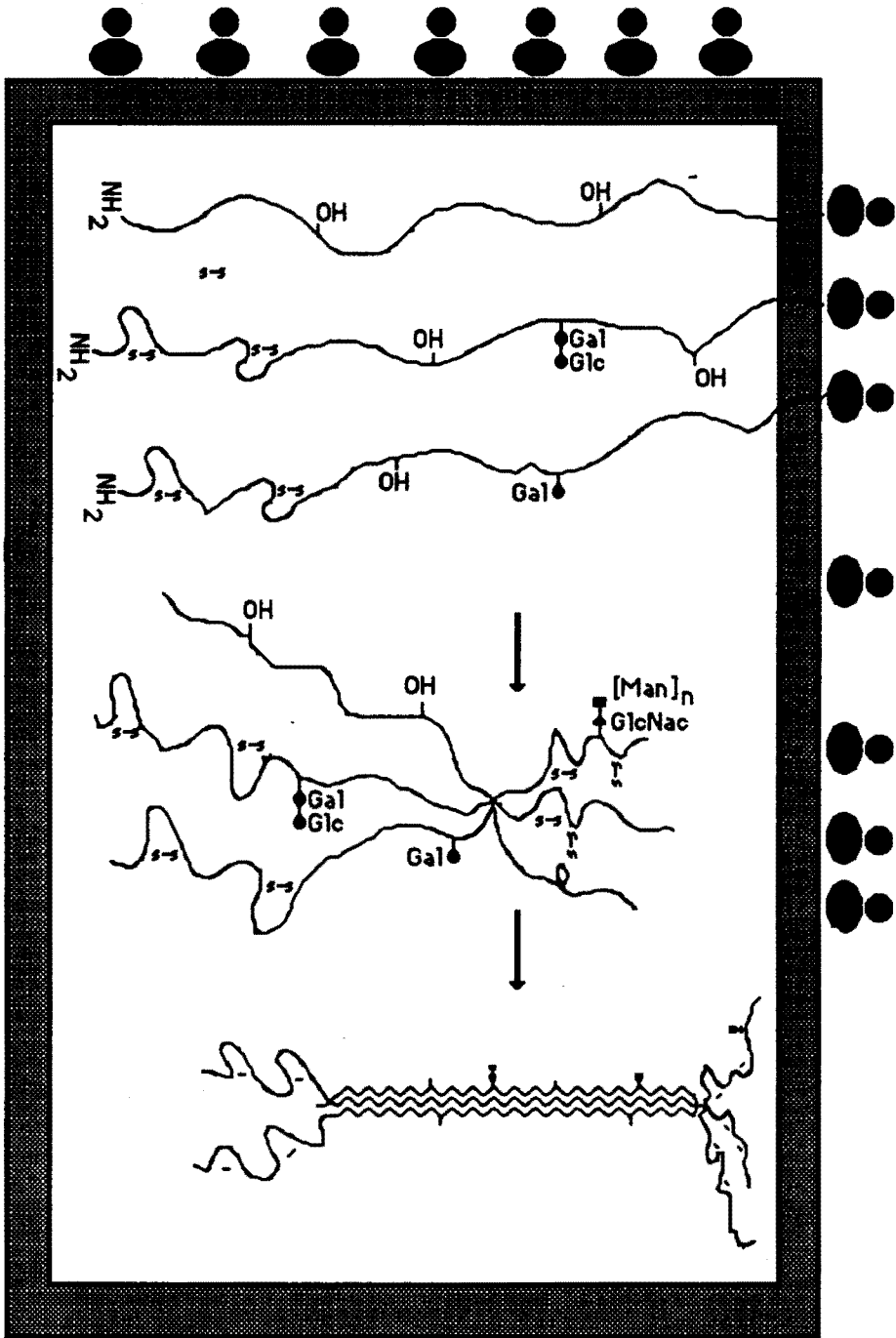
ROUGH ENDOPLASMIC RETICULUM

Figure 3. Protein suicide: Schematic representation of the effect of a mutant $\alpha(I)$ chain on the molecules containing it (pro-$\alpha1^s$). The mutant chain becomes associated with normal pro-α chains into disulfide-linked trimers. The trimers containing mutant chains cannot fold into a stable triple helix at 37°C, and are rapidly degraded. In the presence of one mutant pro-$\alpha1$ allele 75% of the molecules are affected. The presence of one mutant Pro-$\alpha2$ allele, on the other hand, results in 50% defective molecules.

Normal fibrils

Pro-α 1

Pro-α 1^S

Pro-α 2

Degradation
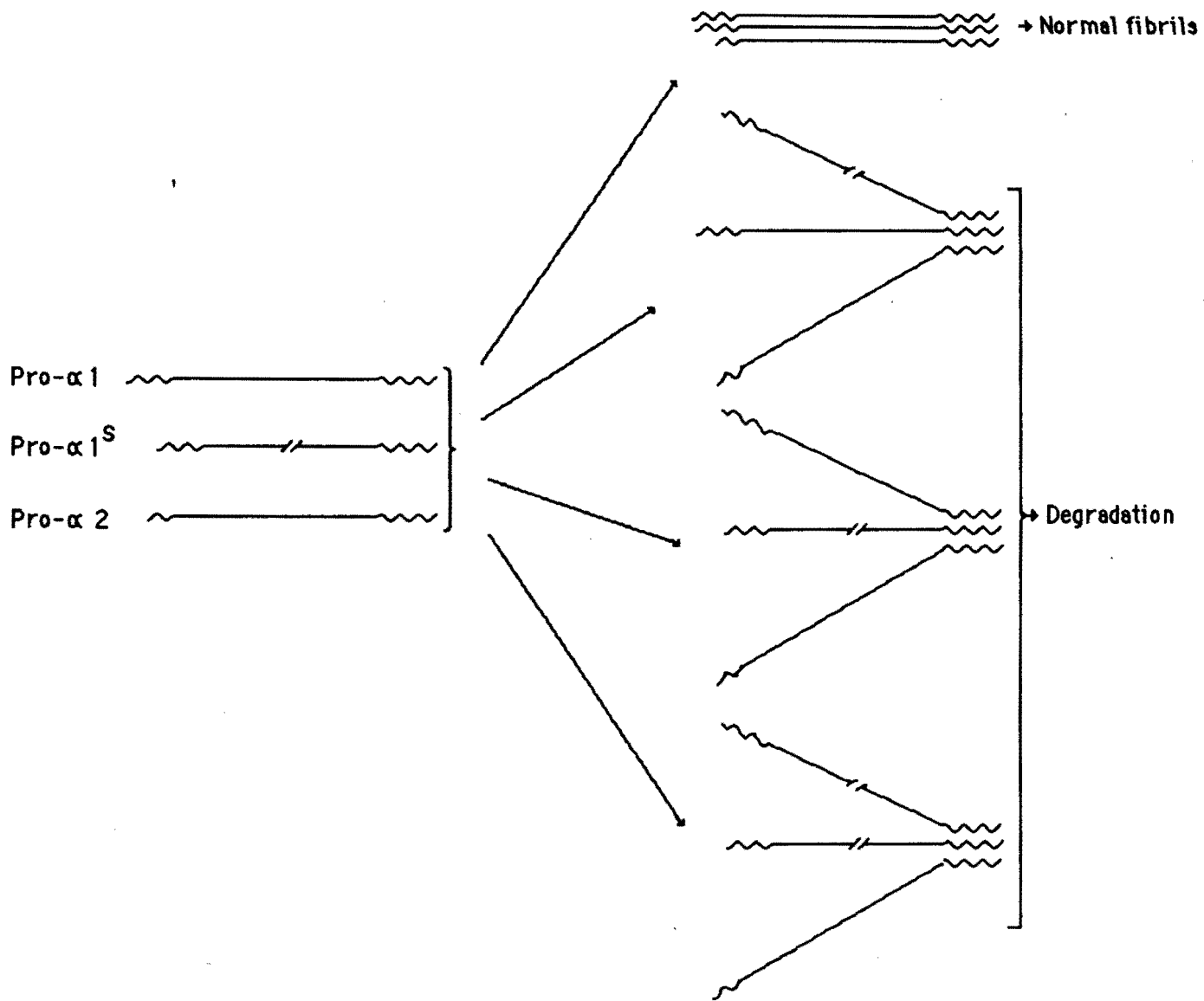
MATERIALS AND METHODS

Source and Preparation of RNA Probes

The collagen cDNA clones encoding pro-$\alpha 1$(I) (Hf404 and Hf677, Chu et al., 1982), pro-$\alpha 2$(I) (Hf32, Myers et al., 1981) and pro-$\alpha 2$(V) (Ok25, Weil et al., 1987) were generously provided by Dr. F. Ramirez. The genomic clones for the 18S and the 28S ribosomal RNA (pXlr-11, Dawid and Wellauer, 1976) were a gift from Dr. I. B. Dawid. The cDNA clones for α tubulin and β actin (pT1 and pA1, respectively, Cleveland et al., 1980) were provided by Dr. P. R. Musich. All of the above cDNAs were cloned in the plasmid vector pBR322. The genomic clones pXlr-12 and pXlr-11 were cloned in the Col E1 vector. The probes used in this study were prepared via either nick translation or in vitro transcription. Prior to nick translation the clones were cut by restriction enzymes to release the insert from the vector. The digested DNA was then used to make ^{32}P labeled probe by the method of Rigby (1977). For the in vitro transcription the insert was first cloned into the pGEM-4Z vector (Promega Biotec, Inc.). This vector contains two promoters, SP6 and T7, flanking a polylinker into which the insert is cloned. The pGEM-4Z clones were linearized by restriction digestion prior to use for in vitro

transcription (see below).

Insert Isolation for Cloning

Hf404 contains a 1.8 kb insert coding for residue 247 to residue 861 in the α chain of α1(I). Hf677 contains a 1.475 kb fragment coding for residue 787 to near the end of the coding region of α1(I). The two α1(I) cDNAs have 74 bps in common. Hf32 contains a 2.2 kb insert coding for residue 450 of the α2(I) chain and extending to the middle of the COOH-terminal propeptide. Ok25 encodes 3.6 kb of α2(V) collagen mRNA extending from the beginning of the α chain to the end of the carboxyl propeptide. pA1 contains a 1.9 kb insert encoding for nearly the full length of ß actin. One hundred micrograms of each of the above plasmids were cut with the appropriate restriction enzyme(s) (EcoR I for Hf404, Hf677, Ok25; Pst I for pA1; EcoR I and Pst I for Hf32) to excise the insert from the vector. The Hf32 cDNA insert was cut into two fragments and the 3' portion (Eco RI/Pst I 1.1 kb fragment), corresponding to amino acid 871 of the α helix to amino acid 1275 of C-propeptide, was used for the subcloning. In all other cases the full insert was used. The product of the digestion reaction was electrophoresed through 1% agarose, by standard procedures (Doering et al., 1982; Sims et al., 1983), to separate the plasmid from the insert DNA (Figure 4). The insert was isolated from the gel using the electroelution method of Dretzen (1981).

Ligation and Transformation

The transcriptional vector pGEM-4Z (Riboprobe Gemini, Promega Biotec, Inc.) used here contains the ampicillin resistance gene and lacZ α-peptide gene. The later gene allows for blue/white color screening of recombinants (Yanisch-Perron et al., 1985). This plasmid was digested with the same restriction enzyme(s) used above for excision of the insert from the original vector. Different molar ratios of plasmid to insert were ligated (Figure 4) overnight at 4°C using 1 unit of T4 DNA ligase in T4 ligation buffer supplied by the manufacturer (BRL, Bethseda Research Laboratories). The ligation reaction was then used to transform E. coli bacterial strain JM109 (Promega Biotec, Inc.) by the calcium chloride transformation method (Cohen et al., 1973). Transformants were selected on ampicillin plates in the presence of 4 mg/ml Bluo gal (BRL, in N,N-Dimethyl formamide) and 1 mg/ml isopropylthio-β-D-galactoside (IPTG, BRL). White colonies (containing recombinant plasmids) were isolated, a replicate prepared, and small-scale plasmid preparations were performed as described below. Restriction digests were then done to determine whether the desired insert was present and identify its orientation. Where possible, plasmids with the insert cloned in both orientations with respect to the SP6 promoter were isolated (see Figure 5).

Ten mls of an overnight culture were centrifuged and the pellet was resuspended in 1 ml of lysis buffer (10 mM Tris-HCl

pH 7.5, 1 mM EDTA, 0.5% SDS). Twenty ug/ml of proteinase K were added to the solution, which was then mixed and incubated at 37°C for 30 minutes. The salt concentration was brought up to 1.0 M by the addition of 370 ul of 4 M NaCl. The mixture was vortexed and incubated on ice for 1 hour. The chromosomal DNA and the cellular debris were pelleted in a 40 K rotor (Beckman) at 30,000 RPM for 30 minutes at 0°C. The supernatant was extracted once with water-saturated phenol and once with ethyl ether. Samples were incubated at 65°C until the residual ether had evaporated. The plasmid DNA was precipitated with 2 volumes of 95% ethanol at -20°C for at least 1 hour. The DNA pellet was washed once in 70% ethanol, vacuum dried and dissolved in 50 ul of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 7.9). Once the recombinant was identified, a large-scale plasmid preparation was carried out by a modification of the cesium chloride-ethidium bromide gradient method (Maniatis et al., 1982).

In Vitro Transcription

The templates for _in vitro_ transcription were prepared by truncation of the plasmids downstream from the cloning site for the inserts. The message and anti-message RNA probes for all of the cDNAs, except $\alpha 2(I)$, were prepared by transcription of plasmids containing the cDNA inserted in the two different orientations relative to the SP6 promoter (for example see Figure 5). Both T7 and SP6 promoters were used for the

synthesis of the sense or the anti-sense strand, respectively, of α2(I) RNA probes. The _in vitro_ transcription was done according to the manufacturer's recommendations (Promega Biotec, Inc.). In some cases, after the _in vitro_ transcription the DNA template was degraded by incubation of the reaction mixture with 1 unit of RNAse-free DNase I (RQ1 DNase, Promega Biotec, Inc.) for 15 minutes at 37°C. The probe was then extracted with water-saturated phenol and purified away from the unincorporated nucleotides by running it through a 5 ml Sephadex G-50-80 (Sigma Chemical Co.) filtration column that was equilibrated and eluted with TE buffer. All solutions used with the RNA probes were made with water that had been treated with diethyl pyrrocarbonate for 12 hours (DEPC, Sigma Chemical Co.) and then autoclaved. The amount of probe synthesized was calculated as follows:

The total counts per minute (CPM) of the probe equals

$$\frac{CPM}{1 \text{ ul of sample}} \text{ X total volume of probe}$$

Using the given specific activity (Ci/mmole) of the radioactive nucleotide (CTP), the number of CTP nucleotides in the transcript, the average molecular weight of ribonucleotides (340 daltons), and the number of decays per minute (dpm) per uCi of radioactive nucleotide (2.2×10^6), the nanograms of transcripts present is calculated using the following formula:

$$\text{Total CPM X } \frac{1 \text{ uCi}}{2.2 \times 10^6 \text{ dpm}} \text{ X } \frac{1 \text{ nmole}}{\text{specific activity of CTP}}$$

$$\text{X } \frac{\text{length of template}}{\text{number of CTPs in template}} \text{ X } \frac{\text{average MW of NTP}}{1 \text{ nmole}}$$

The number of counts per minute for ^{32}P equals the number of disintegration per minute. The specific activity of the radioactive nucleotide equals

$$\frac{\text{daily specific activity of CTP}}{\text{ratio of radioactive to total amount of rCTP used}}$$

RNA Gel Electrophoresis

Radioactively labeled RNA samples were denatured by incubating 0.3 ng of each of the RNA probes in a 10 ul reaction containing 50% formamide, 2.2 M formaldehyde, and 1X MOPS (10X: 0.2 M [3-(N-morpholino) pro-panesulfonic acid], 50 mM sodium acetate, 10 mM EDTA adjusted to pH 7.0 and auto-claved) at 65°C for 15 minutes and then chilling on ice. Prior to loading onto the gel, 2 ul of sterile loading buffer (50% glycerol, 1mM EDTA and 0.4% bromophenol blue) was added. The RNA gel contained 1.0% agarose, 0.66 M formaldehyde and 1X MOPS. All reagents were prepared in DEPC treated water. The gel electrophoresis apparatus was treated with 3% H_2O_2 for 15 minutes prior to use to remove ribonuclease activity.

Preparation of Poly-L-Lysine Coated Glass Fiber Filters

The glass fiber filters (Whatman Glass Microfiber Filters 934-AH) were soaked in 50 ug/ml poly-L-lysine (150-300,000; Sigma Chemical Co.) in 10 mM Tris, pH 8.0, at room temperature for 10 minutes, air dried in a folded piece of Blot Block paper (Schleicher and Schuell, Inc.) and stored with desiccant at room temperature for periods of up to a

month.

Cell Cultures and Fixation

Control human fibroblasts (GM03348B) and fibroblasts from two patients with lethal variants of OI (GM0744, GM02962) were obtained from the National Institute of General Medical Science Human Genetic Mutant Cell Repository. The cells were grown under standard conditions in Dulbecco's Modified Eagle's media (Gibco) containing 10% fetal bovine serum (Gibco), and 50 units/ml of each penicillin and streptomycin (Gibco). The fibroblasts were passaged with 0.06% trypsin (Gibco) in phosphate-buffered saline (PBS, 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, pH 7.0-7.2). Cell cultures between passage 6 and 13 were used exclusively in this study. At confluency, the cells were harvested by trypsinization and washed twice with PBS (de Wet et al., 1983a). The pellet from a 25 cm^2 plastic flask (Falcon) was resuspended, by pipetting, in 1 ml PBS containing either 1% glutaraldehyde (Sigma Chemical Co., Grade I, 25% aqueous solution) or 4% paraformaldehyde (Fisher Scientific, reagent grade). Fibroblast cells suspended in glutaraldehyde were mixed for 30 seconds at room temperature and placed on ice for 1 hour with occasional mixing by pipetting. Fibroblast cells suspended in paraformaldehyde were mixed and incubated at room temperature for five minutes. The cells were then pelleted for 2 minutes in a clinical centrifuge and sequentially dehydrated by washing

twice for 10 minutes in ice-cold water, twice for 15 minutes in 50% ethanol on ice, and once for 25 minutes in 70% ethanol on ice. Cells were finally resuspended in 70% ethanol (1 ml/25 cm^2 flask), counted and stored at -20°C (glutaraldehyde) or 4°C (paraformaldehyde).

^{3}H-labeling of Fibroblast Cells

Human fibroblast cells near confluency were incubated with 5 mls of media containing 25 uCi of ^{3}H-thymidine. The stock solution of the ^{3}H-thymidine was 1 uCi/20 ul of medium. The cells were incubated in this medium for 1 day. The radioactive medium was decanted and the cells washed several times with PBS. The cells were then fixed with 1% glutaraldehyde as described above.

Loading and Treatment of Cells

Filters were placed in a 96 well micro-sample filtration manifold (Schleicher & Schuell, Inc.) and moistened with 10 mM Tris, pH 8.0. The manifold was connected to an aspirator or a vacuum pump and the desired number of cells, in 100 ul of 70% ethanol, was loaded onto the filters under a vacuum of about 40 mm Hg (weak). The filter was then removed and air dried on a piece of Blot Block paper for about 45 minutes. Filters were then incubated with 10 ug/ml (unless stated otherwise) of proteinase K (BRL) in proteinase K buffer (100mM Tris-HCl pH 8.0, 50mM EDTA in non DEPC-treated water) at 37°C

for 40 minutes (unless stated otherwise) in a Zip Lock bag. After the proteinase K treatment the filters were rinsed briefly in proteinase K buffer, immersed in 0.1 M triethanolamine (J. T. Baker Chemical Co., Trolamine) pH 8.0 at room temperature for 10 min, and then in 0.1 M trolamine plus 0.25% acetic anhydride (Baxter Scientific Products, added immediately before use) at room temperature for 10 min. The filters were finally rinsed in proteinase K buffer and allowed to air dry on a piece of Blot Block paper. The rinsing and trolamine treatments were done in a small plastic tray with sufficient volume to cover the filters.

Hybridization Conditions and Southern Blotting

Due to the sequence similarity among the different collagen and structurally similar genes, conditions had to be determined that prevent cross hybridization. This was done using Southern blotting. Several collagen and structural cDNAs were digested with the appropriate enzyme to excise the inserts. The products of the restriction digests were electrophoresed on 1% agarose gels by established methods (Sims et al., 1983). The DNA was transferred to Gene Screen Plus membranes (NEN Research Products) using the alkaline method (Reed and Mann, 1985). The membranes were incubated overnight at 45°C with 35 ng of RNA probe per ml of hybridization solution. A prehybridization step was not included in this procedure (see Results and Discussion). The hybridiza-

tion solution contained 50% formamide (Fluka), 10% dextran sulfate (Pharmacia), 0.05 M Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, 1X Denhardt's solution [50X: 1% Bovine Serum Albumin (Sigma), 1% Ficoll (Pharmacia), 1% Polyvinyl pyrrolidone (Sigma)] and 0.2 M NaCl. After hybridization, the membranes were washed in three changes of 2X SSC (1X; 0.15 M NaCl, 0.015 M sodium citrate, 1 mM EDTA, pH 7.9) for 5 min each at room temperature, two changes of 0.05X SSC for 20 min each at 65°C, and finally with 0.05X SSC for 5 min at room temperature. The membranes were then air dried slightly and exposed to Kodak X ray film with or without Cronex intensifying screens (Doering et al., 1982).

Hybridization to Intact Human Fibroblasts

The hybridization solution used in the _in situ_ hybridization with the cells is identical to that used in the Southern blots except for the elimination of 1% SDS. The filters were placed in Zip Lock bags, the probe was mixed with the hybridization solution at 40 ng/ml, unless stated otherwise, and 0.2 ml/cm^2 of the solution was added to the filters. Hybridization was at 45°C for 16-20 hrs. The washes were the same as those used in the Southern blotting above. The filters were air-dried and autoradiographed. The amount of hybridization was quantitated by punching out of the dots and counting them in a scintillation counter. The hybridization to the cells was adjusted by subtracting the background

hybridization to the filter.

Figure 4. Subcloning: Both the plasmid containing the insert (Hf677) and the vector into which the insert is to be placed (pGEM-4Z) are digested with the same enzyme (i.e., an enzyme that would excise the insert, Eco RI). After the isolation of the insert by electroelution from the agarose gel, it is ligated into the new vector as described in Materials and Methods.
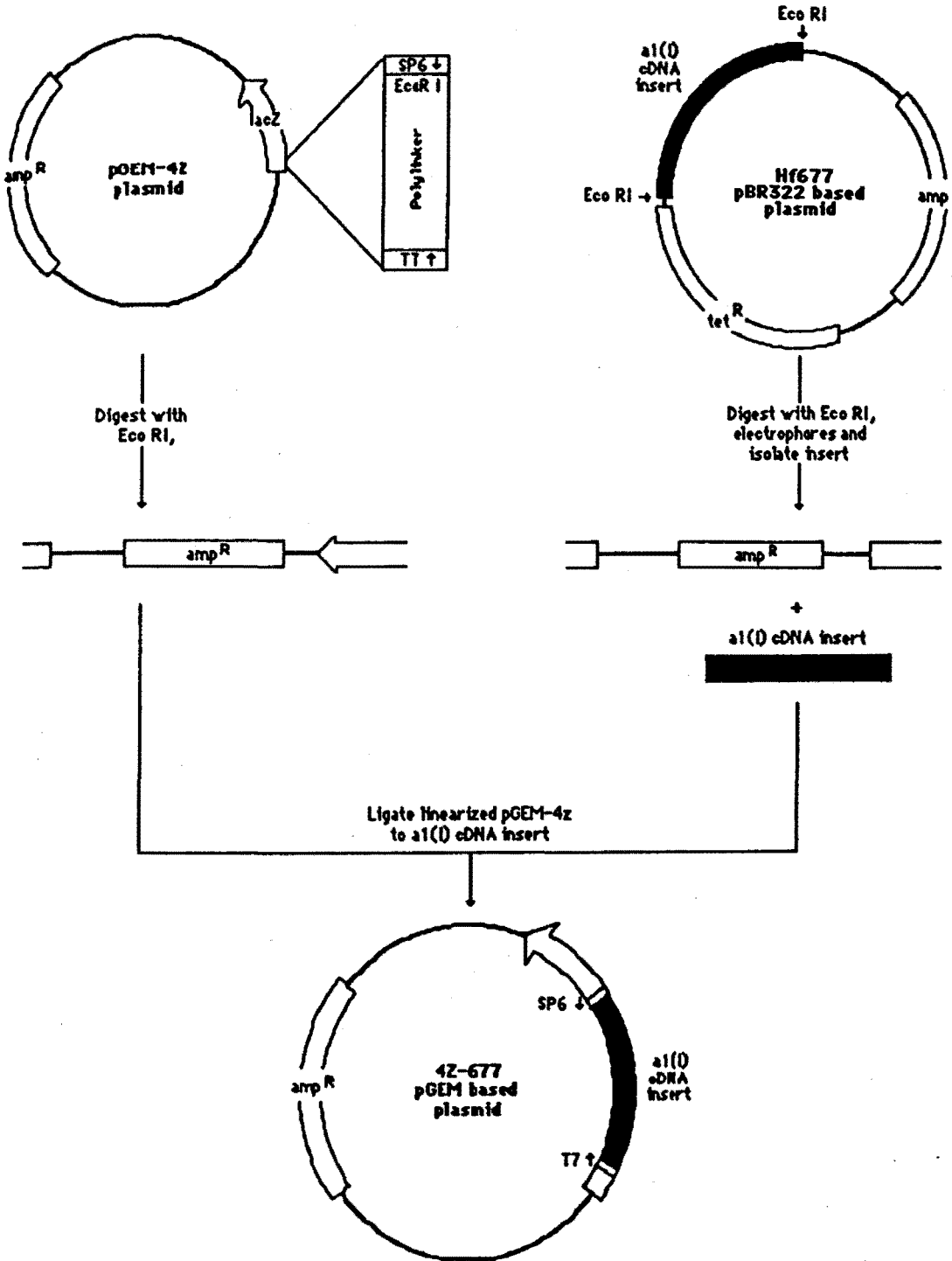
Figure 5. Orientation of insert with respect to the SP6 promoter. After the ligation of the insert into the vector and the transformation into JM109 <u>E. coli</u> bacteria, two plasmids with the insert placed in opposite orientation with respect to the SP6 promoter are isolated. The templates for the <u>in</u> <u>vitro</u> transcription reactions are prepared by the linearization of the plasmids downstream from the insert. Sense or anti-sense transcripts are generated using the SP6 RNA polymerase depending on the orientation of the insert.
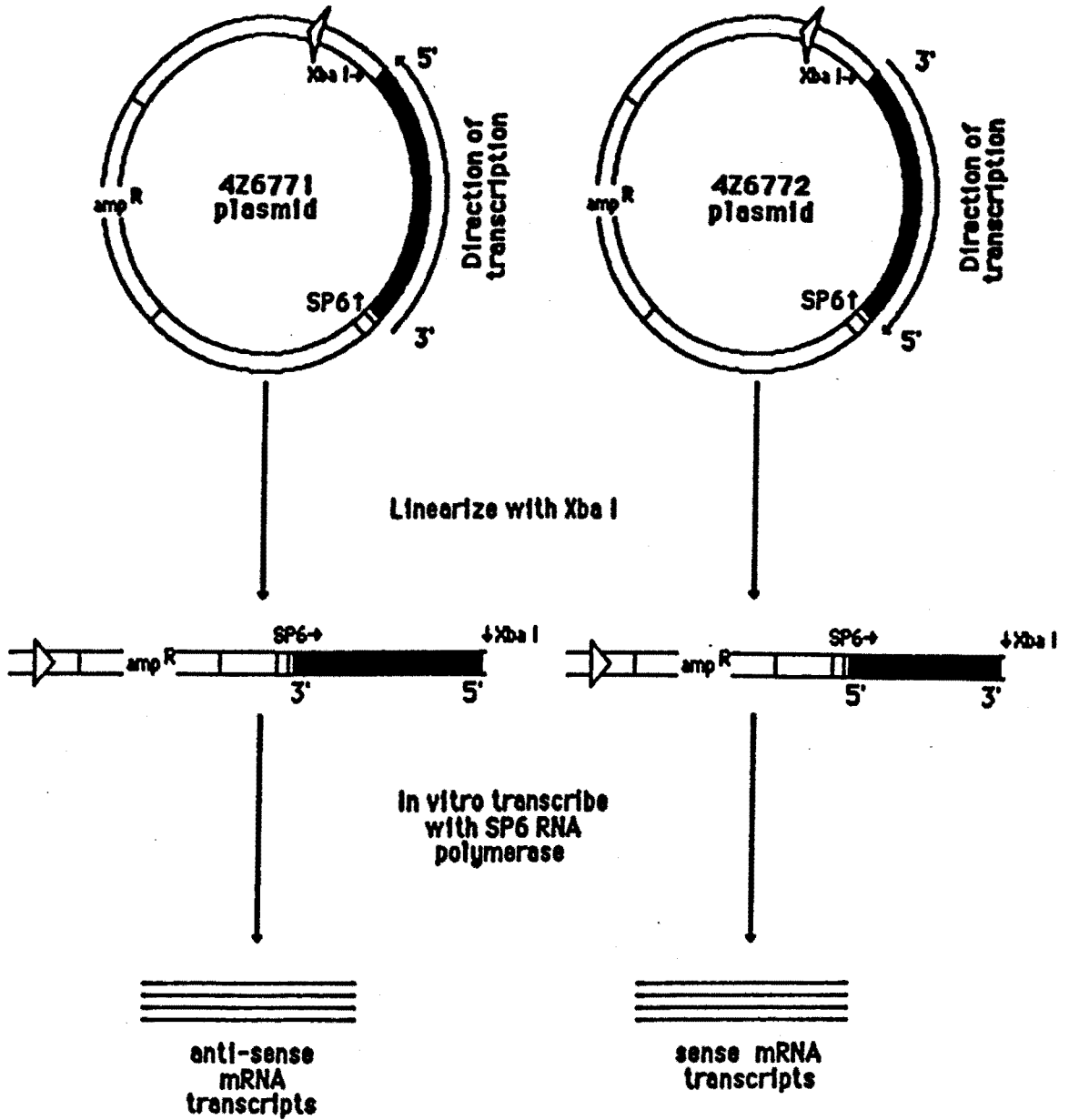
RESULTS AND DISCUSSION

<u>SYNTHESIS OF RNA PROBES</u>

The probes used in this study were cloned in the pGEM4z vector so that both sense and anti-sense RNA transcripts could be generated. The quantity of RNA synthesized was controlled by altering the amount of cold CTP nucleotide used in the transcription reaction. Also, by changing the concentration of the cold nucleotide the specific activity of the probe produced was altered. Thus, increasing the amount of the cold CTP yielded larger quantities of RNA transcripts with a lower specific activity. A typical <u>in</u> <u>vitro</u> transcription reaction contains 0.4 ug of template DNA, 3.5 uM radioactive CTP, 12 uM cold CTP and 10-15 units of RNA polymerase. The RNA yield from such a reaction is about 200 ng. In cases where large quantities of probe are desired, the concentration of the cold nucleotide is increased to 25 uM and the yield obtained is approximately twice as much. Both RNA polymerases, SP6 and T7, produce about the same amount of RNA in comparable reactions.

RNA molecules are very susceptible to degradation by the activity of ubiquitous ribonucleases. Probes synthesized by <u>in</u> <u>vitro</u> transcription may even be more sensitive, presumably

because they lack the 5' cap and the 3' polyadenylation sequences (Sambrook et al., 1989). Therefore, it was important to demonstrate that the synthesized RNA was intact and of correct size. As Figure 6 shows, all of the RNA transcripts used in this study are of the expected size and no degradation is detected. However, two abnormalities can be seen in this autoradiograph. First, the product of transcription of ß actin cDNA (lane 5) shows a major band (A) smaller than the expected size. The synthesis of less-than-full length transcripts can be due to (i) the fortuitous occurrence in the template of sequences that terminate transcription by the DNA-dependent SP6 RNA polymerase used and/or (ii) limiting concentrations of precursor (the radiolabeled nucleotide; Sambrook et al., 1989). The latter condition is unlikely because the band is of a discrete size instead of the smear that would be expected if ^{32}P-CTP was limiting. Premature terminations are probably caused by the formation of a stable secondary structure preventing the RNA polymerase from proceeding to the end of the DNA template (Lewin, 1987, chapter 11). Second, the transcription of the α2(I) collagen sense strand (lane 7), which is the only RNA transcript generated using the T7 RNA polymerase, shows a minor band (B) that is larger (approximately 2.4 kb) than the insert. This band is equal in size to the vector sequence, pGEM4z, indicating that occasionally the T7 RNA polymerase recognizes and binds to the T7 promoter but for some unknown reason it

proceeds in the opposite direction transcribing the wrong strand of the vector sequence instead of the insert (Melton et al., 1984). In the other samples (lanes 1-6) where SP6 RNA polymerase is employed no such extraneous transcripts are seen. Results from work done on _in vitro_ transcription using T7 RNA polymerase show that this enzyme has a higher tendency to initiate transcription at non-specific sites than SP6 RNA polymerase (data not presented; BMBiochemica, 1988) and therefore SP6 RNA polymerase was preferably used whenever possible. These results demonstrate that these RNA probes are basically being synthesized correctly and can thus be used in the present study.

HYBRIDIZATION TO INTACT HUMAN FIBROBLASTS

Fixatives

In the present study, hybridization to RNA in intact cells requires a fixative which provides for the highest RNA retention in the cells and the maximum hybridization signal. These criteria are dependent upon the fixative's ability to inactivate all endogenous ribonucleases that may be present, provide for the maximum diffusion of probe throughout the cytoplasmic matrix, and preserve the cellular RNA. Two of the most frequently used fixatives, glutaraldehyde and paraformal-dehyde, were evaluated to determine which would be more suitable for this work. Normal human fibroblasts (GM03348B) were harvested by trypsinization and split into two halves.

One half was fixed with 1% glutaraldehyde and the other half with 4% paraformaldehyde as described in Materials and Methods. Triplicates of each sample with 5000 cells per dot were loaded onto poly-L-lysine coated glass fiber filters. The filters were then digested with 10 ug/ml of proteinase K at 37 °C for various periods of time followed by triethanol amine and acetic anhydride treatments (see Materials and Methods). The filters were then hybridized to the α1(I) RNA probe. The hybridization intensity to glutaraldehyde-fixed cells increased with increasing time of proteinase K treatment until it reached a plateau at 20 minutes of digestion (Figure 7; see proteinase K concentration and digestion time section). Paraformaldehyde-fixed cells on the other hand, reached a maximum signal after a very brief treatment with proteinase K (2.5 minutes) and the signal intensity declined gradually with increased treatment. The maximum signal obtained with glutaraldehyde-fixed cells is two-fold higher in intensity than that obtained with paraformaldehyde-fixed cells. Two conclusions can be drawn from this experiment. First, glutaraldehyde is apparently a much stronger fixative than paraformaldehyde and thus provides for a tighter crosslinking of the cellular matrix. Consequently, a longer proteinase K digestion is required for optimum probe accessibility to RNA in glutaraldehyde-fixed cells. Second, the weaker signal with paraformaldehyde-fixed cells may indicate that this fixative does not provide for maximum RNA retention in the cell,

resulting in the loss of the cellular RNA with extended proteinase K treatment. It is also possible that paraformaldehyde does not completely inactivate the endogenous ribonucleases, thus resulting in degradation of some cellular RNA. In any case, the stronger signal obtained with glutaraldehyde makes it the preferred fixative for the present study.

A study done by Lawrence and Singer (1985) comparing the fixation of chicken embryo muscle cells with 4% paraformaldehyde and 4% glutaraldehyde yielded results similar to those presented here. They showed that glutaraldehyde provides for a better RNA retention than paraformaldhyde, and upon extended proteinase K treatment, the hybridization signal increased in glutaraldehyde-fixed cells while paraformaldhyde-fixed cells lost more than half of the total cellular RNA. In an apparent contradiction, however, they concluded that paraformaldhyde yields a superior signal-to-noise ratio compared to glutaraldehyde. This was probably due to the fact that their cells were fixed with a higher glutaraldehyde concentration (4% vs 1% used in this study) and so, relative to my results, their proteinase K treatment time was grossly inadequate for maximum hybridization. As a result paraformaldehyde-fixed cells appeared to yield a more intense signal.

RNA vs. Nick Translated Probes

Several studies have shown that RNA probes are preferred over nick-translated probes for _in situ_ hybridization because

they provide for a higher degree of sensitivity (Yu and Gorovsky, 1987; Cox et al., 1984; Deleon et al., 1983). RNA and nick-translated probes were compared to determine their hybridization intensity with glutaraldehyde and paraformaldehyde fixed cells. Comparison of autoradiographs in Figure 8 clearly shows that the signal intensity obtained from the use of RNA probe (A) is vastly higher (about 100-fold) than that obtained from nick-translated probe (B). Consequently, RNA probes were employed in this study. Glutaraldehyde fixation followed by proteinase K treatment gave the strongest signal with either type of probe.

Proteinase K Concentration and Digestion Time

Almost every _in situ_ hybridization procedure includes a step of post-fixation treatment of cells with proteinase in order to improve the hybridization (Brahic and Haase, 1978; Angerer and Angerer, 1981; Edwards and Wood, 1983). The rationale for this is that the partial digestion and removal of some of the cytoskeletal and cytoplasmic proteins allows for a greater penetration of the probe through the cellular matrix and thus provides for a greater accessibility of mRNA for hybridization. The optimum treatment for human cells in the current study was determined using two approaches. The first approach involved the incubation of human fibroblast cells, spotted onto the poly-L-lysine-coated glass filters, with increasing concentrations of proteinase K for a fixed

amount of time. After the hybridization the signal was quantitated and the results plotted as the percent of maximum counts. As shown in Figure 9A the hybridization signal reached a maximum at about 10 ug/ml of proteinase K. Increasing the amount of proteinase K above 15 ug/ml resulted in a gradual decrease of the signal, indicating that the cellular RNA was probably being lost in a similar fashion to that in the proteinase K treatment of cells fixed with paraformaldehyde. In the second approach, the amount of proteinase K was held constant (10 ug/ml) and the incubation time was varied (0 to 70 minutes) to determine the effect of digestion time on signal intensity. The hybridization signal increased dramatically in the first 15 minutes and then leveled off at about 25 minutes (Figure 9B). Further increase in incubation time did not appear to make a significant difference in the hybridization intensity over the length of time tested. Consequently, an incubation period of 40 minutes with 10 ug/ml of proteinase K was chosen as a standard for all experiments.

OPTIMIZING HYBRIDIZATION PARAMETERS

There has been little in situ hybridization work done on cultured cells (Yu and Gorovsky, 1987; Scharffetter et al., 1989; Scharffetter et al., 1989b) and none on hybridization to dissociated human fibroblast cells. Therefore, in order to maximize the hybridization to fibroblasts, a number of parameters, such as cell number, hybridization volume,

hybridization time, probe size and concentration had to be optimized.

Hybridization Volume

The volume of the hybridization solution should be kept low since the kinetics of nucleic acid reassociation are faster in higher concentrations (Sambrook et al., 1989). Moreover, in small volumes the actual amount of probe used for the hybridization is reduced, minimizing the use of radioactive materials. However, it is essential that sufficient solution be present at all times so that the filters remain covered with a film of hybridization solvent. Therefore, the minimum volume of hybridization solution required for maximum signal intensity was investigated. The bar graph in Figure 10 shows that the hybridization signal increased significantly in the first two conditions (0.08 and 0.10 ml/cm^2) followed by only a slight increase at the remaining two volumes (0.20 and 0.30 ml/cm^2). The volume of 0.2 ml/cm^2 was chosen for the rest of the experiments because it was the smallest volume yielding a maximum signal.

Linearity of Signal and Cell Number

The linearity of hybridization signal with increasing number of cells was investigated to determine the maximum number of cells that could be loaded onto the glass fiber filters. Five to thirty thousand cells per dot were spotted

and the filters were treated and hybridized as previously described. The signal intensity increased linearly with increasing number of cells up to twenty thousands cells (Figure 11A). Beyond twenty thousand cells the increase in hybridization was non-linear. Another more detailed study of linearity over the lower range of cell numbers (1-14 thousand cells) was also done (Figure 11B). The hybridization signal obtained was completely linear over this range of cell numbers.

Another experiment was done to determine whether the non-linearity of the signal beyond twenty thousand cells is due to (i) the limited amount of probe available for hybridization or (ii) the fact that the capacity of the filter to retain more cells was exceeded. Fibroblast cells that were labeled with ^{3}H-thymidine served as a marker for the presence of fibroblasts on the glass fiber filters. An experiment similar to that in Figure 11A was done using these cells, except that the probe was not included in the hybridization because the strong signal from the ^{32}P-label would mask the weaker ^{3}H signal. As shown in Figure 11C the curve from this experiment clearly indicates that the capacity of the filter to retain cells is not exceeded. Therefore, the leveling off seen in Figure 11A is due to a limiting amount of probe present in the hybridization solution when large number of cells are used. The maximum number of cells actually required for this protocol is well below the 20 thousand maximum. Most

experiments in this study used cell numbers in the range of 5000 cells per dot.

It is important to point out that the number of cells retained on the filter may vary from one cell type to another. Additionally, the condition of the cells before harvesting may also affect that number. Work done by Yu and Gorovsky (1987), using a procedure similar to that in the present study, established that the maximum number of Tetrahymena thermophilia cells that could be loaded onto the filter was ten thousand. This number increased to three times as much when starved Tetrahymena thermophilia cells were used.

Hybridization Time

The length of hybridization time used in other types of in situ techniques is another parameter that varies greatly among different studies and could range from 3 to 72 hours (Scharffetter et al., 1989; McCabe and Pfaff, 1989; Yu and Gorovsky, 1987). To test the optimum hybridization time, triplicate fibroblast samples were loaded onto the glass fiber filters and hybridized to the $\alpha 1(I)$ RNA probe for different lengths of time (Figure 12A). The hybridization signal intensity plotted against time shows that an incubation period of 16 to 24 hours is required for maximum hybridization signal. The signal intensity gradually declined after 24 hours of hybridization. To determine whether this decline was due to the loss of cells from the filter or the loss of RNA

from the cells, ^{3}H-labeled cells were used again. Tritiated cells were loaded, treated and hybridized as in the experiment above except that the probe was excluded from the hybridization solution. The results (Figure 12B) demonstrate that the number of cells on the filters remains unchanged with time (i.e., the counts were essentially constant across all of the data points). Thus, cells are not being lost with extended periods of hybridization. Rather, it is likely that the cellular RNA is either being degraded or leaking out of the cells at longer hybridization times. Hybridization times of 16-20 hours were used for all of the experiments.

Probe Size

Investigators disagree as to whether smaller or larger probes are more useful for in situ hybridization. Some researchers suggest that the use of larger probe enhances the signal due to the high specific activity of the molecule (Gerhard et al., 1981; Lawrence and Singer, 1985). Others maintain that smaller probe fragments, below 300 base pairs, are essential for successful accessibility of the probe into the cells or tissue (Brahic and Haase, 1978; Angerer and Angerer, 1981). Furthermore, larger probes may be problematic because of difficulties in washing them out of the cells. The effect of probe size on hybridization signal intensity was determined using α1(I) probes of different sizes (1.5 and 0.7 kb). The collagen template was truncated at different

distances from the SP6 promoter and labeled RNA probes of 1.5 kb (4z6671/Xba I) or 0.7 kb (4z6671/Xho I) were synthesized. The GC contents of the two probes did not vary greatly (30% vs 32% respectively) and therefore the specific activity per unit length of the two probes differs only slightly. However, the specific activity per transcript differs by about two fold due to the difference in size. These probes were hybridized to cells spotted onto two identical sets of filters (Figure 13A). One set was washed at 65°C and the other at a lower stringency, 55°C. Filters in each set were hybridized to either equal amounts or equal numbers of transcripts of the two probes (Figure 13A).

The results summarized in Figure 13B clearly show that there is a large difference in the hybridization intensity between the two probe sizes. The 1.5 kb probe gives 8 to 9 (or 13 when equal number of transcripts are compared) fold higher hybridization signal than the 0.7 kb probe. The differences in signal with the two probe sizes are much greater than can be accounted for by their two-fold difference in specific activity per transcript. There is no significant variation in the hybridization intensity between the two washing stringencies (i.e., the ratio of the larger probe to the smaller probe is virtually the same in both washing conditions). Thus, differences in the stability with which the two probes hybridize to cellular mRNA cannot account for the large signal differences. All experiments for testing the

various parameters and conditions of hybridization were done using α1(I) probe of size 1.5 kb. The quantitation experiments utilized the largest possible probe for each gene (see Materials and Methods for sizes).

Probe Saturation

To measure the absolute number of a particular species of mRNA molecule in a cell, it is necessary to hybridize to saturation by using excess amounts of probe. The quantity of probe required for hybridization to saturation was determined by incubating cell-containing filters with increasing amounts of probe. Figure 14 presents the concentration curve obtained for normal human fibroblasts hybridized to the α1(I) collagen probe. The hybridization signal increased sharply with increasing probe concentration up to approximately 2 ug/ml of probe. A plateau was reached at about 3 ug/ml. The background increased somewhat with higher probe concentrations. This saturation concentration is comparable to that seen in other in situ hybridization studies (Yu and Gorovsky, 1987; Lawrence and Singer, 1985; Cox et al., 1984).

The average number of collagen Type I mRNA molecules hybridized per cell can be calculated from the counts per minute of probe hybridized per dot at saturation (42000 CPM), the number of cells per dot (5000 cells), the total amount of probe used in the experiment (5394 ng) and the total counts per minute of the probe (9.0×10^8 CPM). The calculation was

done as follows:

A molecule of $\alpha1(I)$ RNA transcript weighs 8.4×10^{-10} ng.

$$\frac{1.5 \times 10^3 \text{ nucleotide/molecule of } \alpha1(I)}{6 \times 10^{23} \text{ molecules/mole}} \times \frac{340 \text{ gm}}{\text{mole}}$$

The amount of probe bound per sample equals

$$\frac{42000 \text{ CPM/sample} \times 5394 \text{ ng total probe}}{9.0 \times 10^8 \text{ total CPM}}$$

$$= 2.5 \times 10^{-1} \text{ ng/sample}$$

This quantity of probe corresponds to 3×10^8 molecules per sample as calculated below,

$$\frac{2.5 \times 10^{-1} \text{ ng/sample}}{8.4 \times 10^{-10} \text{ ng/molecule of } \alpha1(I) \text{ mRNA}}$$

which in turn equals

$$\frac{3 \times 10^8 \text{ molecules/sample}}{5 \times 10^3 \text{ cell/sample}} = 6 \times 10^4 \text{ molecules of } \alpha1(I) \text{ per cell}$$

These calculations indicate that there are sixty thousand copies of the $\alpha1(I)$ collagen mRNA per fibroblast cell. This number is much higher than expected. According to Hastie and Bishop (1976), the number of the most abundant messages present in any cell type is around 10-12 thousand copies per cell. The number we obtained from our calculation is five times higher, suggesting that multiple molecules of $\alpha1(I)$ RNA probe bind to every target collagen mRNA in the cell (see Conclusion).

Cell Storage

RNA molecules are very susceptible to degradation by

ribonucleases which can be introduced from various sources. To determine the rate of degradation and/or loss of cellular RNA in stored cells, batches of normal human fibroblast cells were harvested and fixed on different dates and stored at - 20°C until hybridization. Triplicates from each sample were loaded onto the glass fiber filter and then treated and hybridized as described in Materials and Methods. As Figure 15 shows, the signal obtained decreased gradually with storage time over the period tested. The signal decreased by about 66% at the end of four months. However, there did not seem to be any significant decrease in the amount of signal for at least two months. Cells that had been stored for less than one month were used in all experiments. Similar experiments performed on fibroblast cells fixed with 4% paraformaldehyde showed a much more rapid loss of signal with storage time (data not shown). Thus, RNA in such cells is more susceptible to degradation and/or loss than it is in glutaraldehyde-fixed cells, further indicating that glutaraldehyde is a much stronger and better fixative than paraformaldehyde.

HYBRIDIZATION CONDITIONS

To obtain the maximum signal-to-noise ratio and the least amount of cross hybridization among different collagen types a number of hybridization parameters had to be studied and optimized.

Dextran Sulfate

A number of studies have shown that in the presence of 10% dextran sulfate, the rate and intensity of hybridization is elevated approximately ten-fold by increasing the effective concentration of the probe in the hybridization solution (Amasino, 1986; Wahl et al., 1979). The effect of dextran sulfate was tested on hybridization to intact human fibroblast cells. Two strips of glass fiber filter with triplicate samples were hybridized to the α1(I) probe in solution with or without dextran sulfate. The signal obtained with cells hybridized in the presence of dextran sulfate was about 2-fold higher than that without it (Figure 16). Furthermore, the background signal was higher in the absence of dextran sulfate. The increase in hybridization signal may be contributed by dextran sulfate's ability to protect the cellular mRNAs. This fact is supported by a study done by Lawrence and Singer (1985) which showed that in the absence of dextran sulfate, the retention of the total cellular RNA was reduced by more than 50%. Consequently, all hybridizations were carried out in solution containing 10% dextran sulfate.

Prehybridization

Standard hybridization requires prehybridization of the filter in hybridization solution lacking probe to reduce background (Sambrook et al., 1989). The necessity of this step was tested for hybridization to RNA in intact cells. The

filters were prehybridized at 45°C in hybridization solution minus the probe. The probe was then added to the hybridization solution and the filters incubated overnight. The results showed no difference in signal-to-noise ratio between prehybridized and non-prehybridized filters (Figure 17). Consequently, no prehybridization step was included in the protocol.

HYBRIDIZATION SPECIFICITY

As for all hybridization procedures, controls to prove the specificity of detection reactions have to be performed. Three different approaches were taken to insure that the cross hybridization to other collagen gene family members, other structurally similar genes, and other cellular components was kept to a minimum.

Cross Hybridization Between Probes

The first approach was to test the extent of cross hybridization of the probes used in this study to other collagen and structurally similar genes. A Gene Screen Plus filter containing all relevant clones was hybridized to each of the probes used in this study; $\alpha1(I)$, $\alpha2(I)$, ß actin, or 28S rRNA (Figure 18A and B). Several hybridization stringencies were tested until one was found that yielded the least cross hybridization signal (less than 10%) without reducing the hybridization to the target cDNA by more than two-fold.

These hybridization conditions were used in the quantitation experiments. The results of cross hybridization between the different probes are summarized in Figure 18C. The RNA probes allow for extreme sensitivity and as a result cross hybridization of less than 1% was easily detectable. The $\alpha1(I)$ probe cross hybridizes to $\alpha1(III)$ and $\alpha2(I)$ cDNAs at about 3.5% each. It also cross hybridized to $\alpha2(V)$, β actin, and α tubulin for a total cross hybridization of 9.5%. Similarly $\alpha2(I)$ cross hybridized with $\alpha1(I)$ only for a total of 8.5%. The β actin cross hybridized to other cDNAs for a total of less than 5%. The 28S rRNA nick translated probe did not cross hybridize to any of the other genes.

Strand Specificity

The second approach was designed to test the probes' faithfulness in hybridization only to the target mRNA and not to non-specific cell components. Fixed cells were loaded onto the poly-L-lysine-coated glass fiber filters and treated as described in Materials and Methods. The filters were then hybridized to either the sense or the anti-sense strand RNA probes. The probes were prepared by the in vitro transcription of the plasmids with inserts cloned in opposite orientations as described earlier (see Materials and Methods and the Results sections on RNA synthesis). The best results were obtained with the $\alpha1(I)$ collagen probe, 4z6771 (Figure 19A). The signal from the hybridization to the anti-sense strand was

15- to 25-fold higher than that obtained from the hybridization to the sense strand. The low background hybridization with the sense strand is inherent to in situ hybridization in general since similar background hybridization is obtained by the hybridization of any sequence (e.g., pBR322 vector sequence) to intact cells (data not shown). Thus to a small extent, the RNA probe binds non-specifically to cellular components and/or it may not be washed out of the cells completely.

The strand specificity of $\alpha2(I)$ and β actin probes is not as good as the $\alpha1(I)$ probe. The difference between the sense and the anti-sense strands is three- to four-fold in these cases (Figure 19A). The intensity of $\alpha2(I)$ sense strand hybridization is higher than that obtained with the sense strand for $\alpha1(I)$, but the signal with the anti-sense strand is lower than the $\alpha1(I)$ counterpart. Therefore, the decrease in the difference between the two strands is partly due to the presence of fewer transcripts for $\alpha2(I)$ per cell in comparison to $\alpha1(I)$ mRNA. There also may be a higher incidence of cross hybridization between the sense strand and other similar mRNAs and cellular component with the $\alpha2(I)$ and β actin probes as shown by the increase in the hybridization signal with that strand.

Ribonuclease A Treatments

The third approach was the treatment of cells with

ribonuclease A either prior to or after hybridization. These experiments were designed to test whether the probe was hybridizing only to its target RNA, and also to test the efficiency of removal of unhybridized probe out of the cells by washing. Fibroblast cells loaded onto the filters were treated with ribonuclease A prior to hybridization to degrade the cellular RNA. The filters were then hybridized to the $\alpha1(I)$ RNA probe. As shown in Figure 19B the hybridization intensity of RNased cells is much lower than that for non-treated cells. No significant difference is seen between cells treated with 5 or 10 ug/ml of RNase A indicating that cellular RNA was easily accessible and was degraded with the lesser concentration of the ribonuclease A. A comparison between untreated cells hybridized in the same bag with treated cells, and untreated cells hybridized in a separate bag show about 2-fold higher signal in the latter case, indicating that the RNase A may have still been slightly active and degraded some of either the RNA probe or the cellular RNA in the untreated cells. In any case, in the absence of cellular RNA, the hybridization signal is very weak, indicating that the probe is not binding non-specifically to other cellular components.

The treatment of cells with ribonuclease A after hybridization degrades all of the unhybridized RNA molecules (both probe and cellular RNA) and also the unpaired (ssRNA) regions of the RNA-RNA hybrid formed between the probe

molecules and the mRNA to which it is hybridized. As can be seen in Figure 19C the untreated cells yield a signal that is 30-60 fold higher than that for the treated cells. This experiment indicates that there are a large number of regions in the RNA-RNA hybrid that are unpaired and are susceptible to the degradative activity of the ribonuclease A. One possibility may be that there is more than one probe molecule bound per target mRNA. In light of results from experiments on the probe saturation and probe size, this situation is likely to be occurring (see Conclusion).

The final treatment and hybridization parameters for the optimum hybridization to the cellular mRNA are summarized in Figure 20. These conditions were used in the quantitation experiments below.

Quantitation of the Relative Amounts of Collagen Type I mRNA in Normal and OI Fibroblasts

After establishing all the parameters and conditions for the optimum hybridization to RNA in intact cells, the quantitation of the $\alpha1(I)$, $\alpha2(I)$, β actin and 28S ribosomal RNA in various cells was done. The purpose of these experiments was to determine if the fibroblasts from two OI individuals differ significantly in the levels of Type I collagen mRNAs from that in the normal fibroblast cells. Two cell lines (GM02962 and GM0744) from individuals with the lethal form of osteogenesis imperfecta and one cell line (GM03348B) from a normal individ-

ual were used. All efforts were made to ensure that the same degree of growth and confluency was reached by all cell lines. However, since OI fibroblasts have a slower rate of growth than normal cells and also because there are differences in the cell size among the different cell lines, OI cultures had to be grown for relatively longer periods of time. Furthermore, in order to eliminate any variation in the harvesting, fixing, and age of the fixed cells among different trials, every OI cell line was harvested and fixed along with a batch of normal cells to which it was compared. The 28S rRNA was also used as an internal control to standardize any variations.

After fixing of the cells, three data points each with increasing number of cells of both OI and the corresponding control cells were treated under identical conditions as described in Materials and Methods. Figure 21 summarizes the results in terms of the slopes of the first order linear regressions. It is apparent that the steady state levels of $\alpha 1$ and $\alpha 2$ could not be compared within the same cell line since the ratio of the two mRNAs is not 2:1 even in the normal fibroblasts. This is not only because of the difference in the size of the two probes but more importantly due to the effect of the binding of more than one probe molecule per target mRNA (see Conclusion for explanation). Therefore, the results here are expressed as the ratio of collagen mRNA to the 28S RNA. The latter was an internal control that served

as a measure of the total RNA in each cell line. It has previously been shown to be a reliable control for such purposes (Leeuw et al., 1989). As can be seen the steady state levels of the total RNA in both of the OI cell lines are higher than in the normal fibroblast cells. It can also be seen that the increase in the total RNA is not correlated with a proportionately equal increase in the amount of $\alpha 1$ and $\alpha 2$ collagen mRNAs in both cell lines (e.g., the ratio of either collagen mRNA to the rRNA in GM0744 OI cells is much lower than that in the normal cells). This may imply that there is a coordinate down regulation of both collagen genes. In cell line GM0744, the ratio of $\alpha 1(I)$ to $\alpha 2(I)$ is the same as the ratio of the two mRNAs in the normal cell line, GM03348B, indicating that the relative ratio of the Type I collagen mRNAs is normal but that they are under-expressed in terms of their absolute amounts. In cell line GM02962 on the other hand, the ratio of the two mRNAs is lower than normal (1.55 vs. 2.7, respectively) and only the $\alpha 1(I)$ gene seems to be under-expressed. The ratio of the $\alpha 2(I)$ mRNA to the 28S rRNA is the same in both GM02962 and the normal cell line.

The results of previous work done by de Wet and his colleagues (1983a, 1983b) on cell line GM0744 indicated that this cell line has the same ratio of $\alpha 1(I): \alpha 2(I)$ mRNA as that found in the normal control fibroblasts, GM03348B. The results presented here also show that the ratio of the two mRNAs in GM0774 is the same as that in the normal cell line,

GM03348B. However, the absolute amounts of the two mRNAs are lower than normal, which the earlier study could not show due to the lack of internal controls. Cell line GM02962, on the other hand, was shown in the earlier studies (de Wet et al., 1983a; de Wet et al., 1983b) to contain three times as much $\alpha1(I)$ as $\alpha2(I)$ mRNA. The results presented here, however, indicate that $\alpha1(I)$ is under-expressed and thus the ratio of $\alpha1(I)$ to $\alpha2(I)$ is less than the normal 2 to 1. The cause of this contradiction is not known. The collagen protein levels have been shown (de Wet et al., 1983a and b) to correlate with the levels of collagen mRNAs present in these cell lines.

There is some disagreement as to whether the collagen mRNA levels vary between cells at different stages of growth. A number of studies have suggested that there is a significant difference in the levels of the Type I collagen mRNA in fibroblasts harvested at different stages of growth confluency (Tolstoshev et al., 1981). Others maintain that there is no appreciable variation (Voss and Bornstein, 1986). Preliminary results (data not shown) studying collagen mRNA levels in cells grown at different cell densities showed a very small amount of variation, certainly not sufficient to account for the differences in the mRNA levels seen between the normal and OI cells. Therefore, these differences reflect the inherent amounts of Type I collagen mRNA in these cell lines. Also, apparent variation in the amount of mRNAs due to differences in susceptibility of each cell line to proteinase K treatment

was ruled out experimentally. OI cells were used in experiments similar to those on proteinase K digestion described above and the difference between OI and normal cells was consistent at a whole range of proteinase K digestion conditions (data not shown).

Figure 6. Size analysis of RNAs synthesized from pGEM4z plasmids. Aliquots of the different radioactive RNAs synthesized by _in_ _vitro_ transcription were electrophoresed in a 1% denaturing agarose gel containing 0.66 M formaldehyde as detailed in Materials and Methods. The gel was dried and the autoradiogram prepared by direct exposure of the gel to the X-ray film. Lane 1 is RNA transcribed from a positive control template of 1386 bp in length. Lanes 2 and 3 are the 1.5 kb anti-sense and sense RNAs for α1(I) made from 4z6771 and 4z6772, respectively. Lanes 4 and 5 are the 1.9 kb sense and anti-sense RNAs for ß actin made from 4zA1 and 4zA2, respectively. Lanes 6 and 7 are the 1.2 kb anti-sense and sense RNAs for α2(I) made from 4z32s using SP6 or T7 promoter, respectively. The SP6 promoter is used for the transcription of all of the inserts except the sense transcripts of α2(I), lane 7, where the T7 promoter is used. The letter A points to a band that is smaller than the expected size of the ß actin transcript. Letter B points to a minor band corresponding to the pGEM-4z vector sequence.

Figure 7. Effects of fixatives on cellular RNA hybridization. Filters with triplicates of 5000 cells per dot, of paraformaldehyde (PF) or glutaraldehyde (GA) fixed cells (GM03348B), were prepared as described in Materials and Methods. The filters were treated with 10 ug/ml of proteinase K at 37°C for the times indicated and hybridized with 40 ng/ml of α1(I) RNA probe in 0.2 ml/cm^2 of hybridization solution for 16-20 hours at 45°C. (A): A representative autoradiograph of these experiments. (B): Graphic representation of the results. The open and closed circles (O, ●) are two independent experiments representing hybridization to glutaraldehyde fixed cells. The open and closed triangles (△, ▲) are two independent experiments representing hybridization to paraformaldehyde fixed cells. Each point is the average of three replicates. The signal is presented as percent of maximum CPM obtained (include all trials for both fixatives). Each curve is the average of two independent experiments.

A

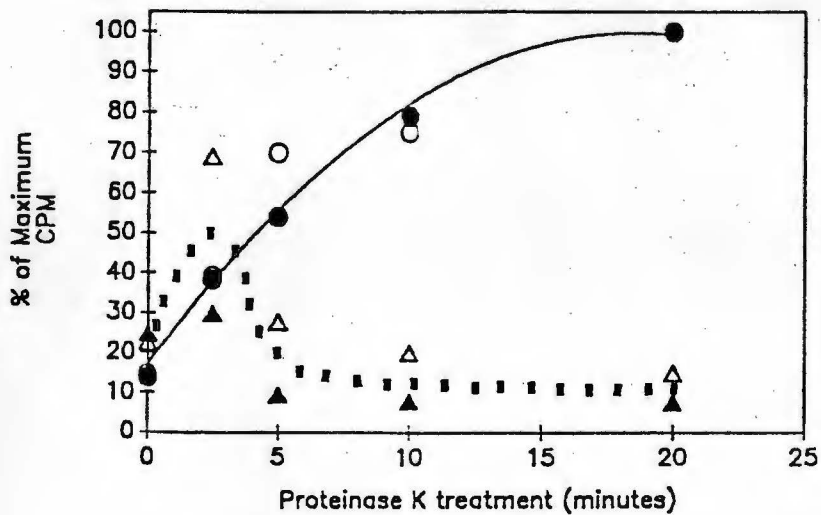TIME (MINUTES)
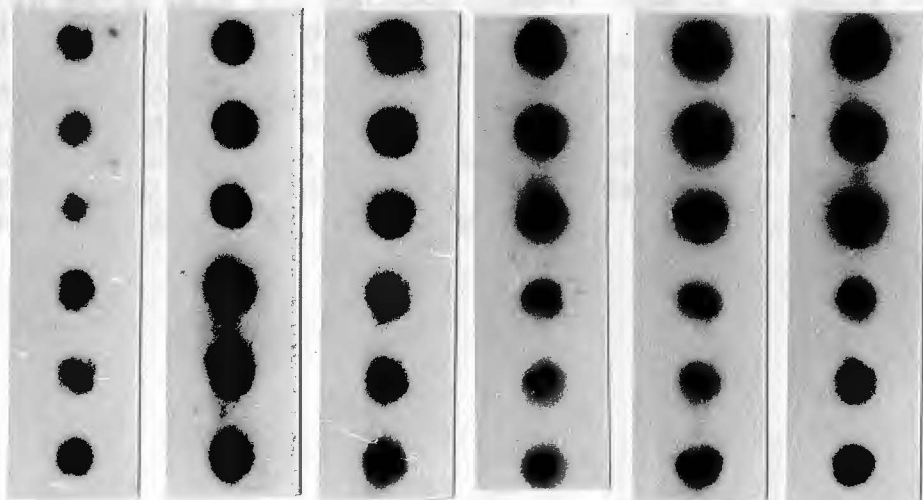
B

Paraformaldehyde vs. Glutaraldehyde

Figure 8. Effects of RNA and nick-translated probes on hybridization to cellular RNA. Two identical sets of filters with triplicates of 5000 cells per dot, of glutaraldehyde- or paraformaldehyde-fixed cells (GM03348B), were prepared as described in Materials and Methods. Both sets of filters were treated with 10 ug/ml of proteinase K for the amount of time (minutes) indicated on the left. Filters in set A were hybridized with 40 ng/ml of $\alpha 1(I)$ RNA probe. Filters in set B were hybridized with 40 ng/ml of nick translated $\alpha 1(I)$ probe. The hybridization was carried out in 0.2 ml/cm^2 of solution for 16-20 hours at 45°C. The filters were washed and exposed to X-ray film for the same amount of time.
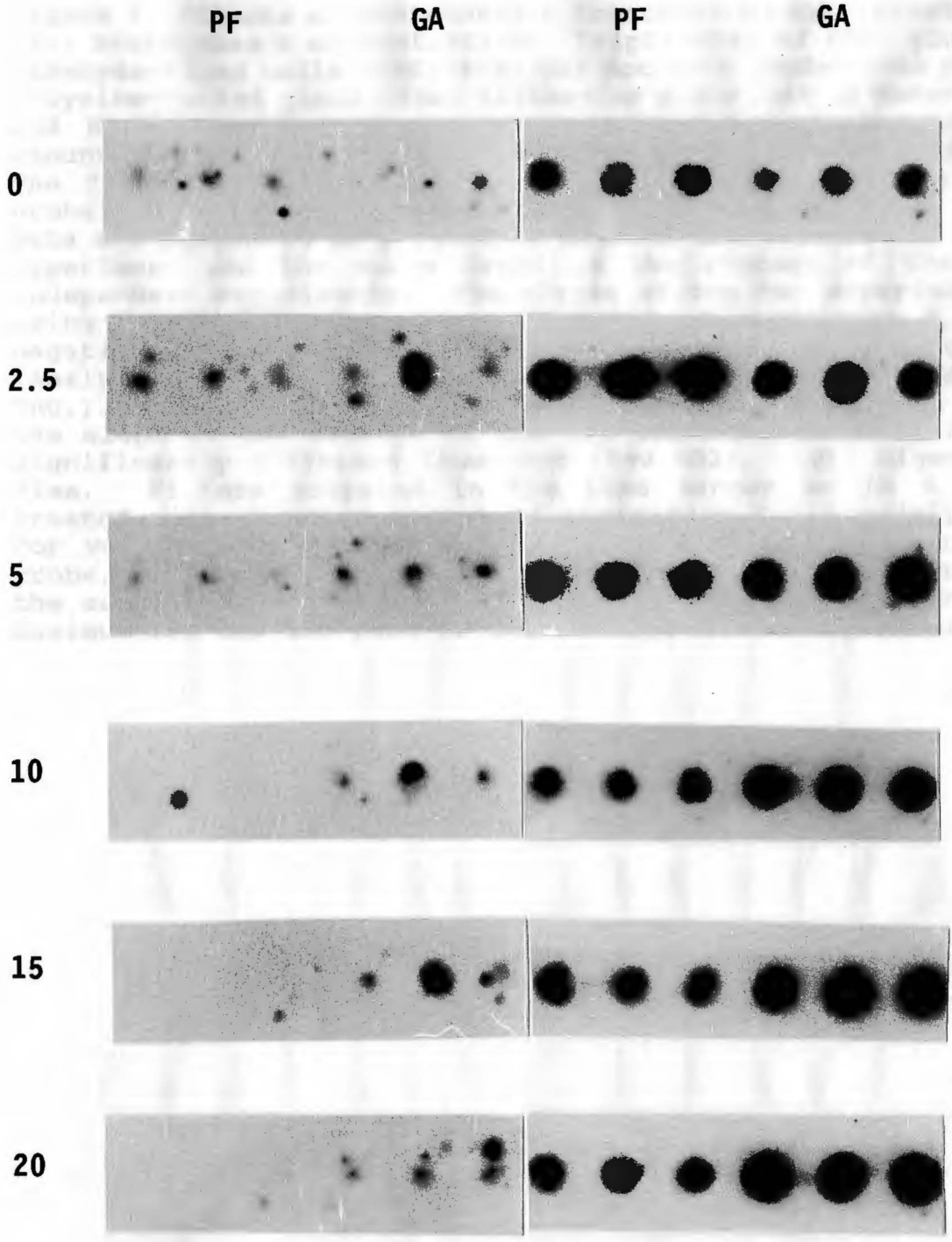
A   B

Figure 9. Effects of proteinase K treatment on hybridization. (A): Proteinase K concentration. Triplicates of 5000 glutaraldehyde-fixed cells (GM03348B) per dot were loaded onto poly-L-lysine-coated glass fiber filters as described in Materials and Methods. The filters were incubated with increasing amounts of proteinase K (0-50 ug/ml) for 40 minutes at 37°C. The filters were then hybridized to 40 ng/ml of $\alpha1(I)$ RNA probe in 0.2 ml/cm^2 of solution for 16-20 hour at 45°C. The data are presented as percent of maximum CPM obtained in each experiment and the curve drawn is the average of the two independent experiments. The slopes of the two experiments, using data points from 10 to 50 ug/ml of proteinase K, are negative but statistically not different from each other (Analysis of co-variance; P=0.923; Systat, 1989, Systat, Inc.). However, the analysis of co-variance test shows that the slope of the average of the two experiments (-0.311) is significantly different from zero (P=0.002). (B): Digestion Time. Filters prepared in the same manner as in A were treated with constant amount of proteinase K (10 ug/ml) but for varying periods of time (0-70 minutes). The amount of probe, hybridization solution and hybridization conditions are the same as above. The results are presented as percent of maximum CPM and the plot is the average of two experiments.

A Proteinase K Concentration
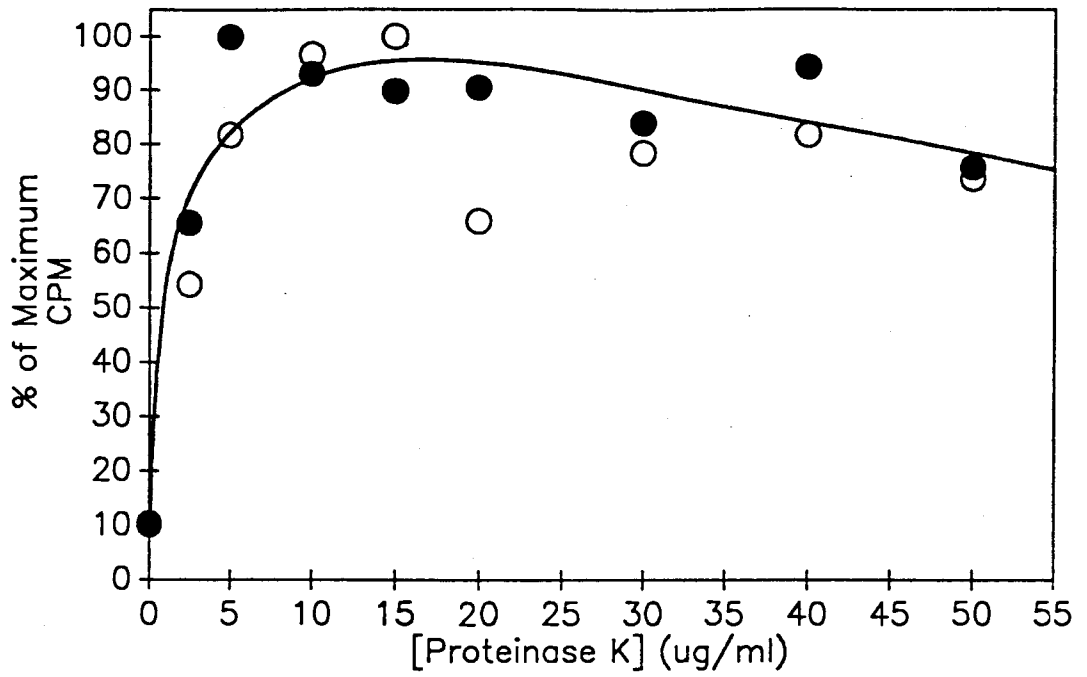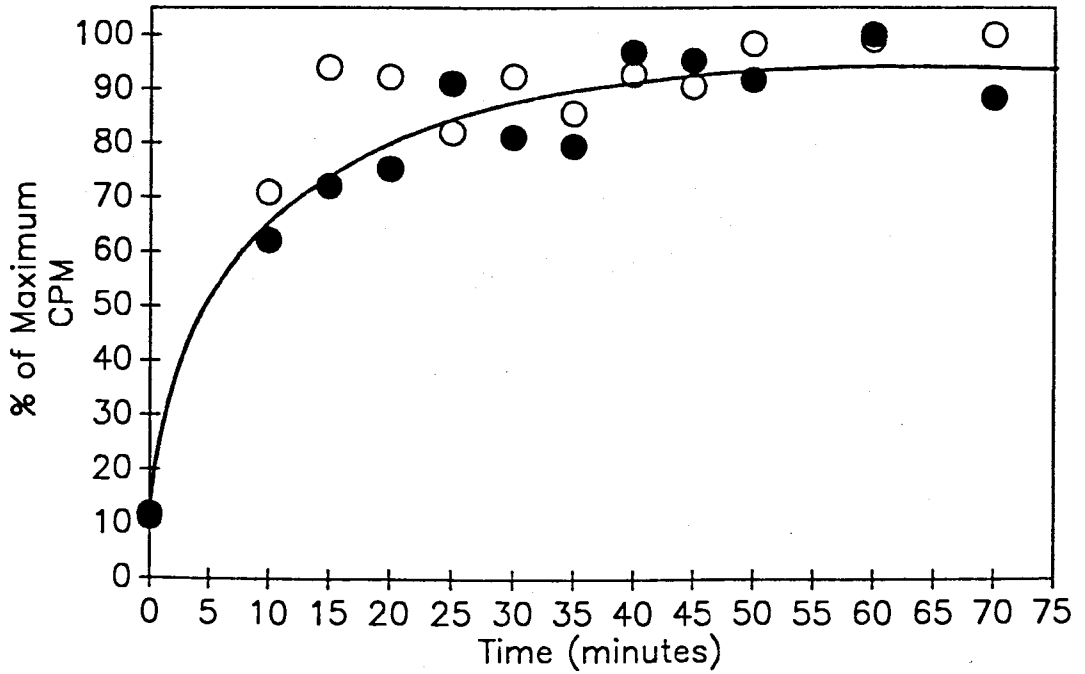
B Proteinase K Digestion Time

Figure 10. Effects of hybridization volume on hybridization. The results of two identical experiments (solid and hatched box) each with four different hybridization volumes are shown. Each bar represents an average of triplicate samples of 5000 cells per dot (Cell line GM03348B) prepared as described in Materials and Methods. The filters were treated with 10 ug/ml of proteinase K for 40 minutes at 37°C. The filters were then hybridized with 40 ng/ml of $\alpha 1(I)$ collagen RNA probe in varying amount of hybridization solution (0.08, 0.10, 0.20, or 0.30 ml/cm^2) for 16-20 hours at 45°C. The data are presented as percent of maximum CPM obtained in each experiment.
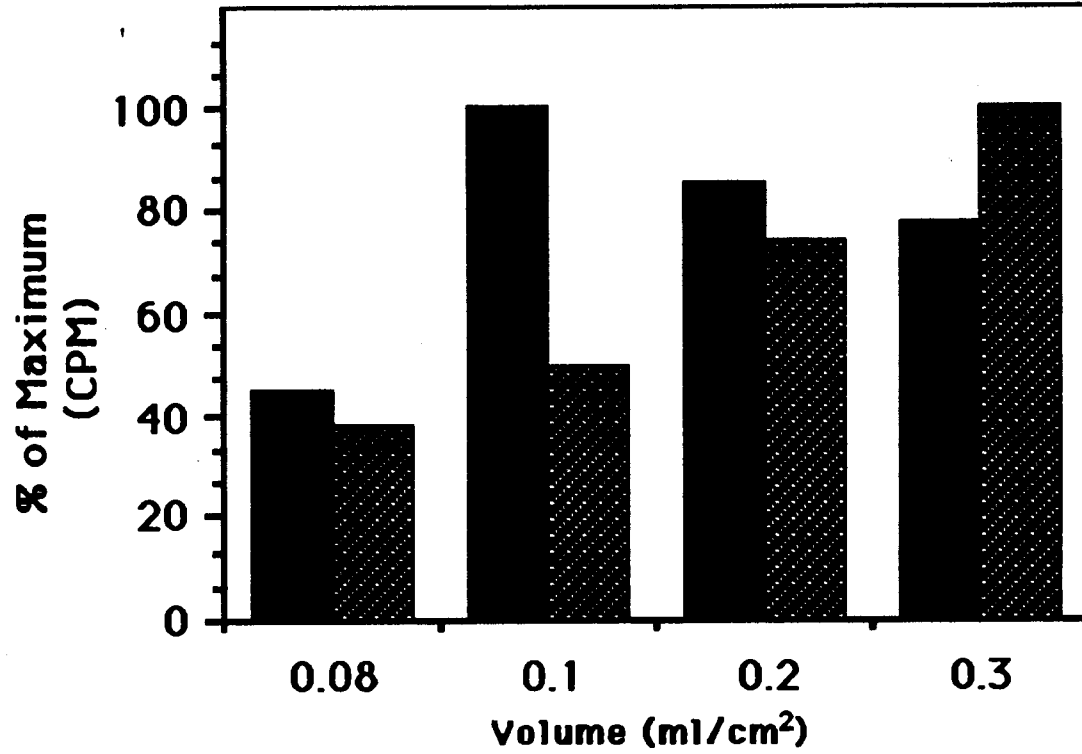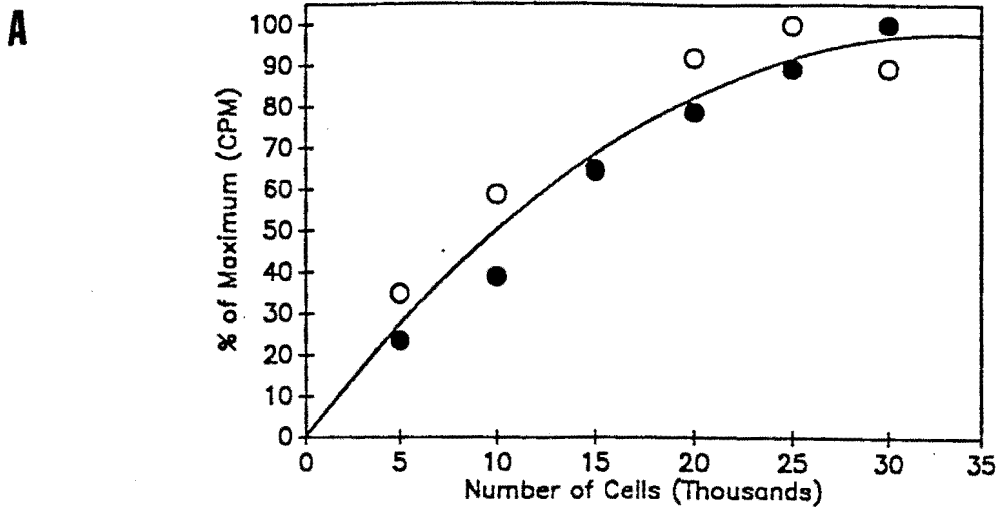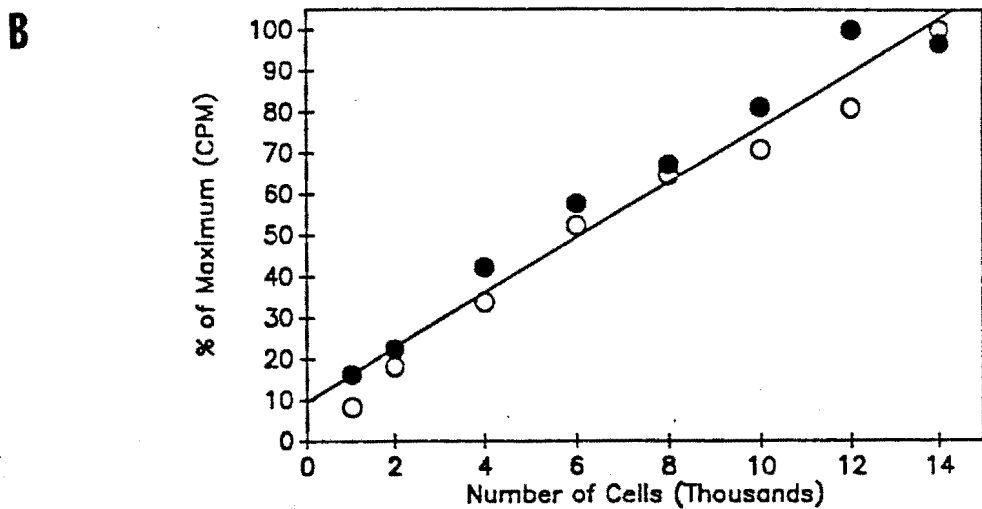
Hybridization Volume

Figure 11. Cell number linearity and retention. (A): Triplicates of 5 to 30 thousand cells (GM03348B) spotted onto poly-L-lysine-coated glass fiber filters were treated and hybridized to the $\alpha1(I)$ collagen RNA probe as described in Materials and Methods. The results are presented as percent of maximum CPM obtained in each experiment. The curve drawn is the second order regression for the two independent experiments (O, ●). (B): A more detailed study of linearity within the usable range, 1-14 thousand cells. The conditions were identical to the above experiments and the curve drawn is the first order regression for the two experiments (△, ▲). (C): Retention of cells onto the glass fiber filters. Human fibroblasts (GM03348B) labeled with ^{3}H-thymidine were used in an experiment identical to that in A except the probe was not included in the hybridization. Again a second order regression line was drawn of CPM vs. the number of cells. The error bars are the standard deviations for each point.

Cell Number Linearity

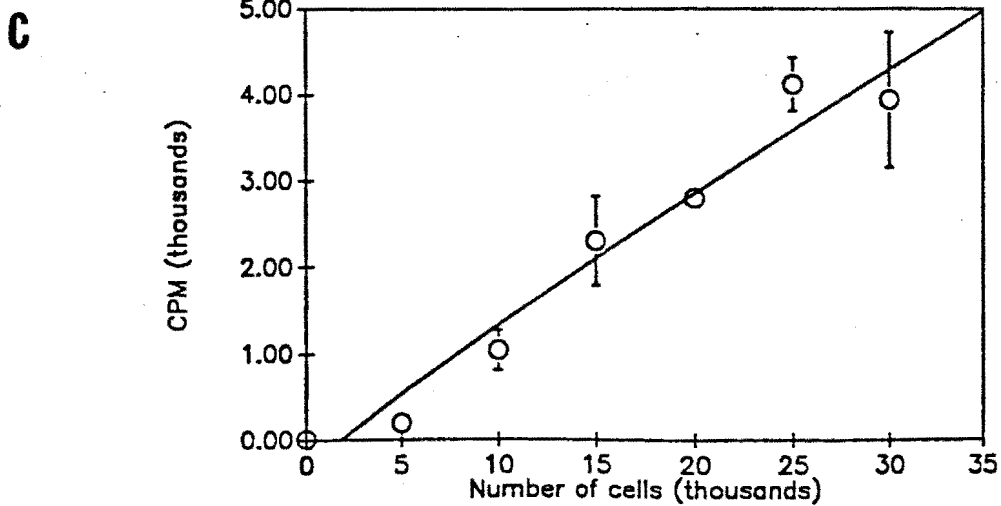Cell Number Linearity

Cell Retention

Figure 12. Time dependence of <u>in</u> <u>situ</u> hybridization and cell retention. (A): Hybridization time. Filters with triplicates of 5000 cells per dot of glutaraldehyde-fixed cells (GM03348B) were prepared and hybridized to the α1(I) RNA probe in 0.2 ml/cm^2 of hybridization solution at 45°C for periods of times indicated on the X-axis. Because the theoretical form of the kinetics is not known, the connection of the points serves to facilitate visualization of the data. (B): Cell retention. Cells labelled with ^{3}H-thymidine were loaded onto the filters, treated and hybridized as in A except the probe was excluded from the hybridization solution. First order regression was drawn to aid in visualization. The error bars are the standard deviations for each point. The correlation coefficient (R) value =4.0872X10^{-1}. Therefore, one point is not significantly different from another, and the line is straight.
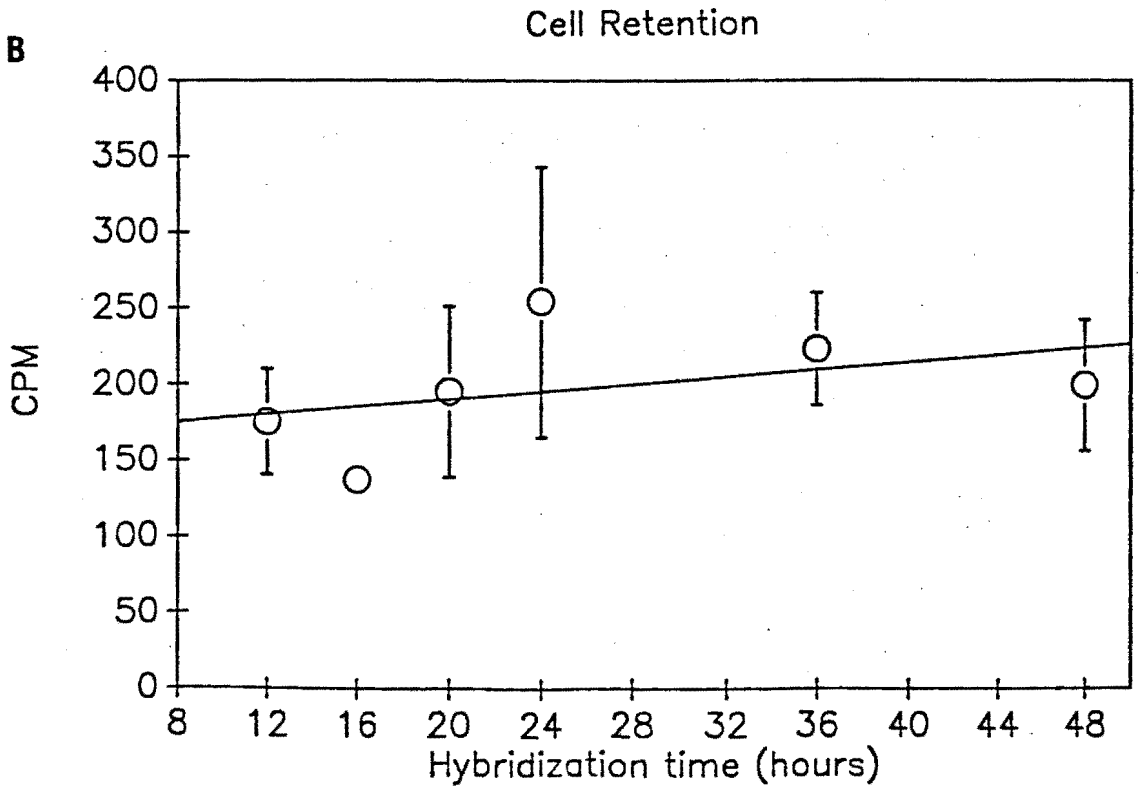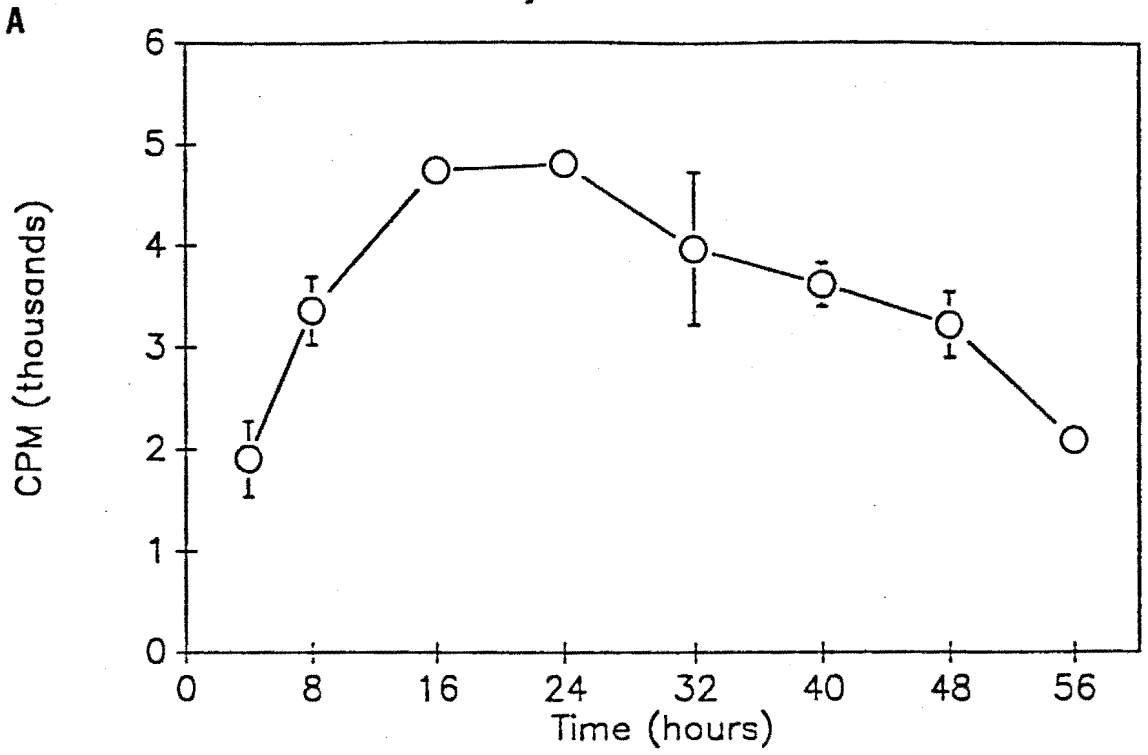
Hybridization Time

Cell Retention

Figure 13. Effects of probe size on hybridization. (A): Triplicates of 5000 normal fibroblast cells (GM03348B) per dot were loaded onto filters and treated as described in Materials and Methods. The filters were hybridized to $\alpha 1$(I) RNA probes of different sizes and amounts (i.e., 40 ng/ml of each probe were used when equal amounts of the two probes were compared, 40 ng/ml of the larger probe and 18 ng/ml of the smaller probe were used when equal numbers of transcripts were compared). The hybridization was carried out in 0.2 ml/cm^2 of solution for 16-20 hours at 45°C. The washing temperatures employed are indicated on top. All filters were exposed to X-ray film for the same amount of time. (B): Summary of the results of the experiment. The results are expressed as the ratio of counts per minute of the larger (4z6771/Xba I, 1.5 kb) probe to that for the smaller (4z6771/Xho I, 0.7 kb) probe.
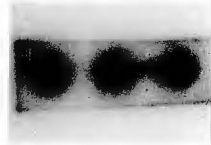
A

55°C   65°C

1.5 KB, 40 NG/ML

0.7 KB, 40 NG/ML

0.7 KB, 18 NG/ML

B
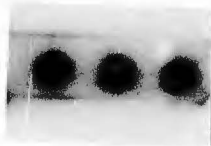
	55°C	65°C
1.5 kb/0.7 kb equal amounts of probe	7.9	9.1
1.5 kb/0.7 kb equal number of transcripts	13.2	12.9

Figure 14. Probe saturation. Three separate experiments (O, ●, ᐃ) each with some different data points are superimposed on each other. Each data point is the average of three samples of 5000 glutaraldehyde-fixed cells (GM03348B) per dot hybridized to the $\alpha 1(I)$ RNA probe at the concentrations indicated on the X-axis. The hybridization was carried out in 0.2 ml/cm^2 of hybridization solution at 45°C for 16-20 hours. The results are expressed as percent of maximum counts per minute obtained in each experiment.

Probe Saturation
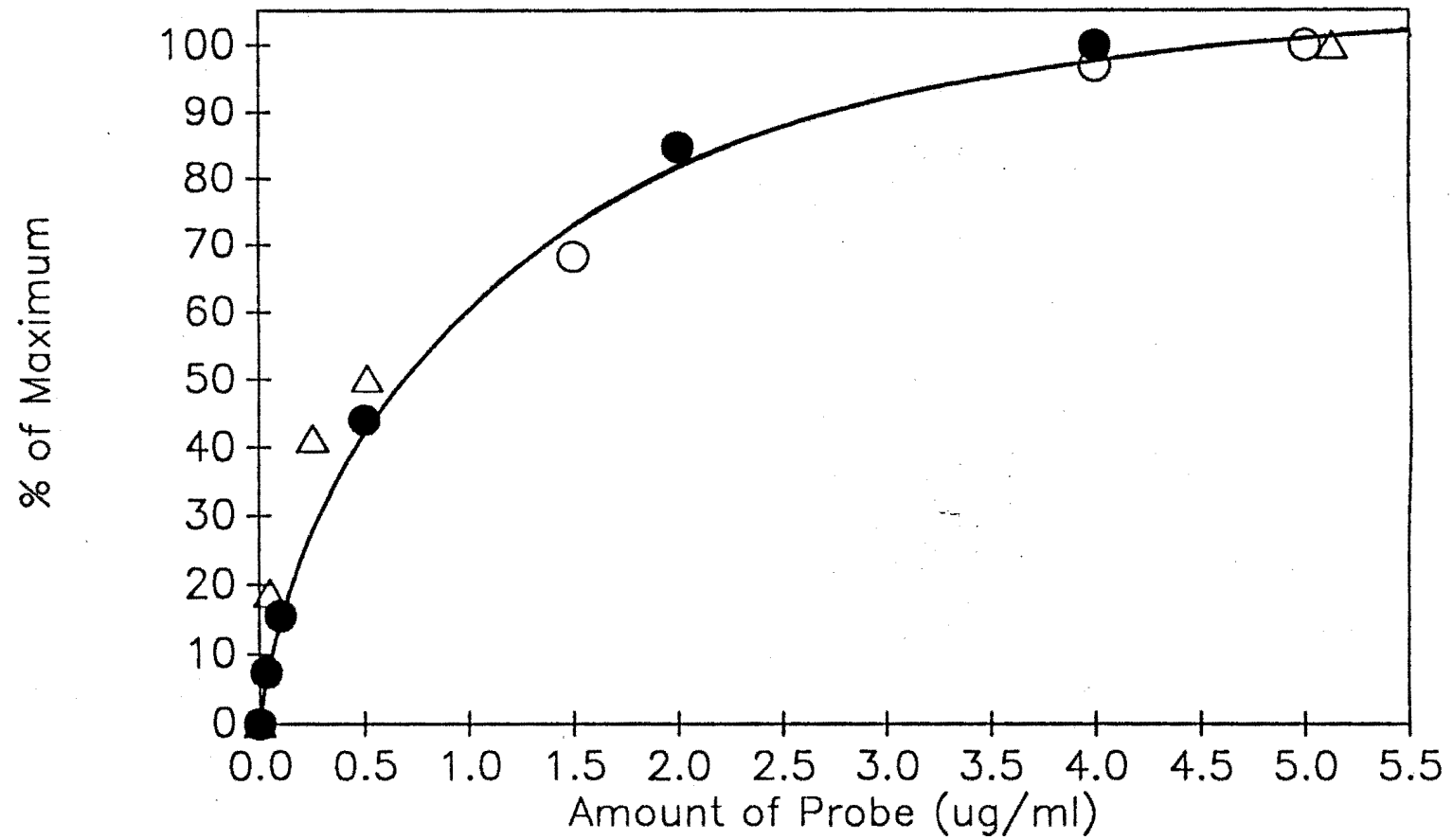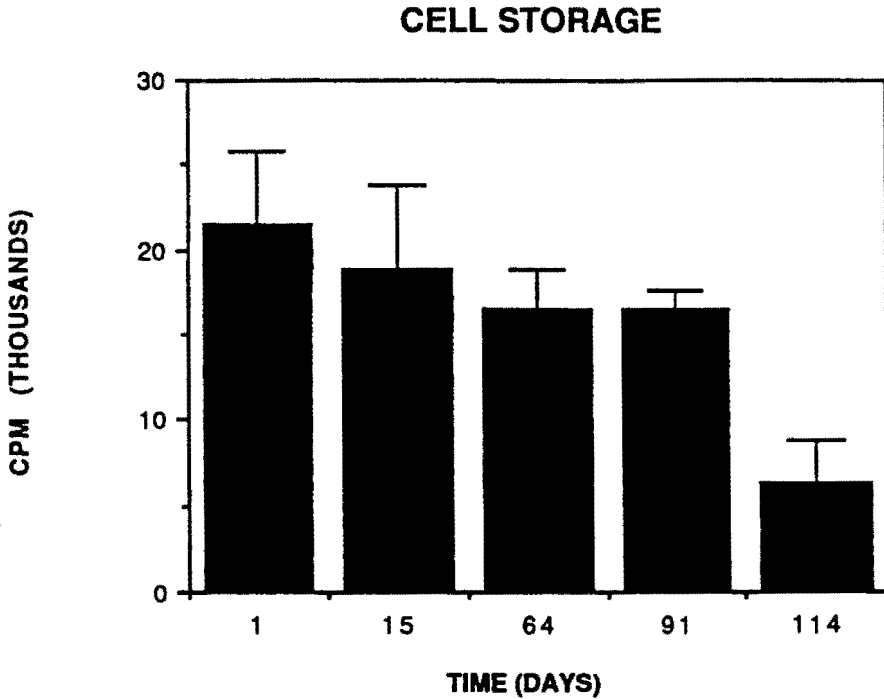
Figure 15. Cell storage: Fibroblasts from cell line GM03348B were harvested and fixed with 1% glutaraldehyde on different dates as outlined in Materials and Methods. The cells were stored for up to four months in 70% ethanol at -20°C before hybridization. Filters with triplicates of each sample (5000 cells per dot) were prepared and hybridized to 40 ng/ml of the $\alpha1(I)$ RNA probe at 45°C for 16-20 hours in a volume of 0.2 ml/cm^2. The filters were washed and the counts per minute of hybridization are presented. The error bars represent the standard deviations between the triplicates in each point.
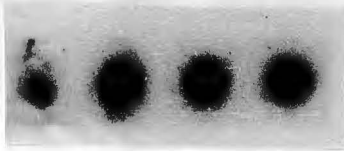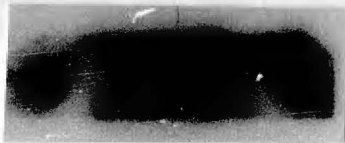
Figure 16. Dextran sulfate. Normal human fibroblast cells (GM03348B) immobilized onto fiber filter at 5000 cells per dot, were hybridized to 40 ng/ml of $\alpha 1(I)$ RNA transcripts in $0.2 \ ml/cm^2$ of hybridization solution with (A) or without (B) 10% dextran sulfate. The hybridization was carried out at 45°C for 16-20 hours. After washing the filters they were exposed to X-ray film for the same amount of time.

A   13730 ± 816

B   14850 ± 724

Figure 17. Effects of prehybridization. Samples of 5000 cells (GM03348B) per dot were loaded onto poly-L-lysine coated glass fiber filters and treated as described in Materials and Methods. Strip A was prehybridized in hybridization solution without the probe for 4 hours at 45°C. Forty ng/ml of $\alpha 1(I)$ RNA probe was added to filter A and at the same time filter B was incubated with hybridization solution containing the probe. The filters were hybridized in 0.2 ml/cm^2 of solution for 16-20 hours at 45°C. The filters were then washed and exposed to X-ray film for the same amount of time. The actual counts per minute and the standard deviation are presented on the left.

Figure 17. Effects of prehybridization. Samples of 5000 cells (GM03348B) per dot were loaded onto poly-L-lysine coated glass fiber filters and treated as described in Materials and Methods. Strip A was prehybridized in hybridization solution without the probe for 4 hours at 45°C. Forty ng/ml of $\alpha1(I)$ RNA probe was added to filter A and at the same time filter B was incubated with hybridization solution containing the probe. The filters were hybridized in 0.2 ml/cm^2 of solution for 16-20 hours at 45°C. The filters were then washed and exposed to X-ray film for the same amount of time. The actual counts per minute and the standard deviation are presented on the left.
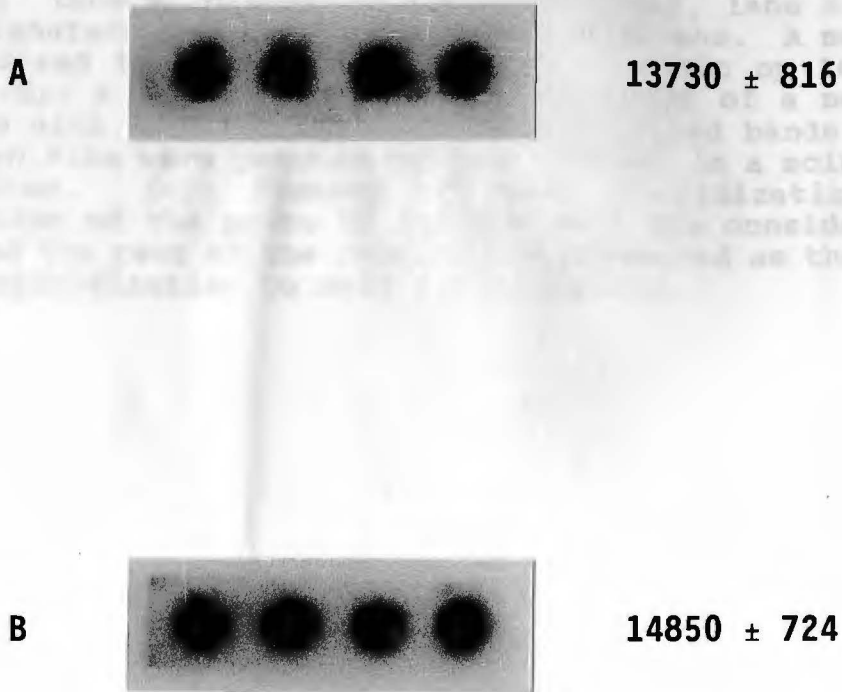
Figure 18. Cross hybridization. (A): A representative agarose gel containing Hf32/Eco RI [α2(I)], lane 1, 4z6771/Eco RI [3' portion of α1(I)], lane 2, 4z4041/Eco RI [5' portion of α1(I)], lane 3, pT1/Pst I (α tubulin), lane 4, pA1/Pst I (β actin), lane 5, pLh14/Eco RI [α1(III)], lane 6, Hf511/Eco RI[α2(V)], lane 7, Ok25/Eco RI [α2(V)], lane 8, pXlr-11/Eco RI (28S rRNA), lane 9, pXlr-12/Eco RI (18S rRNA), lane 10. The DNA was transferred to Gene Screen Plus membrane. A membrane was hybridized to either α1(I), α2(I), β actin or 28S rRNA probes. (B): A representative autoradiograph of a membrane hybridized with the α1(I) probe. The hybridized bands on the Gene Screen Plus were punched out and counted in a scintillation counter. (C): Summary of cross hybridization. The hybridization of the probe to its own cDNA are considered to be 100% and the rest of the results are presented as the ratio of cross hybridization to self hybridization.

C
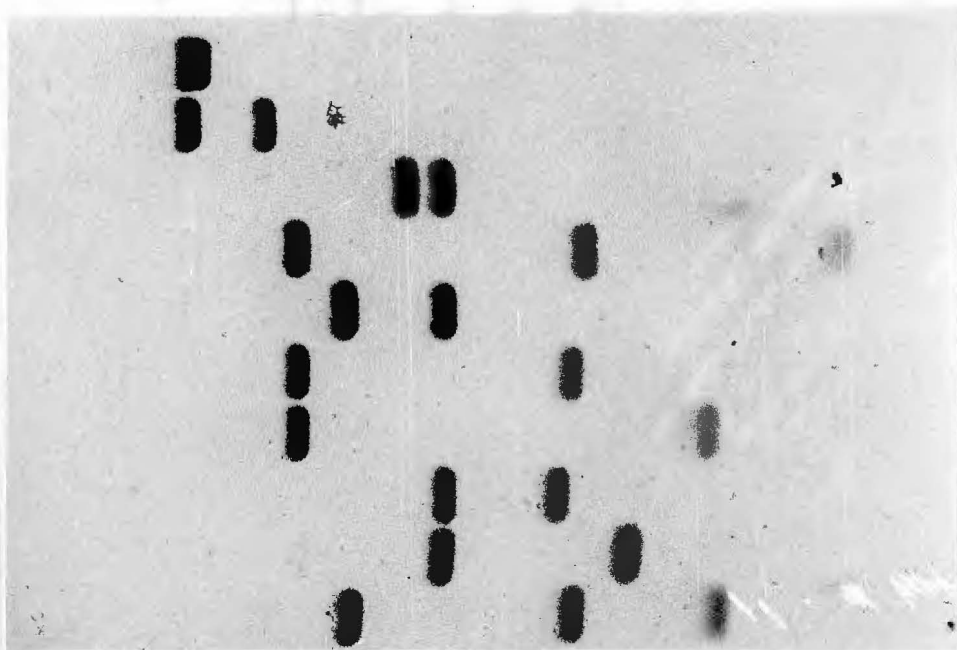
Probes	α1(I)	α2(I)	α1(III)	α2(V)	ß actin	α tubulin	18S	28S	Total
α1(I)	100%	3.6%	3.2%	1.5%	0.5%	0.7%	0%	0%	9.5%
α2(I)	8.5%	100%	0%	0%	0%	0%	0%	0%	8.5%
ß actin	<1%	<1%	<1%	<1%	100%	<1%	0%	0%	<3%
28S	0%	0%	0%	0%	0%	0%	0%	100%	0%

Figure 19. Hybridization specificity. (A): Strand specificity. Triplicates of 5000 cells (GM03348B) per dot were loaded onto the glass fiber filters and treated as described previously. One strip was hybridized to 40 ng/ml of either the sense or the anti-sense strand of $\alpha 1(I)$, $\alpha 2(I)$ or β actin RNA probes. The dots were later punched out and counted in the scintillation counter. The results from representative experiments are presented as counts per minute plus the standard deviation for each probe. (B): Ribonuclease A treatment prior to hybridization. Cells loaded onto the filters were treated as described in text. Filters A and D were treated with 0 ug/ml RNase A for 30 minutes at 37°C. Filter B was treated with 5 ug/ml and filter C with 10 ug/ml RNase A at 37°C for the same length of time as above. Filters B, C, and D were hybridized in one bag. Filter A was hybridized in a separate bag. (C): Ribonuclease A treatment after hybridization. Two strips with triplicates of 5000 cells per dot were treated and hybridized with $\alpha 1(I)$ RNA probe. Only one of the strips (B) was treated with 5 ug/ml of RNase A for 30 minutes at 37°C. In the autoradiograph shown filter B was exposed for four times as long as filter A. The actual counts and the standard deviation for both B and C are presented on the right.

A

	Sense Strand	Anti-Sense Strand	Anti-Sense/Sense
α1(I)	150 ± 14	2841 ± 39	18.9
α2(I)	771 ± 153	1983 ± 483	2.6
ß actin	3202 ± 467	9383 ± 1480	2.9

B

A   15534 ± 2175

B   694 ± 316

C   334 ± 122

D   8946 ± 415

C

A   8223 ± 903

B   268 ± 27

Harvest fibroblast cells at confluency
by trypsinization

Fix with 1% glutaraldehyde in PBS at 4°C for 1 hour

Sequential dehydration of cells with ethanol,
resuspend in 70% ethanol

Load 5000 cell onto poly-L-lysine coated glass fiber
filter under 40 mm Hg vacuum and let air dry

Incubate with 10 ug/ml of proteinase K at 37°C for
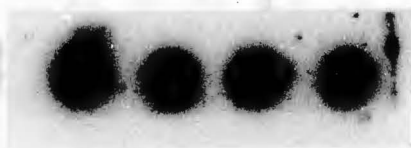40 minutes, followed by triethanol amine and acetic
anhydride treatments, let air dry

Hybridize with 40 ng/ml of RNA probe in 0.2 ml/cm^2 of
hybridization solution containing 10% dextran sulfate
for 16-20 hours at 45°C

Wash with 0.05 X SSC at 65°C, expose to X-ray film,
and count by scintillation counting
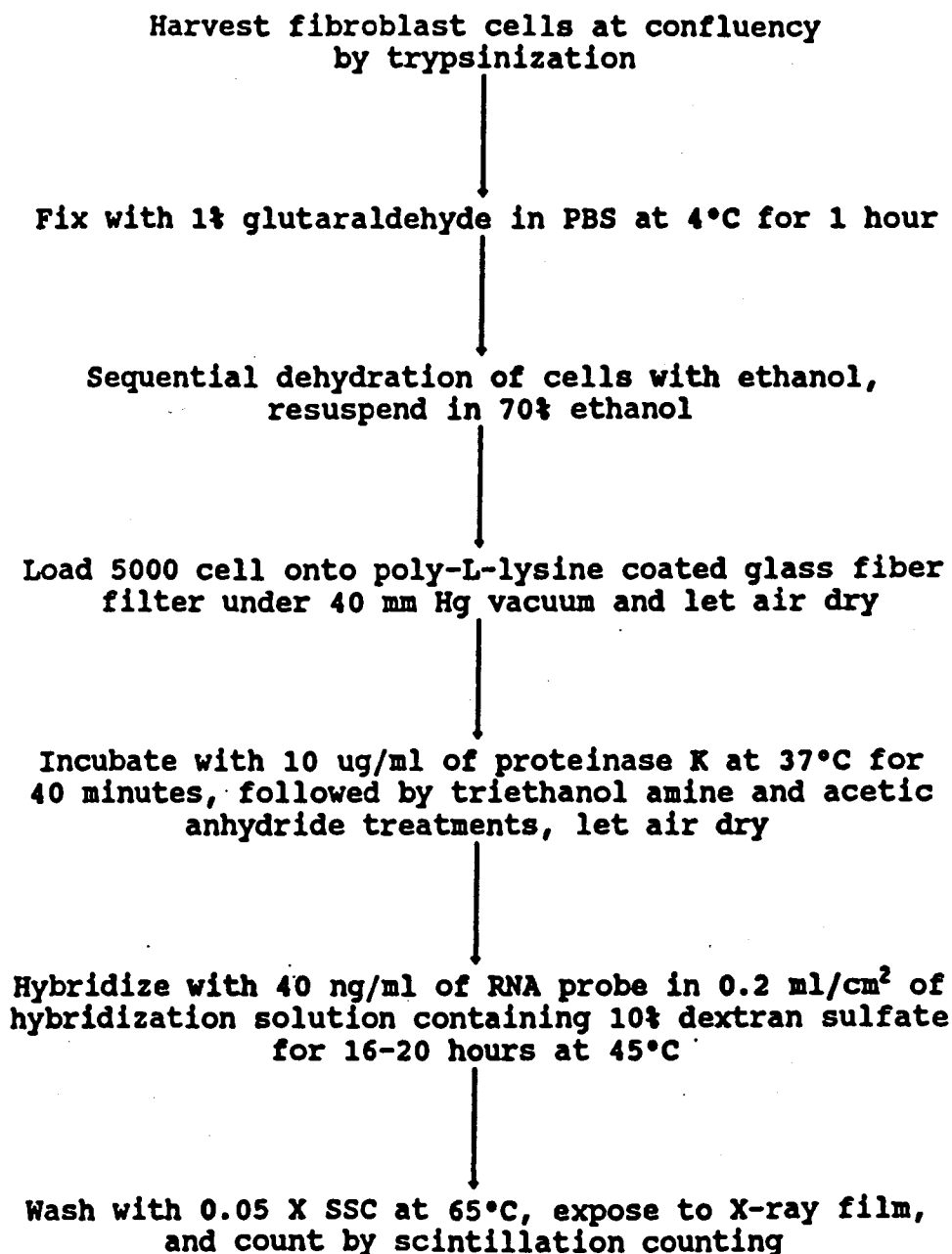
Figure 20. Procedural flow chart based on the optimal treat-
ment and hybridization parameters.

Slopes

	GM0744	GM03348B	GM02962	GM03348B
α1(I)	1.8517	1.3708	2.3701	0.9885
α2(I)	0.5760	0.4355	1.5336	0.3675
rRNA	1.1825	0.2250	1.1550	0.2680

Ratios of Slopes

	GM0744	GM03348B	GM02962	GM03348B
α1/α2	3.2	3.1	1.55	2.7
α1/rRNA	1.7	6.09	2.05	3.7
α2/rRNA	0.5	1.94	1.33	1.37

Figure 21. Quantitation of the relative amounts of Type I collagen and the ribosomal RNA in OI and normal human fibroblasts. Cell lines indicated on the top were fixed with 1% glutaraldehyde, triplicates of 2,4 and 8 thousand cells were loaded onto poly-L-lysine-coated glass fiber filters, and treated as described in Materials and Methods. Each OI cell line (GM0744 and GM02962) was accompanied by normal fibroblasts (GM03348B) which served as a control and a base for comparison. (A): The results are presented as the slopes (counts per cell) of the first order regression line connecting data points of each cell line for each probe. (B): The amount of each collagen species is also presented as the ratio of the collagen slope to the 28S rRNA slope in addition to the ratio of the slopes of the α1(I) to α2(I). The coefficient of variation (V) for two independent trials with cell lines GM03348B, GM02962, and GM0744 are 19%, 35% and 0.16%, respectively.

CONCLUSION

The goal in this study was to develop a quick method for the quantitative determination of the steady state levels of Type I collagen mRNA in fibroblast cells from normal and OI affected individuals. In situ hybridization was chosen because of the advantages it offers: the ease with which this technique is performed, the capability of analyzing a large number of different samples at the same time, and the eliminating of the need for the isolation and handling of the sensitive RNA.

Although in situ hybridization techniques have been known for a long time and a great deal of information is available, not much was known about hybridization to human fibroblast cells grown in cell culture. Consequently, the combination of variables affecting hybridization to messenger RNA in situ had to be worked out. In testing each parameter, maximal hybridization signal, minimal background, and maximal hybridization specificity served as the basic criteria.

The choice of fixative is important so as to povide for the maximum RNA retention and thus the highest hybridization signal. In agreement with the conclusions of other researchers (Lawrence and Singer, 1985; Yu and Gorovsky, 1987; Angerer

and Angerer, 1981) 1% glutaraldehyde crosslinks the cellular matrix extensively and thus ensures maximum RNA retention and a high hybridization signal. However, due to this property of glutaraldehyde, extended digestion with proteinase K is required to achieve maximum penetration of the probe into the cell. Paraformaldehyde, on the other hand, fixes the cells in a more open configuration and thus renders the cellular RNA more accessible to the probe without the need for extensive proteolytic treatment (Lawrence and Singer, 1985), and thus may be more suitable for situations where the preservation of cellular morphology is essential. One disadvantage to the use of paraformaldehyde for fixation is that it does not retain and/or protect the RNA as well as glutaraldehyde and consequently does not provide for the maximum signal. Also as a result paraformaldehyde-fixed cells can not be stored as long as glutaraldehyde-fixed cells. Once glutaraldehyde had been chosen as the fixative the other parameters (i.e., proteinase K treatment, hybridization volume and time, prehybridization, and dextran sulfate etc...) were tested and optimized. Figure 21 shows a flow chart of the final procedure with all of the parameters listed.

The type of probe employed is another independent variable that had to be decided upon. Although nick translated probe could be used for in situ hybridization, single stranded anti-sense RNA probes are clearly superior for several reasons. Aside from their high specific activity and

the constant probe size, RNA-RNA hybrids formed by the pairing of the probe to its target mRNA have a higher thermal stability than RNA-DNA or DNA-DNA hybrids (Casey and Davidson, 1976). This stability contributes to the increased probe specificity and reproducibility of the hybridization reaction. RNA probes are strand-specific and do not contain vector sequences. This prevents non-specific hybridization or competitive hybridization between the complementary strands as occurs with nick-translated probes. The latter fact contributes to the much higher signals obtained with RNA probes than with nick-translated DNA probes. Also, the ability to use ribonucleases to digest away unhybridized and unmatched probe provides for a greater specificity and a very low background which in turn leads to an even higher sensitivity (Zinn et al., 1983).

An unexpected factor that proved to be very critical in the interpretation of quantitation experiments is the potential for the amplification of the signals by RNA probe hyperpolymer formation on target mRNAs. Three pieces of evidence suggest that such events are occurring here. First, experiments comparing probe sizes (Figure 13) showed a 7-fold greater intensity of hybridization with a 1.5 kb probe than one of 0.7 kb. This was not due to differences in the specific activity of the probes or to greater stability of the RNA-RNA hybrids with the longer probe. Second, probe saturation experiments (Figure 14) showed that there are 60000 copies of $\alpha 1(I)$ mRNA per fibroblast cells. As mentioned

earlier this number is several times higher than usual for a high abundance transcript. Third, the treatment of hybridized cells with RNase A (Figure 19C) caused a drop in the hybridization intensity by some 30- to 60-fold, indicating the presence of a great amount of partially hybridized single stranded probe. To explain these events, the following hypothesis is put forth: RNA localized inside a fixed cell is most likely surrounded and/or wrapped around complex molecules such as proteins of the translation machinery. As a result certain regions of the target mRNA are free to hybridize to the probe and others are not. Furthermore, the free regions of the mRNA are separated by large molecules such as ribosomes. This would make every free portion of the mRNA a target for a different probe molecule and results in the binding of more than one molecule of the probe per each Type I collagen mRNA. The number of probe molecules bound to the target RNA is dependent on its size (i.e., larger probes bind at a higher number than smaller probes). However, as can be see from the probe size experiments, doubling the size of the probe causes a seven fold increase in hybridization signal, indicating that the number of molecules binding per target mRNA is not linear. The reason for this is not clear at present. Consequently, results obtained by the use of probes with different sizes can not be compared directly.

Therefore, probe size can play a major role in the quantitation of mRNA in intact cells. In cases where absolute

numbers of transcript are sought, smaller sized probes (Yu and Gorovsky, 1987; Lawrence and Singer, 1985) must be used or RNase A treatment must be a regular part of the washing procedure to eliminate the effects of hyperpolymer formation (Cox et al., 1984). However, when maximum sensitivity is required for detection of a rare transcript, or when the relative ratio of a particular mRNA species between different cell lines is desired, then larger probes may be used.

REFERENCES

Aitchison, K., Ogilvie, D., Honeyman, M., Thompson, E. and Sykes, B. (1988). Homozygous Osteogenesis Imperfecta unlinked to collagen I genes. Hum. Genet. 78,233-236.

Amasino, R. (1986). Acceleration of nucleic acid hybridization rate by poly-ethylene glycol. Anal. Biochem. 152,304.

Angerer, L. and Angerer, R. (1981). Detection of poly A+RNA in sea urchin eggs and embryos by quantitative in situ hybridization. Nucl. Acids Res. 9,2819-2840.

Barsh, G. and Byers, P. (1981). Reduced secretion of structurally abnormal Type I procollagen in a form of Osteogenesis Imperfecta. Proc. Natl. Acad. Sci. US 78,5142-5146.

Barsh, G., David, K. and Byers, P. (1982). Type I Osteogenesis Imperfecta: A nonfunctional allele for pro-a1(I) chains of type I procollagen. Proc. Natl. Acad. Sci. US 79,3838-3842.

Bateman, J., Chan, D., Lamande, S., Mascara, T. and Cole, W. (1988). Biochemical heterogeneity of Type I collagen mutations in Osteogenesis Imperfecta. Ann. N.Y. Acad. Sci. 543,95-105.

Bateman, J., Chan, D., Mascara, T., Rogers, J. and Cole, W. (1986). Collagen defects in lethal perinatal Osteogenesis Imperfecta. Biochem. J. 240,699-708.

Baum, B., Moss, J., Breul, S., Berg, R. and Crystal, R. (1980). Effect of cAMP on the intracellular degradation of newly synthesized collagen. J. Biol. Chem. 255,2843-2847.

Benveniste-Schrode, K., Doering, J., Hauck, W., Schrode, J., Kendra, K. and Drexler, B. (1985). Evolution of chick Type I procollagen genes. J. Mol. Evol. 22,209-219.

Berg, R., Schwartz, M. and Crystal, R. (1980). Regulation of the production of secretory proteins: Intracellular degradation of newly synthesized "defective" collagen. Proc. Natl. Acad. Sci. US 77,4746-4750.

Bienkowski, R., Baum, B. and Crystal, R. (1978). Fibroblasts degrade newly synthesised collagen within the cell before secretion. Nature 276,413-416.

BMBiochemica, (1988). Optimal production of radiolabeled RNA probes. Part II: Template- and polymerase-dependent effects. BMBiochemica 5, No. 4,14-15.

Bonaventure, J., Cohen-Solal, L., Lasselin, C., Allain, J.-C. and Maroteaux, P. (1986). Abnormal procollagen synthesis in fibroblasts from three patients of the same family with a severe form of Osteogenesis Imperfecta (type III). Bioch. et Biophys. Acta 889,23-34.

Bornstein, P. and McKay, J. (1988). The first intron of the a1(I) collagen gene contains several transcriptional regulatory elements. J. Biol. Chem. 263,1603-1606.

Bornstein, P. and Traub, W. (1979). The chemistry and biology of collagen. The proteins. Volume IV. New York, Academic press, USA.

Brahic, M. and Haase, A. (1978). Detection of viral sequences of low reiteration frequency by in situ hybridization. Proc. Natl. Acad. Sci. US 75,6125-6129.

Brenner, D., Rippe, R. and Veloz, L. (1989). Analysis of the collagen a1(I) promoter. Nucl. Acids Res. 17,6055-6064.

Byers, P. (1989). Inherited disorders of collagen gene structure and expression. Am. J. Med. Genet. 34,72-80.

Byers, P., Bonadio, J., Cohn, D., Starman, B., Wenstrup, R. and Willing, M. (1988a). Osteogenesis Imperfecta: The molecular basis of clinical heterogeneity. Ann. N.Y. Acad. Sci. 543,117-128.

Byers, P., Bonadio, J. and Steinmann, B. (1984). Osteogenesis Imperfecta: Update and perspective. Am. J. Med. Genet. 17,429-435.

Byers, P., Starman, B., Cohn, D. and Horwitz, A. (1988b). A novel mutation causesa perinatal lethal form of osteogenesis imperfecta. J. Biol. Chem. 263,7855-7861.

Byers, P., Wenstrup, R., Bonadio, J., Starman, B. and Cohn, D. (1987). Molecular basis of inherited disorders of collagen biosynthesis: Implications for prenatal diagnosis. Curr. Probl. Derm. 16,158-174.

Capco, D. and Jeffery, W. (1978). Differential distribution of Poly(A)-containing RNA in the embryonic cells of Oncopeltus fasciatus. Develop. biol. 67,137-151.

Casey, J. and Davidson, N. (1976). Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentration of formamide. Nuc. Acid. Res.

4,1539-1552.

Cheah, K. (1985). Collagen genes and inherited connective tissue disease. Biochem. J. 299,287-303.

Chu, M.-L., Gargiulo, V., Williams, C. and Ramirez, F. (1985b). Multiexon deletion in an Osteogenesis Imperfecta variant with increased Type III collagen mRNA. J. Biol. Chem. 260,691-694.

Chu, M.-L., Myers, J., Bernard, M., Ding, J.-F. and Ramirez, F. (1982). Cloning and characterization of five overlapping cDNAs specific for the human pro-a1(I) collagen chain. Nucl. Acids Res. 10,5925-5934.

Chu, M.-L., Wet, W. d, Bernard, M. and Ramirez, F. (1985a). Fine structural analysis of the human pro-a1(I) collagen gene. J. Biol. Chem. 260,2315-2320.

Cleveland, D., Lopata, M., Macdonald, R., Cowan, N., Rutter, W. and Kirschner, M. (1980). Number and evolutionary conservation of alpha and beta tubulin and cytoplasmic beta and gamma actin genes using specific cloned cDNA probes. Cell 20,95-105.

Cohen, S., Chang, A. and Hsu, L. (1973). Non-chromosomal antibiotic resistance in bacteria: geneic transformation of E. Coli by R-factor DNA. Proc. Natl. Acad. Sci. US 69,2110.

Cohn, D., Apone, S., Eyer, D., Starman, B., Andreassen, P., Charbonneau, H., Nicholls, A., Pope, F. and Byers, P. (1988b). Substitution of cysteine for glycine within the carboxyl-terminal telopeptide of the a1 chain of Type I collagen produces mild osteogenesis imperfecta. J. Biol. Chem. 263,14605-14607.

Cohn, D., Wenstrup, R. and Willing, M. (1988a). General strategies for isolating the genes encoding Type I collagen and for characterizing mutations which produce osteogenesis imperfecta. Ann. N.Y. Acad. Sci. 543,129-135.

Cox, K., Deleon, D., Angerer, L. and Angerer, R. (1984). Detection of mRNAs in Sea Urchin embryos by in situ hybridization using asymmetric RNA probes. Develop. Biol. 101,485-502.

Crombrugghe, B. d and Pastan, I. (1982). Structure and regulation of collagen gene. TIBS 11-13.

Dawid, I. and Wellauer, P. (1976). A reinvestigation of 5' to

3' polarity in 40S ribosomal RNA precursor of Xenopus laevis. <u>Cell</u> 8,443-448.

Deleon, D., Cox, K., Angerer, L. and Angerer, R. (1983). Most early-variant histone mRNA is contained in the pronucleus of Sea Urchin eggs. <u>Develop. Biol.</u> 100,197-206.

de Wet, W. d, Bernard, M., Benson-Chanda, V., Chu, M.-L., Dickson, L., Weil, D. and Ramirez, F. (1987). Organization of the Human pro-a2(I) collagen gene. <u>J. Biol. Chem.</u> 262,16032-16036.

de Wet, W. d, Chu, M.-L. and Prockop, D. (1983a). The mRNAs for the pro-a1(I) and pro-a2(I) chains of Type I procollagen are translated at the same rate in normal human fibroblasts and in fibroblasts from two variants of OI with altered steady state ratios of the two mRNAs. <u>J. Biol. Chem.</u> 258,14385-14389.

de Wet, W. d, Pihlajaniemi, T., Myers, J., Kelly, T. and Prockop, D. (1983b). Synthesis of a shortened pro-a1(I) chain and decreased synthesis of pro-a2(I) chains in a proband with osteogenesis imperfecta. <u>J. Biol. Chem.</u> 258,7721-7728.

Dickson, I., Millar, E. and Veis, A. (1975). Evidence for abnormality of bone-matrix proteins in osteogenesis imperfecta. <u>Lancet</u> 586-587.

Dickson, L., Pihlajaniemi, T., Deak, S., Pope, F., Nicholls, A., Prockop, D. and Myers, J. (1984). Nuclease S1 mapping of a homozygous mutation in the carboxyl-propeptide-coding region of the pro-a2(I) collagen gene in a patient with Osteogenesis Imperfecta. <u>Proc. Natl. Acad. Sci. US</u> 81,4525-4528.

Doering, J., Burket, A. and Vogel, L. (1987). Collagen gene deletions in osteogenesis imperfecta patients. <u>J. Cell. Biol.</u> 105,213a.

Doering, J., Jelachich, M. and Hanlon, K. (1982). Identification and genomic organization of human tRNA Lys genes. <u>FEBS Lett.</u> 146,47-51.

Dretzen, D., Bellard, M., Corsi, P.S. and Chambon, P. (1981). Anal. Biochem. 112,295.

Dunn, M., Rojkind, M., Warren, K., Hait, P., Rifas, L. and Seifter, S. (1977). Liver collagen synthesis in murine Schistosomiasis. <u>J. Clin. Invest.</u> 59,666-674.

Edwards, M. and Wood, W. (1983). A cytological hybridization

method for detecting specific messenger RNAs in embryos and tissues of Caenorhabditis elegans. <u>Dev. Biol.</u> 97,375-390.

Ehrinpreis, M., Giambrone, M. and Rojkind, M. (1980). Liver proline oxidase activity and collagen synthesis in rats with cirrhosis induced by carbon tetrachloride. <u>Biochim. Biophys. Acta.</u> 629,184-193.

Falk, C., Schwartz, R., Ramirez, F. and Tsipouras, P. (1986). Use of molecular haplotypes specific for the human pro-a2(I) collagen gene in linkage analysis of the mild autosomal dominant forms of Osteogenesis Imperfecta. <u>Am. J. Hum. Genet.</u> 38,269-279.

Gall, J. and Pardue, M. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. <u>Proc. Natl. Acad. Sci. US</u> 63,378-383.

Gerhard, D., Kawasaki, E., Bancroft, F. and Szabo, P. (1981). Localization of a unique gene by direct hybridization in situ. <u>Proc. Natl. Acad. Sci.</u> 78,3755-3759.

Hastie, N. and Bishop, J. (1976). The expression of three abundance classes of mRNA in mouse tissues. <u>Cell</u> 9,761-774.

Hollister, D. and Holbrook, P.B.K. (1984). Genetic disorders of collagen metabolism. <u>Adv. Hum. Genet.</u> 12,1-87.

Huerre, C., Junien, C., Weil, D., Chu, M.-L., Morabito, M., Cong, N. Va, Myers, J., Foubert, C., Gross, M.-S., Prockop, D., Boue, A., Kaplan, J.-C., Chapelle, A.d. l and Ramirez, F. (1982). Proc. Natl. Acad. Sci. US 79,6627.

Junien, C., Weil, D., Myers, J., Cong, N. Va, Chu, M._., Foubert, C., Gross, M.-S., Prockop, D., Kaplan, J.-C. and Ramirez, F. (1982). Assignment of the human proa2(I) collagen structural gene (COLIA2) to chromosome 7 by molecular hybridization. <u>Am. J. Hum. Genet.</u> 34,381-387.

Kadonaga, J., A, K., Tjian, J. and Tjian, R. (1986). Promoter-specific activation of RNA polymerase II transcription by Sp1. <u>TIBS</u> 11,20-23.

Karsenty, G., Golumbek, P. and Crombrugghe, B. d (1988). Point mutations and small substitution mutations in three different upstream elements inhibit the activity of the mouse a2(I) collagen promoter. <u>J. Biol. Chem.</u> 263,13909-13915.

Khillan, J., Schmidt, A., Overbeek, P., Crombrugghe, B. d and Westphal, H. (1986). Developmental and tissue-specific expression directed by the a2 type I collagen promoter in transgenic mice. Proc. Natl. Acad. Sci. US 83,725-729.

Kivirikko, K. and Myllyla, R. (1982). Posttranslational enzymes in the biosynthesis of collagen: intracellular enzymes. Methods Enzymol. 82,245-304.

Kuivaniemi, H., Sabol, C., Tromp, G., Sippola-Thiele, M. and Prockop, D. (1988). A 19-base pair deletion in the pro-a2(I) gene of Type I procollagen that causes in-frame RNA splicing from exon 10 to exon 12 in a proband with atypical osteogenesis imperfecta and in his asymptomatic mother. J. Biol. Chem. 263,11407-11413.

Labhard, M., Wirtz, M., Pope, F., Nicholls, A. and Hollister, D. (1988). A cysteine for glycine substitution at position 1017 in an a1(I) chain of Type I collagen in a patient with mild dominantly inherited osteogenesis imperfecta. Mol. Biol. Med. 5,197-207.

Lawrence, J. and Singer, R. (1985). Quantitative analysis of in situ hybridization methods for the detection of actin gene expression. Nucl. Acids Res. 13,1777-1799.

Leeuw, W. d, Slagboom, P. and Vijg, J. (1989). Quantitative comparison of mRNA levels in mammalian tissues: 28S ribosomal RNA level as an accurate internal control. Nucl. Acids Res. 17,10137-10138.

Lewin, B. (1987). Genes III. Third ed. 183-216.

Maniatis, T., Fritsch, E. and Sambrook, J. (1982). Molecular Cloning.

Marini, J., Grange, D., Gottesman, G., Lewis, M. and Koeplin, D. (1989). Osteogenesis imperfecta type IV: detection of a point mutation in one a1(I) collagen allele (COL1A1) by RNA/RNA hybrid analysis. J. Biol. Chem. 264,11893-11900.

Mayne, R. and Burgeson, R. (1987). Structure and function of collagen types. Academic press, Inc., New York, USA.

McCabe, J. and Pfaff, D. (1989). In situ hybridization: A methodological guide. Meth. Neurosci. 1,98-126.

McKusick, V. (1972). Heritable disorders of connective tissue. St Louis: CV Mosby.

Melton, D., Krieg, P., Rebagliati, M., Maniatix, T., Zinn, K. and Green, M. (1984). Efficient in vitro synthesis of

biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nuc. Acid. Res. 12,7035-7056.

Myers, J., Chu, M.-L., Faro, S., Clark, W., prockop, D. and

Ramirez, F. (1981). Cloning a cDNA for the pro-a2 chain of human Type I collagen. proc. Natl. Acad. Sci. US 78,5316-3520.

Pack, M., Constantinou, C., Kalia, K., Nielsen, K. and Prockop, D. (1989). Substitution of serine for a1(I)-glycine 844 in a severe variant of osteogenesis imperfecta minimallly destabilizes the triple helix of type I procollagen. J. Biol. Chem. 264,19694-19699.

Paglia, L., Wiestner, M., Duchene, M., Ouellette, L., Horlein, D., Martin, G. and Muller, P. (1981). Effects of procollagen peptides on the translation of type II collagen mRNA and on collagen biosynthesis in chondrocytes. Biochemistry 20,3525-3527.

Paglia, L., Wilczek, J., Leon, L. d, Martin, G., Horlein, D. and Muller, P. (1979). Inhibition of procollagen cell-free synthesis by amino-terminal extension peptides. Biochemistry 18,5030-5034.

Penttinen, R., Jr., Lichtenstein, G.M. and McKusick, V. (1975). Abnormal collagen matabolism in cultured cells in Osteogenesis imperfecta. Proc. Natl. Acad. Sci. US 72,586-589.

Pihlajaniemi, T., Dickson, L., Pope, F., Korhonen, V., Nicholls, A., Prockop, D. and myers, J. (1984). Osteogenesis Imperfecta: Cloning of a pro-a2(I) collagen gene with a frameshift mutation. J. Biol. Chem. 259,12041-12944.

Privalov, P. (1982). Stability of proteins: proteins which do not present a single cooperative system. Adv. Prot. Chem. 35,1-104.

Prockop, D. (1984). Osteogenesis Imperfecta: phenotypic heterogeneity, protein suicide, short and long collagen. Am. J. Hum. Genet. 36,499-505.

Prockop, D., Constantinou, C., Dombrowski, K., Hojima, Y., Kadler, K., Kuivaniemi, H., Tromp, G. and Vogel, B. (1989). Type I procollagen: the gene-protein system that harbors most of the mutations causing osteogenesis imperfecta and probably more common heritable disorders of connective tissue. Am. J. Med. Genet. 34,60-67.

Prockop, D., Kivirikko, K., Tuderman, L. and Guzman, N. (1979). The biosynthesis of collagen and its disorders. N. Engl. J. Med. 301,13-23.

Prockop, D. and Kivirikko, K.I. (1984). Heritable diseases of collagen. N. Engl. J. Med. 311,376-386.

Prockop, D. and Tuderman, L. (1982). Posttranslational enzymes in the biosynthesis of collagen: extracellular enzymes. Methods Enzymol. 82,305-318.

Reed, K. and Mann, D. (1985). Rapid transfer of DNA from agarose gels to nylon. Nucl. Acids Res. 13,7207-7221.

Rigby, P., Dieckmann, M., Rhodes, C. and Berg, P. (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113,237-251.

Rohde, H., Vargas, L., Hahn, E., Kalbflesisch, H., Brugera, M. and Timpl, R. (1979). Radioimmunoassay for type III procollagen peptide and its application to human liver disease. Eur. J. Clin. Invest. 9,451-459.

Rojkind, M. and Leon, L. d (1970). Collagen biosynthesis in cirrhotic rat liver slices: a regulatory mechanism. Biochim. Biophys. Acta 217,512-522.

Rossi, P. and Crombrugghe, B. d (1987). Identification of a cell-specific transcriptional enhancer in the first intron of the mouse a2(I) collagen gene. Proc. Natl. Acad. Sci. US 84,5590-5594.

Rossouw, C., Vergeer, W., Plooy, S.d., Bernard, M., Ramirez, F. and Wet, W. d (1987). DNA sequences in the first intron of the human pro-a1(I) collagen gene enhance transcription. J. Biol. Chem. 262,15151-15157.

Rowe, D., Jr., Shapiro, M.P. and Schlesinger, S. (1985). Diminished type I collagen synthesis and reduced alpha 1(I) collagen messenger RNA in cultured fibroblasts from patients with dominantly inherited (type I) osteogenesis imperfecta. J. Clin. Invest. 76,604-611.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989). Molecular cloning, a laboratory manual. 2nd ed.

Scharffetter, K., Kulozik, M., Stolz, W., Lankat-Buttgereit, B., Hatamochi, T., Sohnchen, R. and Krieg, T. (1989b). Localization of collagen a1(I) gene expression during wound healing by in situ hybridization. J. Invest.

<u>Dermat.</u> 93,405-412.

Scharffetter, K., Stolz, W., Lankat-Buttgereit, B., Mauch, C., Kulozik, M. and Krieg, T. (1989a). In situ hybridization-a useful tool for studies on collagen gene expression in cell culture as well as in normal and altered tissue. <u>Cell Path.</u> 56,299-306.

Schmidt, A., Rossi, P. and Crombrugghe, B. d (1986). Transcriptional control of the mouse a2(I) collagen gene: functional deletion analysis of the promoter and evidence for cell-specific expression. <u>Mol. Cell. Biol.</u> 6,347-354.

Sillence, D., Rimoin, D. and Danks, D. (1979a). Clinical variability in Osteogenesis Imperfecta: Variable expressivity or genetic heterogeneity. <u>Birth Defects</u> XV,113-129.

Sillence, D., Senn, A. and Danks, D. (1979b). Genetic heterogeneity in Osteogenesis Imperfecta. <u>J. Med. Gen.</u> 16,101-116.

Sims, M., Doering, J. and Hoyle, H. (1983). DNA methylation patterns in the 5S DNAs of Xenopus laevis. <u>Nucl. Acids Res.</u> 11,277-290.

Sippola, M., Kaffe, S. and Prockop, D. (1984). A heterozygous defect for structurally altered pro-a2 chain of Type I procollagen in a mild variant of Osteogenesis Imperfecta. <u>J. Biol. Chem.</u> 259,14094-14100.

Steinmann, B., Rao, V., Vogel, A., Bruckner, P., Gitzelmann, R. and Byers, P. (1984). Cysteine in the triple-helical domain of one allelic product of the a1(I) gene of Type I collagen produces a lethal form of Osteogenesis Imperfecta. <u>J. Biol. Chem.</u> 259,11129-11138.

Steinmann, B., Nicholls, A. and Pope, F. (1986). Clinical variability of Osteogenesis Imperfecta reflecting molecular heterogeneity: Cysteine substitutions in the a1(I) collagen chain producing lethal and Mild forms. <u>J. Biol. Chem.</u> 261,8958-8964.

Sykes, B., Ogilvie, D., Anderson, P.W.J. and Jones, N. (1986). Osteogenesis Imperfecta is linked to both Type I collagen structural genes. <u>Lancet</u> 12,69-72.

Sykes, B., Ogilvie, D., Wordsworth, P., Willis, G., Mathew, C., Beighton, P., Nicholls, A., Pope, F., Thompson, E., Tsipouras, P., Schwartz, R., Jensson, O., Arnason, A., Borresen, A.-L., Heiberg, A., Frey, D. and Steinmann, B.

(1990). Consistent linkage of dominantly inherited osteogenesis imperfecta to the Type I collagen loci: COL1A1 and COL1A2. Am. J. Hum. Genet. 46,293-307.

Sykes, B. and Solomon, E. (1978). Assignment of a type I collagen structural gene to human chromosone 7. Nature 272,548-549.

Tolstoshev, P., Berg, R., Rennard, S., Bradley, K., Trapnell, B. and Crystal, R. (1981). Procollagen production and procollagen messenger RNA levels and activity in human lung fibroblasts during periods of rapid and stationary growth. J. Biol. Chem. 256,3135-3140.

Tsipouras, P., Borresen, A.-L., Dickson, L., Berg, K., Prockop, D. and Ramirez, F. (1984). Molecular heterogeneity in the mild autosomal dominant forms of osteogenesis imperfecta. Am. J. Hum. Genet. 36,1172-1179.

Voss, T. and P. Bornstein (1986). Regulation of type I collagen mRNA levels in fibroblasts. Eur. J. Biochem. 157,433-439.

Wahl, G., Stern, M. and Stark, G. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. US. 76,3683-3687.

Weil, D., Bernard, M., Gargano, S. and Ramirez, F. (1987). The proa2(V) collagen gene is evolutionarily related to the major fibrillar-forming collagens. Nucl. Acids Res. 15,181-198.

Wenstrup, R., Cohn, D., Cohen, T. and Byers, P. (1988). Arginine for glycine substitution in the triple-helical domain of the product of one a2(I) collagen allele (COL1A1) produces the osteogenesis imperfecta type IV phenotype. J. Biol. Chem. 263,7734-7740.

Wenstrup, R., Tsipouras, P. and Byers, P. (1986). Osteogenesis imperfecta type IV: biochemical confirmation of genetic linkage of human Type I collagen. J. Clin. Invest. 78,1449-1455.

Wiestner, M., Krieg, T., Horlein, D., Glanville, R., Fietzek, P. and Muller, P. (1979). Inhibiting effect of procollagen peptides on collagen biosynthesis in fibroblast cultures. J. Biol. Chem. 254,7016-7023.

Williams, C. and Prockop, D. (1983). Synthesis and processing of a Type I procollagen containing shortened pro-a1(I)

chains by fibroblasts from a patient with Osteogenesis Imperfecta. J. Biol. Chem. 258,5915-5921.

Willing, M., Cohn, D., Starman, B., Holbrook, K., Greenberg, C. and Byers, P. (1988). Heterozygosity for a large deletion in the a2(I) collagen gene has a dramatic effect on Type I collagen secretion and produces perinatal lethal osteogenesis imperfecta. J. Biol. Chem. 263, 8398-8404.

Wozney, J., Hanahan, D., Tate, V., Boedtker, H. and Doty, P. (1981). Structure of the pro a2(I) collagen gene. Nature 294,129-135.

Yamada, Y., Avvedimento, V., Mudryi, M., Ohkubo, H., Vogeli, G., Irani, M., Pastan, I. and Crombrugghe, B. d (1980). The collagen dene: evidence for its evolutionary assembly by amplification of a DNA segment containing an exon of 54 pb. Cell 22,887-892.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33,103.

Yu, S.-M. and Gorovsky, M. (1987). In situ dot blots: quantitation of mRNA in intact cells. Nucl. Acids Res. 14,7597-7615.

Zinn, K., DiMaio, D. and Maniatis, T. (1983). Identification of two distinct regulatory regions adjacent to the human b-interferon gene. Cell 34,865.

THESIS APPROVAL SHEET

The thesis submitted by Edward Touma has been read and approved by the following committee:

   Dr. Jeffrey L. Doering, Director
   Associate Professor, Biology
   Loyola University of Chicago

   Dr. Diane Suter
   Assistant Professor, Biology
   Loyola University of Chicago

   Dr. John Smarrelli, Jr.
   Associate Professor, Biology
   Loyola University of Chicago

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given the final approval by the committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

1-29-91
_____   _____
      Date   Director's Signature