Quantification of aggrecan and link-protein mRNA in human articular cartilage of different ages by competitive reverse transcriptase–PCR

Mark C. BOLTON, Jayesh DUDHIA and Michael T. BAYLISS*

Biochemistry Division, Kennedy Institute of Rheumatology, 6 Bute Gardens, Hammersmith, London W6 7DW, U.K.

A competitive reverse transcriptase–PCR (RT–PCR) assay has been developed for the quantification of particular mRNA species in human articular cartilage. Competitor RNA species were synthesized that differed from the amplified target sequence only by the central insertion of an *EcoRI* restriction site. By using known amounts of synthetic target and competitor RNA, it was shown that competitor RNA molecules designed in this way are reverse-transcribed and amplified with equal efficiency to the target of interest. Furthermore quantification could be performed during the plateau phase of the PCR, which was necessary when using ethidium bromide fluorescence as a detection system. The inhibition of aggrecan and link-protein mRNA expression by interleukin 1 or tumour necrosis factor in monolayers of human articular chondrocytes quantified by this competitive RT–PCR method compared favourably with Northern hybridization studies. The main advantage of this technique is that it can be

used to quantify levels of mRNA with RNA extracted directly from 100 mg wet weight of human articular cartilage. Age-related changes in aggrecan and link-protein mRNA were therefore quantified in human articular cartilage directly after dissection from the joint. The concentration of link-protein mRNA was higher in immature cartilage than in mature cartilage when expressed relative to the amount of glyceraldehyde-3-phosphate dehydrogenase mRNA, but no age-related changes were observed in aggrecan mRNA expression. The ratio of aggrecan to link-protein mRNA was higher in mature cartilage than in immature tissue. These age-related differences in the molecular stoichiometry of aggrecan and link-protein mRNA might have implications with respect to the regulation of the formation and the stability of the proteoglycan aggregates in cartilage.

INTRODUCTION

Chondrocytes in articular cartilage are embedded in an extracellular matrix composed principally of collagen type II and proteoglycan aggregates. The large aggregating proteoglycan, aggrecan, forms a non-covalent interaction via its G1 binding domain with hyaluronic acid, which is stabilized by association with link protein [1,2]. The proteoglycan component of cartilage is subject to continuous turnover and the tissue composition is dictated by the balance of synthesis of macromolecules by the chondrocytes, their retention in the matrix by the formation of aggregates, and proteolytic degradation and release from the tissue. An imbalance of these processes is a feature of diseases such as osteoarthritis and rheumatoid arthritis but the mechanisms of regulation of balanced turnover are still not clearly understood. Age-related changes in the structure and glycosylation patterns of aggrecan [3,4] and link protein [5] have been observed in human articular cartilage, but the expression of aggrecan and link-protein mRNA has not been studied in this tissue.

Studies of the regulation of cartilage matrix turnover at the level of mRNA expression are hampered by the difficulty in extracting RNA from articular cartilage [6]. The tissue has a low cell content [7] and the proteoglycans tend to co-purify with the RNA because they too are large, highly negatively charged molecules. Previous studies have therefore mostly involved isolation of the chondrocytes from the extracellular matrix with subsequent primary culture before RNA extraction. During

monolayer culture, however, the cells undergo a gradual de-differentiation and exhibit a switch from expression of type II collagen to type I collagen [8–11]. Even when RNA is extracted from chondrocytes immediately after proteolytic digestion of the extracellular matrix [12], it is still possible that the digestion procedure could have had undesirable effects on the levels of individual mRNA species. Furthermore cell adhesion molecules such as the integrins, which have been detected in human articular cartilage [13,14], mediate signalling between components of the extracellular matrix and the intracellular environment. Isolation of RNA directly from cartilage explants without disruption of the extracellular matrix is therefore preferable for measurement of mRNA levels found *in vivo*.

Although RNA has been successfully extracted from canine articular cartilage for analysis by Northern hybridization [15,16], these studies required large amounts of tissue. The sensitivity of reverse transcriptase–PCR (RT–PCR) makes this technique particularly suitable for the analysis of gene expression in human articular cartilage, which is often in limited supply and in which the cells account for only 1–2 % of the total tissue mass. However, a number of theoretical considerations must be addressed before RT–PCR can be justified as a means of quantifying gene expression [17,18]. We have developed a competitive RT–PCR method for measuring gene expression by using RNA extracted directly from small amounts of human articular cartilage. An RNA competitor template, which differed from the amplified target sequence only by the central insertion of an *EcoRI* restriction site, was used to account for differences between

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1 β , interleukin 1 β ; MMLV, Moloney Murine Leukaemia Virus; RT–PCR, reverse transcriptase–PCR; TNF α , tumour necrosis factor α .

* To whom correspondence should be addressed.

samples in the efficiency of both reverse transcription and of the PCR itself. Competitor RNA molecules were constructed for aggrecan, link protein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were used to quantify the mRNA levels for these gene products in human articular cartilage of differing ages.

MATERIALS AND METHODS

RNA extraction directly from human articular cartilage

Human articular cartilage was obtained from mid-thigh and hindquarter amputations at the time of surgery and was macroscopically normal. Full-thickness cartilage was removed from the total joint surface and at least three random pieces of approx. 100 mg were transferred to Eppendorf tubes containing 500 μ l of Solution D [4 M guanidinium isothiocyanate/25 mM sodium citrate/0.5% (w/v) *N*-lauroylsarcosine (sarcosyl)/0.1 M 2-mercaptoethanol]. The tubes were left on ice for at least 5 min and then stored at -70°C unless used immediately for RNA extraction. The cartilage pieces in Solution D were transferred to a small Petri dish, finely chopped and transferred back to the same Eppendorf tube. Samples were vortex-mixed and the Solution D (approx. 400 μ l) was removed from the tissue debris to a fresh tube. The tissue debris was extracted with a further 100 μ l of Solution D and this was pooled with the original extract to give a final volume of 500 μ l. Total RNA was isolated by the acid/phenol/chloroform method [19] and the final RNA pellet was dissolved in diethyl pyrocarbonate-treated water (1 μ l per mg wet weight of original tissue). RNA was stored in aliquots at -70°C before use.

RT-PCR

Aliquots of RNA (10 μ l) and competitor RNA templates (5 μ l) were heated to 65°C for 5 min and cooled on ice. Reverse transcription was performed at 37°C for 1 h in 50 mM Tris/HCl, pH 8.3, containing 75 mM KCl, 10 mM dithiothreitol, 0.5 mM dNTPs (Pharmacia, St. Albans, Herts., U.K.) and 2.7 nM specific reverse primers (92/B, 92/H and 95/B; see Table 1) with 8 units

of RNAGuard (Pharmacia) and 400 units of Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.) in a total volume of 30 μ l. The resulting cDNA was heated to 95°C for 5 min, cooled on ice and 9 μ l used for PCR with 10 mM Tris/HCl, pH 8.3, containing 50 mM KCl, 2.5 mM MgCl_2 , 0.25 mM dNTPs, 1.6 μ M each of forward and reverse primer, 5 μ g of poly(inosinic acid) and 1 unit of AmpliTaq DNA polymerase (Applied Biosystems, Warrington, Cheshire, U.K.) in a total volume of 25 μ l. After an initial denaturation step at 94°C for 1 min, amplification was performed at 92°C for 40 s, 56°C for 45 s and 72°C for 30 s for 30 cycles, with a final 5 min extension step at 72°C . An Omnigene thermal cycler (Hybaid, Teddington, Middlesex, U.K.) was used for all experiments. Reagents for reverse transcription and PCR were always prepared as a single reaction mix and then sampled into separate tubes. Recommended precautions were taken to reduce the risk of contamination [20] and negative controls (no added reverse transcriptase) were conducted in all experiments.

RT-PCR using *rTth* DNA polymerase

Use of the Perkin Elmer EZ *rTth* RNA PCR kit enables RT-PCR to be performed in a single reaction tube using the same thermostable DNA polymerase and buffer conditions for both reactions. Reactions were set up individually for each mRNA of interest in a total volume of 25 μ l containing 3 μ l of total cartilage RNA and 5 μ l of the appropriate competitor RNA in 50 mM Bicine/115 mM potassium acetate/8% (w/v) glycerol (pH 8.2)/2.5 mM manganese acetate/0.30 mM dNTPs, with 1.6 μ M each of forward and reverse primer and 8 units of RNAGuard and 2.5 units of *rTth* DNA polymerase. Reverse transcription was performed at 60°C for 30 min, followed by an initial denaturation step at 95°C for 2 min. Amplification was performed with a two-step protocol at 93°C for 1 min and 60°C for 1 min for 30 cycles, with a final 7 min extension step at 60°C . Negative controls were conducted by keeping reactions containing all components on ice during the reverse transcription step and placing them in the thermal cycler 1 min before the initial denaturation step.

Table 1 Primers used for measurement of gene expression and for the synthesis of competitor sequences

Nucleotide positions are from published sequences for aggrecan [21], link protein [24] and GAPDH [25]. The primer names are shown in parentheses after the sequence. Final PCR product sizes for the wild-type sequences are 213 bp for aggrecan (primers 95/A and 95/B), 177 bp for link protein (primers 92/A and 92/B) and 150 bp for GAPDH (primers 92/G and 92/H). The *EcoRI* site to be incorporated into competitor sequences is shown underlined in bold type.

Target protein	Sequence (5'–3') (Primer name)	Primer position
Forward primers (Primer A in Figure 1)		
Aggrecan	GGGTCAACAGTGCCTATCAG (95/A)	344–363
Link protein	CTCACTCTGGAAGATTATGGG (92/A)	385–405
GAPDH	GCATCTTCTTTGCGTCGCC (92/G)	31–50
Reverse primers (Primer b in Figure 1)		
Aggrecan	GGGTGTAGCGTGTAGAGATG (95/B)	537–556
Link protein	CACAGCATCCTGGTCCAG (92/B)	544–561
GAPDH	GTCATTGATGGCAACATATCC (92/H)	2033–2054
Forward primers for incorporation of <i>EcoRI</i> site (Primer C in Figure 1)		
Aggrecan	TGGGGT GAATTC CTACCGCTGCGAGGTGATG (95/C)	444–468
Link protein	TACAAG GAATTC GTGTGGTATTCCTTACTTTC (94/F)	467–493
GAPDH	AACGGA GAATTC TTTGGTCTATTGGGCGCC (94/D)	(340–344) + (1979–1998)
Reverse primers for incorporation of <i>EcoRI</i> site (Primer d in Figure 1)		
Aggrecan	CGGTAG GAATTC ACCCAGAGTCATTGGAGCG (95/D)	430–455
Link protein	CCACAG GAATTC CTTGAAGTCCAGTGCTACC (94/G)	453–478
GAPDH	ACCAAA GAATTC CCGTTGACTCCGACCTTC (94/E)	(327–340) + (1979–1985)

Figure 1 Schematic representation of the synthesis of competitor sequences for use in competitive RT-PCR

The scheme is designed to illustrate how design of the primers C and d (see Table 1) results in the annealing of 18 base pairs for overlap extension. The sequence defined by the letters A–P represents the central portion of the sequence to be amplified by primers A and b, the centre itself being between bases H and I. Capital letters indicate sense sequence and lower-case letters indicate anti-sense sequence. The chequered area indicates the *EcoRI* sequence to be incorporated. The positions of annealing of primers A, b, C and d are shown but only primers A and C (sense primers) will anneal to the anti-sense cDNA. Primer d will bind to the sense strand synthesized from primer A during the first cycle of PCR and primer b will anneal to the sense strand synthesized from primer C during the first cycle of PCR.

Analysis of PCR products

To 10 μ l of PCR products were added 1 μ l of 10 $\times$ React buffer 2 (500 mM Tris/HCl, pH 8.0, containing 100 mM MgCl₂ and 500 mM NaCl) and 5 units of *EcoRI* (Gibco BRL) and digestion at 37 °C was performed for 3 h. Restricted products were subjected to electrophoresis in 1 $\times$ TBE buffer (89 mM Tris/HCl, pH 8.30, containing 89 mM boric acid and 2 mM EDTA) at room temperature at 100 V on a 3% (w/v) NuSieve agarose gel (FMC BioProducts, Cambridge, U.K.) and detected by ethidium bromide fluorescence. Quantification of bands was performed either by densitometry (volume analysis) of a Polaroid negative of the gel with a Model GS-670 Scanning Densitometer (Bio-Rad, Hemel Hempstead, Herts., U.K.) or by the direct measurement of ethidium bromide fluorescence with the Bio-Rad Gel Doc 1000 system. The choice of instrument for analysis was not critical as both systems have a similar linear range of detection, but the latter instrument was preferable because saturation of band intensity is automatically detected as the gel is analysed. Quantitative analysis was performed with Molecular Analyst

software (Bio-Rad) and background correction made for each band individually by using an area next to each band.

Design of oligonucleotides

Oligonucleotide sequences and PCR product sizes used for quantification of gene expression are shown in Table 1. Primers for aggrecan were designed in the G1-domain-coding region [21] spanning intron 3 between the immunoglobulin fold domain (exon 3) and the first B loop domain (exon 4) as published for the human gene structure [22]. For link protein, one primer was designed in the immunoglobulin fold domain and the other in the first proteoglycan tandem repeat domain, thereby spanning an intron [23,24]. Primers for GAPDH were designed in exon 1 and exon 3, thereby spanning two known introns [25]. The PCR product sizes are 213, 177 and 150 base pairs for aggrecan, link protein and GAPDH respectively. The additional oligonucleotides required for the synthesis of competitor sequences are also shown in Table 1.

Synthesis of competitor RNA templates

Chondrocytes were isolated from 19-year-old human articular cartilage [26] and total RNA was extracted by the acid/phenol/chloroform method [19]. Total RNA (100 ng) was reverse-transcribed and competitor DNA templates were synthesized from the cDNA by overlap extension [27–29]. Two separate PCRs were performed, one with primers A and d and the other with primers b and C (Figure 1 and Table 1) with the parameters as described above. The two PCR products were diluted 100-fold, mixed 1:1, and used in a fresh PCR for 30 cycles. Two cycles of amplification were performed before the addition of primers A and b to encourage overlap extension to occur in preference to amplification. The final PCR product differed from the amplified target sequence only by the central insertion of an *EcoRI* restriction site. This competitor PCR product was gel-purified with the SpinBind kit (FMC BioProducts) and cloned by T/A overlap ligation in pBlueScript KS vector (Stratagene) that had been previously restricted with *SmaI* for 3'-deoxythymidine addition by a standard protocol [30]. The orientation of the insert was determined by restriction analysis and sequencing. Clones were chosen, from which sense RNA was transcribed *in vitro* with T7 RNA polymerase (Stratagene) from plasmid linearized with *XhoI*. Plasmid DNA was removed by the addition of RNase-free DNase (Pharmacia) and incubation at 37 °C for 30 min. The final RNA product was purified by acid/phenol/chloroform extraction, and precipitated three times to ensure complete removal of free nucleotides. The competitor RNA was then quantified by spectrophotometry followed by dilution with diethyl pyrocarbonate-treated water containing 2.5 μ g/ μ l poly(inosinic acid) (Pharmacia) as carrier and storage as aliquots at –70 °C. For use, aliquots of 5 μ g/ μ l were diluted to appropriate concentrations such that the final concentration of poly(inosinic acid) in the reverse transcription reaction was 0.016 μ g/ μ l. Amounts of competitor RNA species were converted to copy numbers by using the known molecular masses. Competitor RNA molecules were named according to the size of their respective PCR product: Agg219 for aggrecan, link183 for link protein and GAPDH156 for GAPDH. The sizes of the RNA competitor molecules were 321, 283 and 258 bases for aggrecan, link protein and GAPDH respectively. In addition, a synthetic RNA for GAPDH with no *EcoRI* site (GAPDH150) was prepared in the same way as the competitor RNA species but

without the initial overlap extension. This was used for competitive RT-PCR studies with GAPDH156 competitor RNA to validate the methodology.

RESULTS

Identification of heteroduplex formation after RT-PCR of target and competitor RNA

Co-amplification of target and competitor sequences consistently resulted in the presence of one or two additional PCR products that migrated more slowly than the expected PCR products of correct size during gel electrophoresis. They were thought to be heteroduplex molecules formed from the annealing of a target sequence with its complementary competitor sequence. The non-base-paired *Eco*RI sequence in the heteroduplex can form a hairpin loop resulting in a secondary structure that delays the migration of the heteroduplex relative to the homoduplex bands. It was demonstrated that these products were heteroduplex molecules by using GAPDH150 and GAPDH156 synthetic RNA (Figure 2, upper panel). The products were formed only when GAPDH150 and GAPDH156 sequences were amplified together and not when each of them was amplified alone (Figure 2, upper panel, lanes 1–3). When the PCR products for GAPDH150 and GAPDH156 (amplified separately) were mixed, heated to 95 °C for 5 min and allowed to cool slowly to room temperature, two heteroduplex bands formed (Figure 2, upper panel, lane 4) and these could not be restricted with *Eco*RI (Figure 2, upper panel, lane 5). The bands were, however, susceptible to digestion by S1 nuclease [31] as shown by the shift in their electrophoretic mobility towards that of the homoduplex target band (Figure 2,

upper panel, lane 7). This observation was consistent with removal of the hairpin loop in the heteroduplex molecule by the S1 nuclease, resulting in a product that migrated closer to the target PCR product. The formation of two distinct heteroduplex species is thought to be due to the presence of a complementary base pair (A-T) either side of the *Eco*RI restriction site incorporated into the GAPDH sequence. These two bases might base-pair either as part of the *Eco*RI hairpin structure or they might contribute to the reannealing of the target and competitor sequence (Figure 2, lower panel). This prediction also explains why there is only a single heteroduplex formed after competitive RT-PCR for aggrecan and link protein because the competitor RNA species for these two target genes do not have a complementary base pair either side of the *Eco*RI restriction site.

For all quantitative studies the densitometric values were obtained for the heteroduplex band(s) and were used to correct the values obtained for target and competitor bands. This was done by adding half of the densitometric value obtained for the heteroduplexes to each of the target and competitor values, because the heteroduplex by definition is made up of equal numbers of target and competitor strands. The final densitometric values obtained are referred to as the corrected volumes for the target and competitor PCR products. Although correction for the heteroduplex will not affect the ratio of target to competitor when their concentrations are equal, it will substantially alter any other target-to-competitor ratio.

Validation of the competitive RT-PCR procedure

Accurate quantification of target mRNA molecules by competitive RT-PCR requires the fulfilment of certain criteria to validate the method. The theoretical basis of competitive RT-PCR requires that both target and competitor sequences are amplified with equal efficiency. This means that the ratio of target to competitor molecules must remain constant throughout the cycles during which measurements are made. To test this requirement experimentally, equal quantities (1.44×10^7 copies) of GAPDH150 RNA (target sequence) and GAPDH156 RNA (competitor sequence) were subjected to RT-PCR for increasing numbers of cycles (Figure 3A). The ratio of PCR products for GAPDH150 to GAPDH156 remained constant throughout cycles 25 to 35 (1.23 ± 0.14 ; (mean $\pm$ S.D.; $n = 11$) (Figure 3B). The apparent higher corrected volume ratios at cycles 23 and 24 resulted from difficulty in measurement of the low-intensity bands. The numerical values for the efficiency of amplification (R) as described by the equation $Y = A(1 + R)^n$ (where Y is the yield of PCR product, A the initial number of substrate molecules and n the cycle number) [32] can be obtained from the slope of the plot of $\log Y$ against n [slope = $\log(1 + R)$] between two consecutive cycles. These calculated values gradually decreased from a maximum of 0.6 to 0 between cycles 24 and 35 (the maximum possible value for R is 1, which would indicate a 2-fold increase in product at each cycle) and indicated that the reaction had already started to plateau before detection of the PCR products by ethidium bromide fluorescence. This observation is consistent with the appearance of the heteroduplex molecules. For the competitive PCR studies described here, measurements can be made at any cycle number because the ratio of target to competitor product remains constant even as the plateau phase progresses. An efficiency curve was also performed at an initial ratio of GAPDH150 RNA to GAPDH156 RNA of 2:1 and showed that this ratio did not change throughout cycles 24–35 (2.20 ± 0.24 ; mean $\pm$ S.D.; $n = 12$; results not shown).

Competitive RT-PCR analysis usually involves titration of the amount of competitor with a constant amount of sample RNA.

Figure 2 Identification of heteroduplex molecules formed after competitive RT-PCR of GAPDH150 and GAPDH156 sequences

Upper panel: GAPDH150 synthetic RNA and GAPDH156 competitor RNA (1.44×10^7 copies of each) were subjected to RT-PCR either alone or in the same reaction. The heteroduplex molecules are labelled H1 and H2. The positions of the target band (GAPDH150; 150 base pairs) and the competitor band (GAPDH156 restricted with *Eco*RI; 78 base pairs) are denoted by T and C respectively. Lane 1, GAPDH150 + GAPDH156 PCR products restricted with *Eco*RI; lane 2, GAPDH150 PCR product (non-restricted); lane 3, GAPDH156 PCR product (non-restricted); lane 4, PCR products shown in lanes 2 and 3 were mixed, heated to 95 °C for 5 min and cooled slowly to room temperature; lane 5, products from lane 4 restricted with *Eco*RI; lane 6, products from lane 4 subjected to digestion conditions for S1 nuclease but without addition of the enzyme; lane 7, products from lane 4 digested with S1 nuclease; lane 8, *Pst*I digest of lambda DNA. Lower panel: two possible forms of heteroduplex for GAPDH due to the complementary A-T base pair either side of the *Eco*RI restriction site.

Figure 3 Efficiency curves for the co-amplification of GAPDH150 and GAPDH156 sequences

GAPDH150 synthetic RNA and GAPDH156 competitor RNA were used together in a 150 μ l reverse transcription reaction. For PCR, a master mix of all reaction components was added to the cDNA and 25 μ l aliquots were removed to separate tubes. All samples were subjected to the usual PCR parameters but one was removed from the thermocycler after the extension step of cycle 23 and after each subsequent cycle until the end of the extension step of cycle 35. (A) Ethidium bromide-stained agarose gel of RT-PCR products with GAPDH150 synthetic RNA and GAPDH156 competitor RNA at an initial ratio of 1:1 (7.2×10^7 copies of each per 150 μ l of reverse transcription reaction). H1, H2, T and C denote heteroduplex, target and competitor bands as described in Figure 2. (B) Densitometric analysis of the results shown in (A); the ratio of corrected volumes for GAPDH150 (■) to GAPDH156 (□) is shown in the inset.

A plot of the log of the ratio of target to competitor product against the log of the competitor concentration should give a straight line with a slope of -1 [17,18]. The amount of competitor used at which the ratio of target to competitor PCR products is equal to unity (log ratio = 0; the equivalence point) can be used to calculate the initial number of molecules of target sequence. Such studies were performed with 1.44×10^7 copies of GAPDH150 RNA titrated with GAPDH156 RNA in the range 3.6×10^6 to 5.76×10^7 copies (Figure 4A). A plot of the log of the ratio of the densitometric values for GAPDH150 to GAPDH156 PCR products against the log of the number of copies of GAPDH156 RNA added was linear with a slope of -0.93 ± 0.13 (mean $\pm$ S.D.; $n = 3$) (Figure 4B). The ratio of the corrected volumes for GAPDH150 to GAPDH156 was 1:1 (log ratio = 0) at a value of $(1.55 \pm 0.138) \times 10^7$ copies of GAPDH156 competitor RNA originally added (mean $\pm$ S.D.; $n = 3$).

Figure 4 Competitive RT-PCR of GAPDH150 synthetic RNA against GAPDH156 competitor RNA

A constant amount of GAPDH150 synthetic RNA (1.44×10^7 copies) was titrated with 2-fold serial dilutions of GAPDH156 competitor RNA (3.6×10^6 to 5.76×10^7 copies) in five separate 30 μ l reverse transcription reactions. All samples were subjected to RT-PCR and *Eco*RI restriction as described. (A) Ethidium bromide-stained agarose gel showing the characteristic pattern of competitive RT-PCR products. M denotes the marker lane (lambda DNA restricted with *Pst*I); H1, H2, T and C denote heteroduplex, target and competitor bands as described in Figure 2. (B) Densitometric results from three experiments plotted as the log of the ratio of the corrected densitometric volumes for target to competitor PCR products (GAPDH150 to GAPDH156; mean $\pm$ S.D.; $n = 3$) against the log of copies of GAPDH156 competitor RNA used.

Comparison of Northern hybridization and competitive RT-PCR to measure aggrecan, link-protein and GAPDH mRNA in cultured human chondrocytes

Extraction of total RNA from human chondrocytes grown in monolayer culture yields sufficient amounts of pure RNA for analysis of gene expression by Northern hybridization. Inhibition of aggrecan and link-protein mRNA expression by interleukin 1 (IL-1) or tumour necrosis factor (TNF) in monolayer cultures of freshly isolated human chondrocytes was therefore used as a system in which to quantify changes in gene expression by competitive RT-PCR for comparison with Northern hybridization results. Total RNA was extracted from the chondrocytes after 24 h of treatment with either IL-1 β or TNF α in serum-free Dulbecco's modified Eagle's medium. Dilutions (10-fold) of competitor RNA species (Agg219, Link183 or GAPDH156) were initially titrated with 5 ng of total RNA to estimate the number of copies of each specific mRNA present (results not shown). For precise quantification, seven serial dilutions (2-fold) of competitor RNA species were used that spanned the estimated

Table 2 Comparison of specific mRNA expression by chondrocyte cultures measured by competitive RT-PCR and Northern hybridization

Total RNA was extracted from chondrocytes (isolated from a 16-year-old specimen) in monolayer culture after a 24 h treatment with or without IL-1 β (1 ng/ml) or TNF α (10 ng/ml). Competitive RT-PCR titrations were performed with serial 2-fold dilutions of Agg219, Link183 and GAPDH156 competitor RNA species and the final bands obtained after RT-PCR and *EcoRI* restriction quantified by densitometry of a Polaroid negative of the ethidium bromide-stained gel. The log of the ratio of the corrected densitometric values of target to competitor PCR products was plotted against the log of the number of copies of competitor RNA originally added. These plots were linear with the correlation coefficients (r^2) shown. Values for the range of competitor RNA used represent 10⁻³ copies of competitor RNA per 30 μ l of reverse transcription reaction (containing 5 ng of chondrocyte total RNA) and were used in 2-fold increments. The equivalence point (EP) for each mRNA is the number of copies of competitor RNA required at which the ratio of target PCR product to competitor PCR product is unity. The absolute copy numbers for aggrecan and link protein were normalized relative to the number of copies of GAPDH mRNA before calculating the percentage inhibition. For Northern hybridization (Figure 5), percentage inhibitions were calculated from the arbitrary ratios obtained for the band intensities of aggrecan or link protein relative to GAPDH and are shown in parentheses for comparison with the RT-PCR values.

Treatment	Target mRNA	Range of competitor RNA used	Gradient (r^2)	EP (10 ⁻³ copies per 5 ng of total RNA)	Copies per copy of GAPDH mRNA	% Inhibition of control (cf. Northern analysis)
Control	Aggrecan	7.25–464	–0.97 (0.98)	48.79	0.0184	–
IL1 β	Aggrecan	7.25–464	–0.92 (0.99)	9.04	0.0058	68 (72)
TNF α	Aggrecan	7.25–464	–0.95 (0.97)	15.06	0.0060	67(51)
Control	Link protein	8.15–522	–0.97 (0.95)	54.81	0.0221	–
IL1 β	Link protein	8.15–522	–1.05 (0.99)	27.10	0.0173	22 (22)
TNF α	Link protein	8.15–522	–1.00 (0.97)	30.12	0.0136	38 (20)
Control	GAPDH	360–23040	–0.84 (0.98)	2599	–	–
IL1 β	GAPDH	360–23040	–0.83 (0.99)	1539	–	–
TNF α	GAPDH	360–23040	–0.92 (0.99)	2291	–	–

Figure 5 Northern hybridization for aggrecan, link protein and GAPDH with RNA extracted from isolated chondrocytes

Chondrocytes were extracted from 16-year-old human femoral condyle cartilage as described previously [26] and cultured for 24 h in serum-free Dulbecco's modified Eagle's medium containing Glutamax I (Gibco BRL; catalogue number 31966-021) and supplemented with 1% (v/v) non-essential amino acids and antibiotics (100 i.u. of penicillin and 100 mg of streptomycin per ml of medium). The cells were incubated for a further 24 h with or without IL-1 β (1 ng/ml) or TNF α (10 ng/ml) and total RNA was extracted by the acid/phenol/chloroform method [19]. Aliquots of 5 μ g of total RNA per lane were subjected to electrophoresis through 1.2% (w/v) agarose gels with 2.2 M formaldehyde and transferred to nylon membrane (Hybond N, Amersham). Blots were sequentially hybridized to anti-sense riboprobes for link protein, aggrecan and GAPDH generated by transcription *in vitro* with [α -³²P]UTP from the plasmids containing the cloned PCR products Link183, Agg219 and GAPDH150 respectively. Post-hybridization washes were under stringent conditions (0.05 $\times$ SSC at 80 $^{\circ}$ C for link protein, 0.05 $\times$ SSC at 75 $^{\circ}$ C for aggrecan and 0.1 $\times$ SSC at 65 $^{\circ}$ C for GAPDH) and were followed by exposure to Hyperfilm MP (Amersham) for various times up to 24 h for quantification within the linear range of the film by densitometry. Exposure times for blots shown were 23 h for link protein, 14 h for aggrecan and 7.5 h for GAPDH. Lane 1, control; lane 2: IL-1 β ; lane 3: TNF α . Sizes in kb of the marker RNA ladder (Gibco BRL) are shown at the left of the blots.

equivalence points for each mRNA of interest. The results for these competitive RT-PCR analyses were quantified as described for Figure 4 and the final results are shown in Table 2. To quantify the inhibition of aggrecan and link-protein mRNA

expression by IL-1 β or TNF α , the copy numbers for these mRNA species were normalized per copy of GAPDH mRNA (see Table 2). Similarly, the band intensities for individual mRNA species obtained from the Northern analysis (Figure 5) were normalized relative to the values for GAPDH, before calculating the percentage inhibition of mRNA expression induced by each cytokine (Table 2). IL-1 β inhibited the expression of aggrecan mRNA and link-protein mRNA by 70% and 22% respectively, when determined by either competitive RT-PCR or Northern hybridization. When these two methods of analysis were compared by using TNF α -treated cells the results were more variable, but in both cases the expression of aggrecan mRNA was inhibited to a greater extent than that of link-protein mRNA.

Competitive RT-PCR to measure aggrecan, link-protein and GAPDH mRNA in intact human cartilage

Competitive RT-PCR assays were performed with RNA isolated directly from femoral condyle cartilage of individuals aged 16 and 35 years. Preliminary titrations with 4-fold dilutions of competitor RNA were performed to estimate the equivalence points (results not shown). Dilutions (2-fold) spanning the estimated equivalence point were then used for more accurate quantification (Figure 6). Plots of the log of the ratio of the target to competitor PCR products against the log of the copies of competitor RNA used had gradients close to -1 (Table 3). The equivalence point was calculated for each mRNA from the graphs and the results are shown in Table 3. For normalization of the results, values for aggrecan and link protein were expressed relative to GAPDH mRNA levels (Table 3). The ratio of aggrecan mRNA to GAPDH mRNA was 2.4 times higher in the 16-year-old than in the 35-year-old cartilage. The link-protein mRNA-to-GAPDH mRNA ratio was 5.8 times higher in the 16-year-old than in the 35-year-old cartilage. Thus mRNA for both aggrecan and link protein decreased with age, but link protein decreased to a greater extent than aggrecan. The stoichiometry of link-protein mRNA to aggrecan mRNA was 3.15 for the 16-year-old and 1.28 for the 35-year-old. To further investigate changes in aggrecan and link-protein mRNA with ageing, a larger number

Figure 6 Competitive RT-PCR for aggrecan, link protein and GAPDH with RNA isolated directly from human articular cartilage

Total RNA was isolated directly from human articular cartilage (femoral condyle from 16-year-old and 35-year-old specimens) and 10 μ l (equivalent to 10 mg of tissue) used per reverse transcription reaction for competitive RT-PCR as described. Five serial dilutions (2-fold) of competitor RNA were used for each sample. Lane 1 indicates the highest amount of competitor RNA used and lane 5 the lowest amount. The range of copies of competitor RNA used for each age and mRNA are shown in Table 3. Negative controls in which the reverse transcriptase was omitted from the reverse transcription reaction are shown in Lane 6. M denotes the marker lane (Lambda DNA restricted with *Pst*I).

Table 3 Competitive RT-PCR with RNA extracted directly from human articular cartilage

Competitive RT-PCR titrations were performed as shown in Figure 6 and the PCR products quantified by densitometry of a Polaroid negative of the ethidium bromide-stained gel. The log of the ratio of the corrected densitometric values of target to competitor PCR products was plotted against the log of the number of copies of competitor RNA originally added. These plots were linear with the correlation coefficients (r^2) shown. Values for the range of competitor RNA used represent 10^{-3} copies of competitor RNA per 30 μ l of reverse transcription reaction and were used in 2-fold increments. The equivalence point (EP) for each mRNA is the number of copies of competitor RNA required at which the ratio of target PCR product to competitor PCR product was unity.

Age (years)	Target mRNA	Range of competitor RNA	Gradient	r^2	EP	Copies per copy of GAPDH mRNA
16	Aggrecan	43.5–696	–0.87	0.91	196	0.26
16	Link Protein	163–2608	–0.95	0.99	619	0.81
16	GAPDH	180–2880	–0.92	0.99	761	–
35	Aggrecan	9.0–144	–0.97	0.77	69	0.11
35	Link protein	12.2–195.2	–1.10	0.99	89	0.14
35	GAPDH	90–1440	–1.09	0.99	655	–

of samples were analysed, but there was considerable variation when triplicate samples from the same total RNA preparation were measured, especially for aggrecan. It was considered that differences in the efficiency of reverse transcription between target mRNA and competitor RNA, due to the formation of secondary structures within the much larger target mRNA species, could explain these results. This possibility was therefore tested by using a thermostable DNA polymerase (*rTth* polymerase) that also has reverse transcriptase activity, allowing this reaction to be performed at an elevated temperature to reduce the formation of secondary structures.

RNA extracted from the 16-year-old and 35-year-old specimens used for the experiment shown in Figure 6 were reanalysed with *rTth* DNA polymerase and the EZ buffer system. The calculated equivalence points for the 16-year-old were 5.1×10^6 , 2.6×10^6 and 9.5×10^5 copies per 10 μ l of total RNA and for the 35-year-old they were 1.8×10^6 , 2.8×10^5 and 8.1×10^5 copies per 10 μ l of total RNA for aggrecan, link protein and GAPDH respectively. The equivalence point for aggrecan was therefore approx. 26 times higher than that obtained with the MMLV reverse transcriptase (compare with results in Table 3), confirming that use of the latter enzyme resulted in inefficient reverse transcription of aggrecan mRNA. The equivalence points for link protein and GAPDH were increased by approx. 3.5-fold and 1.2-fold respectively.

Age-related changes in aggrecan and link-protein mRNA in human articular cartilage measured with *rTth* DNA polymerase for RT-PCR

Total RNA was extracted from 22 samples of human articular cartilage of different ages and subjected to RT-PCR with the EZ *rTth* RNA PCR kit. Individual reactions were performed for aggrecan, link protein and GAPDH in triplicate with a single concentration of the appropriate competitor RNA: 700000 copies of Agg219 RNA, 250000 copies of Link183 RNA or 280000 copies of GAPDH156 RNA per 3 μ l of cartilage total RNA. These were selected empirically by using the results obtained from the analysis of the 16-year-old and 35-year-old total RNA and other preliminary experiments (results not shown). The mean coefficients of variation of the 22 individual specimens analysed were $8 \pm 5\%$, $9 \pm 5\%$ and $9 \pm 7\%$ (means $\pm$ S.D.; $n = 22$) for aggrecan, link protein and GAPDH respectively. The age-related changes measured with the *rTth* DNA polymerase for aggrecan, link protein and GAPDH are shown in Figure 7. There was no clear age-related pattern for expression of aggrecan mRNA (Figure 7A). There was, however, a decrease in the level of link-protein mRNA with increasing age (Figure 7B). The ratio of aggrecan mRNA to link-protein mRNA ranged from 1 to 3 in immature cartilage and from 3 to 10 after 19 years of age (Figure 7C).

Figure 7 Age-related changes of aggrecan and link-protein mRNA in human articular cartilage

Total RNA was isolated directly from cartilage of different ages and 3 μ l (equivalent to 3 mg of tissue) used for RT-PCR (in triplicate), using only a single copy number of each competitor RNA (7×10^5 copies of Agg219, 2.5×10^5 copies of Link183 and 2.8×10^5 copies of GAPDH156). Final band intensities were measured with the Bio-Rad Gel Doc 1000 system and Molecular Analyst software, and the ratio of corrected volumes for target to competitor PCR products was used to calculate the amount of each mRNA per 3 μ l sample RNA. (A) Relative levels of aggrecan mRNA to GAPDH mRNA; (B) relative levels of link-protein mRNA to GAPDH mRNA; (C) stoichiometry of aggrecan mRNA to link-protein mRNA.

DISCUSSION

Human articular cartilage has a very low cell content and the amount available for analysis is often limited, especially from

diseased joints. These are compelling reasons for using RT-PCR to provide a sensitive measure of the expression of specific mRNA species. Careful consideration must, however, be given to the design of an RT-PCR assay if it is to be used for quantitative purposes. Some quantitative PCR procedures require products to be measured exclusively during the logarithmic phase of the reaction before the plateau phase is reached. For example, this is a mandatory condition when the target gene of interest and an endogenous, constitutively expressed gene are amplified in the same tube and quantified relative to each other. However, if one sequence is amplified with a higher efficiency and is present at a higher initial concentration, it could reach the plateau phase of the reaction before the other sequence has been amplified to detectable levels [33]. Some workers have circumvented this problem by delaying the addition of primers for more abundant mRNA species until a later cycle in the PCR [34,35], but such an approach is very laborious when multiple samples are analysed. Another approach has been to change the concentrations of individual primer pairs to adjust amplification efficiencies so that the logarithmic phase occurs simultaneously for each sequence [36]. Such optimization procedures can also be very labour-intensive and may have to be performed each time a different sample is analysed. A further limitation of this co-amplification procedure is that changes in amplification efficiencies between different samples may not be proportionate for PCR products amplified with different primer pairs. We have therefore selected competitive RT-PCR as the method for quantifying the amount of a specific mRNA in human articular cartilage. Although DNA competitor molecules were used in the original assay described by Gilliland et al. [37], we have prepared competitor RNA molecules that are added to the sample RNA before cDNA synthesis to account for changes in the efficiency of both the reverse transcription and the PCR [38]. One problem with this approach is that it must be assumed that during reverse transcription the shorter RNA competitor templates behave similarly to the much larger mRNA molecules. During the development of this assay, we have shown that this assumption is not necessarily true because reverse transcription at elevated temperature using *rTth* DNA polymerase (a thermostable enzyme that also has reverse transcriptase activity) reverse-transcribed aggrecan mRNA 26 times more efficiently than did MMLV reverse transcriptase. This observation showed that secondary structure formation within the large mRNA species can significantly affect the absolute values obtained for mRNA levels when measured by competitive RT-PCR and therefore reverse transcription at elevated temperature is highly recommended.

Competitor sequences can be synthesized so that they differ from the target sequence either by their size or by the presence (or absence) of a restriction site [37]. Because differences in product size have been shown to affect amplification efficiency [39], we chose to use a similar-sized competitor that could be distinguished from the target sequence, after PCR, by restriction digestion. The competitor sequence incorporated between the primers can either be almost identical with [37], or completely different from [38,40], the target sequence of interest. When sequences are different, quantification has to be performed during the logarithmic phase [41] because a reaction plateau caused by reannealing of PCR products would not necessarily occur at the same cycle number for target and competitor sequences alike. In contrast, when the target and competitor sequences are almost identical, complementary strands from target and competitor PCR products can anneal in all combinations (homoduplex and heteroduplex) and therefore they reach a plateau simultaneously. Thus target-to-competitor ratios remain constant throughout all

cycles of PCR. By designing a competitor that differed from the target sequence only by the insertion of an *EcoRI* restriction site, we were able to distinguish heteroduplex formation and quantify mRNA levels during the plateau phase of the reaction. This is an important feature of the method because the use of ethidium bromide fluorescence was not sensitive enough to detect PCR products during the logarithmic phase of the reaction. In addition, the central insertion of the *EcoRI* restriction site enabled the competitor PCR product to be cleaved into two equal-sized fragments, which improved the sensitivity of their detection. It is a theoretical requirement of a competitive RT-PCR assay of this kind that the plot generated from a competitor titration experiment should have a gradient of -1 [17]. Fulfilment of this criterion is considered to be a good indication that target and competitor have been amplified with equal efficiency [18]. In our experiments, known quantities of synthetic RNA (GAPDH150 RNA and GAPDH156 competitor RNA) were used to ensure not only that a gradient of -1 was achieved but also that amplification efficiencies of target and competitor sequences were the same regardless of the cycle number of the PCR.

The RNA extracted directly from small amounts of human articular cartilage is not of sufficient purity or yield for analysis by Northern hybridization. Treatment of human articular chondrocytes in monolayer culture with IL-1 β or TNF α was therefore used as a system in which to compare changes in mRNA levels measured by either competitive RT-PCR or Northern hybridization. The extent of inhibition of aggrecan and link-protein mRNA expression by these cytokines compared favourably when measured by either method of quantification. These results further validate the use of this competitive RT-PCR assay to quantify changes in mRNA expression and illustrate the advantages of this technique over Northern hybridization in that it provides information on absolute levels of specific mRNA species and requires less initial total RNA. The main advantage of this competitive RT-PCR assay, however, is that particular mRNA species can be quantified with RNA extracted directly from cartilage tissue. RT-PCR products have been detected previously for aggrecan and versican by using RNA extracted directly from small amounts of human articular cartilage [42], but quantification was not performed in this study. More recently, Re et al. [43] described a non-competitive RT-PCR procedure for the quantification of aggrecan and link-protein mRNA in bovine articular cartilage. DNA standard templates were prepared by cloning PCR products, derived from the tissue RNA, into a plasmid vector; dilutions of these standards were amplified to produce a standard curve. The tissue cDNA was amplified separately and quantified by reference to the standard curve. Separate amplification of target and standard is a major disadvantage of the method, because it is not possible to account for changes in the amplification efficiency of different RNA samples that may occur. One way of doing this is to include competitor molecules with the sample in the same tube in the form of a competitive RT-PCR assay. A quantitative analysis of competitive RT-PCR titrations, performed with RNA extracted directly from 16-year-old and 35-year-old femoral condyle cartilage, generated graphs with gradients of -1 [17,18]. It was also essential to relate levels of target mRNA to an endogenously expressed transcript, because both the purity and quantity of RNA isolated directly from cartilage were too low for spectrophotometric determination of RNA concentration. Amounts of aggrecan and link-protein mRNA were therefore normalized relative to the amount of GAPDH mRNA. Analysis of RNA from 22 individuals showed that the expression of link-protein mRNA relative to GAPDH mRNA was generally higher in

immature cartilage (9–20 years) than in mature cartilage, but no age-related pattern was observed for aggrecan mRNA.

Link protein and aggrecan are components of the proteoglycan aggregate formed when they interact with hyaluronan in the extracellular matrix of cartilage [2], and experiments with Swarm rat chondrosarcoma cells have suggested that these two proteins are assembled in an equimolar ratio [44]. It might therefore be expected that the expression of aggrecan and link protein would be co-ordinately regulated, but this was certainly not the case with respect to age-related changes in mRNA expression in human articular cartilage, indicating that they are not necessarily subject to the same regulatory mechanisms. These might include differences in the rates of transcription or in the stability of the mRNA. Whether or not these age-related changes in mRNA expression result in corresponding changes in the level of newly synthesized protein still has to be determined, but it is interesting to speculate that they may be responsible, in part, for the age-related increase in the molar ratio of aggrecan to link protein which has been measured in extracts of human cartilage [3]. These findings suggest that the molecular stoichiometry of aggregates in human articular cartilage is changing during maturation and aging of the tissue and they also predict that this will compromise the stability of the complex. Recent studies provide additional support for this hypothesis by confirming that there is a higher proportion of link-protein-deficient, less stable aggregates in mature cartilage [45,46]. Many of these age-related changes are usually attributed to proteolytic events occurring in the extracellular matrix. However, to achieve a comprehensive evaluation of the mechanisms that regulate the turnover of matrix components it is necessary to measure gene expression at both the transcriptional and the translational levels. The competitive RT-PCR assay described here provides a sensitive and quantitative method for assessing how the regulation of chondrocyte mRNA levels contribute to cartilage matrix homeostasis.

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REFERENCES

- Hardingham, T. E. and Fosang, A. J. (1992) *FASEB J.* **6**, 861–870
- Morgelin, M., Heinegard, D., Engel, J. and Paulsson, M. (1994) *Biophys. Chem.* **50**, 113–128
- Hardingham, T. and Bayliss, M. T. (1990) *Semin. Arthritis Rheum.* **20**, 12–33
- Buckwalter, J. A., Roughley, P. J. and Rosenberg, L. C. (1994) *Microsc. Res. Tech.* **28**, 398–408
- Mort, J. S., Poole, A. R. and Roughley, P. J. (1983) *Biochem. J.* **214**, 269–272
- Sandell, L. J. (1995) in *Extracellular Matrix: A Practical Approach* (Haralson, M. A. and Hassell, J. R., eds.), pp. 329–351, Oxford University Press, Oxford
- Stockwell, R. A. (1979) in *Biology of Cartilage Cells* (Harrison, R. J. and McMin, R. M. H., eds.), pp. 32–80, Cambridge University Press, Cambridge
- Benay, P. D. and Brown, P. D. (1986) in *Articular Cartilage Biochemistry* (Kuettnner, K. E., Schleyerbach, R. and Hascall, V. C., eds.), pp. 219–233, Raven Press, New York
- Hering, T. M., Kollar, J., Huynh, T. D., Varelas, J. B. and Sandell, L. J. (1994) *Arch. Biochem. Biophys.* **314**, 90–98
- Watt, F. M. and Dudhia, J. (1988) *Differentiation* **38**, 140–147
- Goldring, M. B., Sandell, L. J., Stephenson, M. L. and Krane, S. M. (1986) *J. Biol. Chem.* **261**, 9049–9055
- Curtis, A. J., Devenish, R. J. and Handley, C. J. (1992) *Biochem. J.* **288**, 721–726
- Woods, Jr., V. L., Schreck, P. J., Gesink, D. S., Pacheco, H. O., Amiel, D., Akeson, W. H. and Lotz, M. (1994) *Arthritis Rheum.* **37**, 537–544
- Salter, D. M., Hughes, D. E., Simpson, R. and Gardner, D. L. (1992) *Br. J. Rheumatol.* **31**, 231–234
- Adams, M. E., Huang, D. Q., Yao, L. Y. and Sandell, L. J. (1992) *Anal. Biochem.* **202**, 89–95
- Matyas, J. R., Adams, M. E., Huang, D. and Sandell, L. J. (1995) *Arthritis Rheum.* **38**, 420–425
- Raeymaekers, L. (1993) *Anal. Biochem.* **214**, 582–585

- 18 Raeymaekers, L. (1995) *Genome Res.* **5**, 91–94
- 19 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 20 Kwok, S. and Higuchi, R. (1989) *Nature (London)* **339**, 237–238
- 21 Doege, K. J., Sasaki, M., Kimura, T. and Yamada, Y. (1991) *J. Biol. Chem.* **266**, 894–902
- 22 Valhmu, W. B., Palmer, G. D., Rivers, P. A., Ebara, S., Cheng, J. F., Fischer, S. and Ratcliffe, A. (1995) *Biochem. J.* **309**, 535–542
- 23 Dudhia, J., Bayliss, M. T. and Hardingham, T. E. (1994) *Biochem. J.* **303**, 329–333
- 24 Dudhia, J. and Hardingham, T. E. (1990) *Nucleic Acids Res.* **18**, 1292
- 25 Ercolani, L., Florence, B., Denaro, M. and Alexander, M. (1988) *J. Biol. Chem.* **263**, 15335–15341
- 26 Palmer, R. M. J., Hickery, M. S., Charles, I. G., Moncada, S. and Bayliss, M. T. (1993) *Biochem. Biophys. Res. Commun.* **193**, 398–405
- 27 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) *Gene* **77**, 51–59
- 28 Higuchi, R., Krummel, B. and Saiki, R. K. (1988) *Nucleic Acids Res.* **16**, 7351–7367
- 29 de Kant, E., Rochlitz, C. F. and Herrmann, R. (1994) *Biotechniques* **17**, 934–942
- 30 Finney, M. (1994) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), pp. 15.7.1–15.7.6, John Wiley and Sons, New York
- 31 Tabor, S. and Struhl, K. (1989) in *Current Protocols in Molecular Biology* (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), pp. 3.12.1–3.12.6, John Wiley and Sons, New York
- 32 Chelly, J., Kaplan, J. C., Maire, P., Gautron, S. and Kahn, A. (1988) *Nature (London)* **333**, 858–860
- 33 Murphy, L. D., Herzog, C. E., Rudick, J. B., Fojo, A. T. and Bates, S. E. (1990) *Biochemistry* **29**, 10351–10356
- 34 Kinoshita, T., Imamura, J., Nagai, H. and Shimotohno, K. (1992) *Anal. Biochem.* **206**, 231–235
- 35 Wong, H., Anderson, W. D., Cheng, T. and Riabowol, K. T. (1994) *Anal. Biochem.* **223**, 251–258
- 36 Dukas, K., Sarfati, P., Vaysse, N. and Pradayrol, L. (1993) *Anal. Biochem.* **215**, 66–72
- 37 Gilliland, G., Perrin, S., Blanchard, K. and Bunn, H. F. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2725–2729
- 38 Wang, A. M., Doyle, M. V. and Mark, D. F. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9717–9721
- 39 Chelly, J., Montarras, D., Pinset, C., Berwald-Netter, Y., Kaplan, J. C. and Kahn, A. (1990) *Eur. J. Biochem.* **187**, 691–698
- 40 Siebert, P. D. and Larrick, J. W. (1992) *Nature (London)* **359**, 557–558
- 41 Pannetier, C., Delassus, S., Darche, S., Saucier, C. and Kourilsky, P. (1993) *Nucleic Acids Res.* **21**, 577–583
- 42 Grover, J. and Roughley, P. J. (1993) *Biochem. J.* **291**, 361–367
- 43 Re, P., Valhmu, W. B., Vostrejs, M., Howell, D. S., Fischer, S. G. and Ratcliffe, A. (1995) *Anal. Biochem.* **225**, 356–360
- 44 Kimura, J. H., Hardingham, T. E. and Hascall, V. C. (1980) *J. Biol. Chem.* **255**, 7134–7143
- 45 Wells, T., Morgelin, M., Davidson, C. and Bayliss, M. T. (1993) *Trans. Orthop. Res. Soc.* **18**, 640 (abstract)
- 46 Wells, T. (1994) Ph.D. Thesis, University of London