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# *In situ* hybridization reveals differential spatial distribution of mRNAs for type I and type II collagen in the chick limb bud

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## Summary

**During limb development, type I collagen disappears from the region where cartilage develops and synthesis of type II collagen, which is characteristic of cartilage, begins. *In situ* hybridization using antisense RNA probes was used to investigate the spatial localization of type I and type II collagen mRNAs. The distribution of the mRNA for type II collagen corresponded well with the pattern of type II collagen synthesis, suggesting control at the level of transcription and mRNA accumulation. In contrast, the pattern of mRNA for type I collagen remained more or less uniform and did not correspond with the synthesis of the protein, suggesting control primarily at the level of translation or of RNA processing.**

Key words: collagen, chick limb bud, *in situ* hybridization.

## Introduction

The early chick limb bud comprises a morphologically homogeneous population of mesenchymal cells within an ectodermal jacket. The cartilage elements develop from the cells at the core of the mesenchyme. As the wing bud grows out, a region at the tip called the progress zone maintains its undifferentiated character and has a high proliferative index (Hornbruch & Wolpert, 1970; Summerbell & Lewis, 1975). Proximally, the cells in the core undergo condensation and differentiate into cartilage. The cartilage elements are laid down in a proximodistal sequence: humerus, radius and ulna, wrist, hand. Type II collagen is one of the molecular components of cartilage whereas type I collagen is characteristic of fibroblasts. Transient expression of type I collagen and fibronectin, detected using fluorescent antibodies, has been associated with the condensation of mesenchyme that occurs at the beginning of cartilage condensation (Linsenmayer *et al.* 1973; Von der Mark *et al.* 1976*a*,*b*; Vertel & Dorfman, 1978; Von der Mark, 1980; Dessau *et al.* 1980). Levels of type I collagen then decrease, and there is an increase in levels of type II collagen in the core region as cells begin to differentiate into chondrocytes around stage 25. The temporal expression of the mRNAs encoding type I and type II collagen has been studied. Kravis & Upholt (1985) showed that after the time of condensation there is a continuous increase in the amount of mRNA for type II collagen both *in vivo* and in micromass cultures of limb bud mesenchyme. This has been confirmed for micromass cultures by Kosher *et al.* (1986) who also reported a low level of type II collagen mRNA in mesenchyme of limb buds from stages as early as 18/19, which is some 24 h earlier than the time of condensation. They also suggest that there is translational control of type I collagen since the significant levels of type I collagen mRNA present at early stages are maintained in 7-day limb cartilage, when type I collagen itself is barely detectable.

We have used *in situ* hybridization of specific RNA probes for collagen type I ($\alpha 2$) and type II ($\alpha 1$) mRNAs to determine their spatial and temporal distribution during chick limb development. We were particularly interested in the relative levels of collagen II mRNA in the progress zone and during the early stages of cartilage differentiation because we have shown that this zone has a high capacity for cartilage differentiation in micromass culture (Cottrill *et al.* 1987). In fact, the pattern of cartilage formation

may be viewed in terms of the inhibition of cartilage differentiation as the cells leave the progress zone, so that cartilage differentiation becomes restricted to the core of the bud (Solursh, 1984; Cottrill *et al.* 1987).

Here, we demonstrate that during early cartilage formation transcription and accumulation of $\alpha$1(II) collagen mRNA is initiated in regions where cartilage is developing. In contrast, levels of $\alpha$2(I) collagen mRNA remain relatively low throughout the limb bud at all stages of development examined and expression of protein may therefore be regulated at the level of translation or of RNA processing.

## Materials and methods

### *Tissue preparation*

Fertilized chicken eggs were obtained from Poyndon Farm, Waltham Cross, Herts, and incubated at 38 ± 1°C. The embryos were staged according to Hamburger & Hamilton (1951) and the limb buds dissected and fixed in fresh PBS containing 4% (w/v) paraformaldehyde. Following dehydration and light staining with eosin, the tissue was embedded in paraffin wax (melting point 56°C) and 7 $\mu$m sections were cut using an American Optical 820 rotary microtome. Glass slides were acid washed, coated with a 1 mg ml$^{-1}$ solution of poly-L-lysine (Sigma) and sections mounted on a droplet of water and left to adhere overnight at 37°C in a dust-free atmosphere.

### *Probe preparation*

Chicken collagen gene probes were gifts from Anna Maria Frischauf [pCg45; type I ($\alpha$2) cDNA clone; Lehrach *et al.* (1979)] and Linda Sandell [p1.7; type II ($\alpha$1) subgenomic clone; Sandell *et al.* (1984)]. Restriction fragments exhibiting low cross homology (Fig. 1) were isolated by gel electrophoresis and subcloned into the SP6/T7 RNA polymerase vector pGEM 1 (Promega Biotec). Plasmid templates were linearized and antisense (cRNA) or sense (mRNA) transcripts were synthesized by incubation of 1 $\mu$g linearized template DNA at 37°C for 2 h in 40 mM-Tris–HCl (pH 7·5) containing 2 mM-spermidine, 20 mM-dithiothreitol, 0·43 mM-UTP, 0·43 mM-ATP, 0·43 mM-GTP, 5 $\mu$M-CTP, 1 unit $\mu$l$^{-1}$ RNAse inhibitor (BCL Ltd), 70 $\mu$Ci $^{32}$P-CTP (NEN Ltd), 0·2 units $\mu$l$^{-1}$ SP6 or T7 RNA polymerase (BCL Ltd) and, for T7 RNA polymerase, 10 mM-sodium chloride. Template was digested by incubation with 1 $\mu$g RNase-free DNase (Miles) at 37°C for 10 min and unincorporated nucleotides were removed by gel filtration on Sephadex G-50 (medium). Labelled probe was concentrated by ethanol precipitation.

### In situ *hybridization*

The method used for hybridization was a modification of that described by Hoefler *et al.* (1986). Wax was removed from the slides by two washes in xylene and the sections were rehydrated. Following a 5 min wash in PBS (pH 7·2) the slides were immersed in 0·1 M-glycine/PBS for 5 min, then in 0·3% (v/v) Triton X-100/PBS for 15 min, then washed (two times 3 min) in PBS and incubated for 30 min at 37°C in 0·1 M-Tris–HCl (pH 8·0) containing 1 $\mu$g ml$^{-1}$ proteinase K and 50 mM-EDTA. Sections were fixed in 4% (w/v) paraformaldehyde/PBS for 5 min, washed (two times 3 min) in fresh PBS and immersed in 0·1 M-triethanolamine (pH 8·0) containing 0·25% (v/v) acetic anhydride for 10 min. Finally, the slides were prehybridized in 50% (v/v) formamide/2×SSC at 37°C for at least 15 min.

$^{32}$P-labelled probe ($10^8$ cts min$^{-1}$ ml$^{-1}$) was applied to each section in 10 $\mu$l hybridization buffer (250 mM-Tris–HCl (pH 7·6) containing 50% (v/v) formamide, 2×SSC, 0·5% (w/v) SDS, 10% (w/v) dextran sulphate, 0·25% (w/v) BSA, 0·25% (w/v) ficoll, 0·25% (w/v) polyvinylpyrollidone, 0·5% (w/v) sodium pyrophosphate and 250 $\mu$g ml$^{-1}$ denatured herring testes DNA). Sections were then covered with a siliconized, baked coverslip and incubated for 16–20 h in a humidified chamber at 43°C.

Coverslips were floated off in 4×SSC and excess probe was removed by three 20 min washes in 4×SSC, one 30 min wash in 10 mM-Tris–HCl (pH 8·0) containing 20 $\mu$g ml$^{-1}$ RNase A (Sigma), 0·5 M-NaCl and 1 mM-EDTA, one 30 min wash in 2×SSC and one 30 min wash in 0·1×SSC. All washes were performed at 60°C. The sections were dehydrated by passage through 70, 95 and 100% (v/v) ethanol with 0·3 M-ammonium acetate and then air dried.

### *Detection*

Kodak K-5 emulsion was melted in an equal volume of 2% (v/v) glycerol at 45°C and slides were dipped, cooled on an aluminium tray over ice for 5 min and allowed to dry at room temperature. After exposure at 4°C for 4–9 days in boxes containing desiccant, the slides were developed for 2·5 min at 20°C in Kodak D-19 developer, stopped in 1% (v/v) glacial acetic acid and fixed in 5% (w/v) sodium thiosulphate. Sections were then stained with haematoxylin and eosin, dehydrated, cleared and mounted.

### *Purification of polyadenylated RNA*

Total RNA was purified from whole legs of 10-day-old chick embryos using the guanidinium isothiocyanate method of Chirgwin *et al.* (1979). Polyadenylated RNA was isolated by affinity chromatography on oligo(dT)-cellulose (Collaborative Research Ltd) according to Craig *et al.* (1976).

### *Northern blotting*

Polyadenylated RNA (2 $\mu$g per track) was electrophoresed on a 1% (w/v) agarose MOPS–formaldehyde gel and blotted onto Biodyne membrane (PALL) essentially as described by Taylor *et al.* (1984). Filters were probed with $^{32}$P-labelled cRNA prepared as above and washed to 0·1×SSC at 65°C.

## Results

### *Probe selection*

Type I and type II collagen are fibrillar proteins with a triple helical structure. Type I collagen is a heterotrimer [$\alpha$1(I)]$_2$ $\alpha$2(I)] and type II collagen is a homotrimer [$\alpha$1(II)]$_3$. The similarity in structure of these

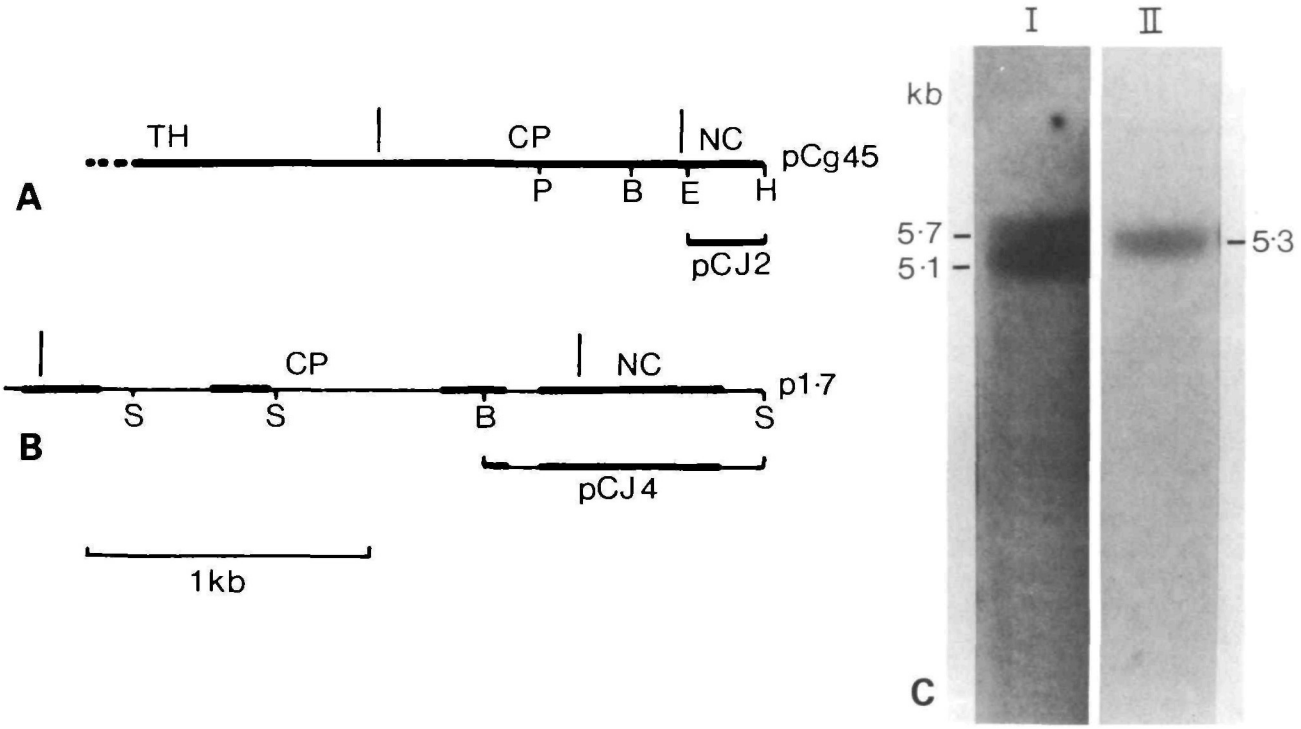


**Fig. 1.** Fragments of chick collagen gene clones selected for use as probes. (A) Type I ($\alpha$2) collagen cDNA pCg45. A 341 bp *Eco*RI–*Hin*dIII restriction fragment was subcloned into RNA transcription vector pGEM 1. (B) Type II ($\alpha$1) collagen subgenomic clone p1.7. A 1 kb *Bam*HI–*Sma*I restriction fragment was subcloned into pGEM 1. TH, triple helical-coding region; CP, carboxyl telopeptide and propeptide-coding region; NC, 3′ noncoding region; P, *Pst*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I; (——), exons; (——), introns. (C) Northern blot of polyadenylated RNA isolated from 10-day-old chick embryo legs. Filters hybridized with $^{32}$P-labelled antisense RNA prepared from (I) subclone specific to type I collagen gene, (II) subclone specific to type II collagen gene. Sizes of transcripts are indicated in kilobases.

proteins is reflected in the nucleic acid sequences of the genes encoding them. Thus, nucleic acid homology between the chicken $\alpha$2(I) and $\alpha$1(II) chain genes rises to 70% in the triple-helix coding region and 67% in the region encoding the carboxyl terminal telopeptide and propeptide (Sandell *et al.* 1984).

In order to localize and distinguish between the mRNAs for type I and type II collagen *in situ*, we required probes which exhibited lowest cross-homology. Analysis of the available sequences for the collagen $\alpha$2(I) and $\alpha$1(II) genes (Fuller & Boedtker, 1981; Sandell *et al.* 1984) using the Beckman Micro-Genie sequence analysis programme (Queen & Korn, 1984) enabled us to select restriction fragments with homology of less than 45% (Fig. 1A,B). These were a 341bp *Eco*RI–*Hin*dIII fragment of the $\alpha$2(I) cDNA clone pCg45 and a 1 kb *Bam*HI–*Sma*I fragment of the $\alpha$1(II) genomic clone p1.7. Fragments were isolated and subcloned into the RNA transcription vector pGEM 1, yielding plasmids pCJ2 ($\alpha$2(I) collagen) and pCJ4 ($\alpha$1(II) collagen) from which single-stranded antisense (cRNA) or sense (mRNA) probes could be synthesized.

To test the specificity of the probes a Northern blot of polyadenylated RNA, isolated from 10-day chick embryo legs, was hybridized with $^{32}$P-labelled antisense RNA (Fig. 1C). After high-stringency washing, transcripts of 5·7 kb and 5·1 kb were detected with the type I collagen probe whereas the type II collagen probe hybridized to a single transcript of 5·3 kb. These are the expected sizes of the respective mRNAs (Merlino *et al.* 1983).

### *Localization of collagen mRNAs in 10-day chick embryo legs*

Both type I and II collagen are abundant in 10-day-old (stage-36) chick leg tissues (Von der Mark *et al.* 1976*a*,*b*), so in each *in situ* experiment we included sections of 10-day-old legs as positive controls. Typical hybridization patterns obtained with antisense and sense probes are shown in Fig. 2. The type I collagen antisense probe hybridized mainly with tendons and perichondrium and to some extent to the ectoderm, whilst the type II collagen antisense probe gave a strong signal over the cartilage elements alone. The differences in the distribution of hybridization signal with these two probes is so striking that they act

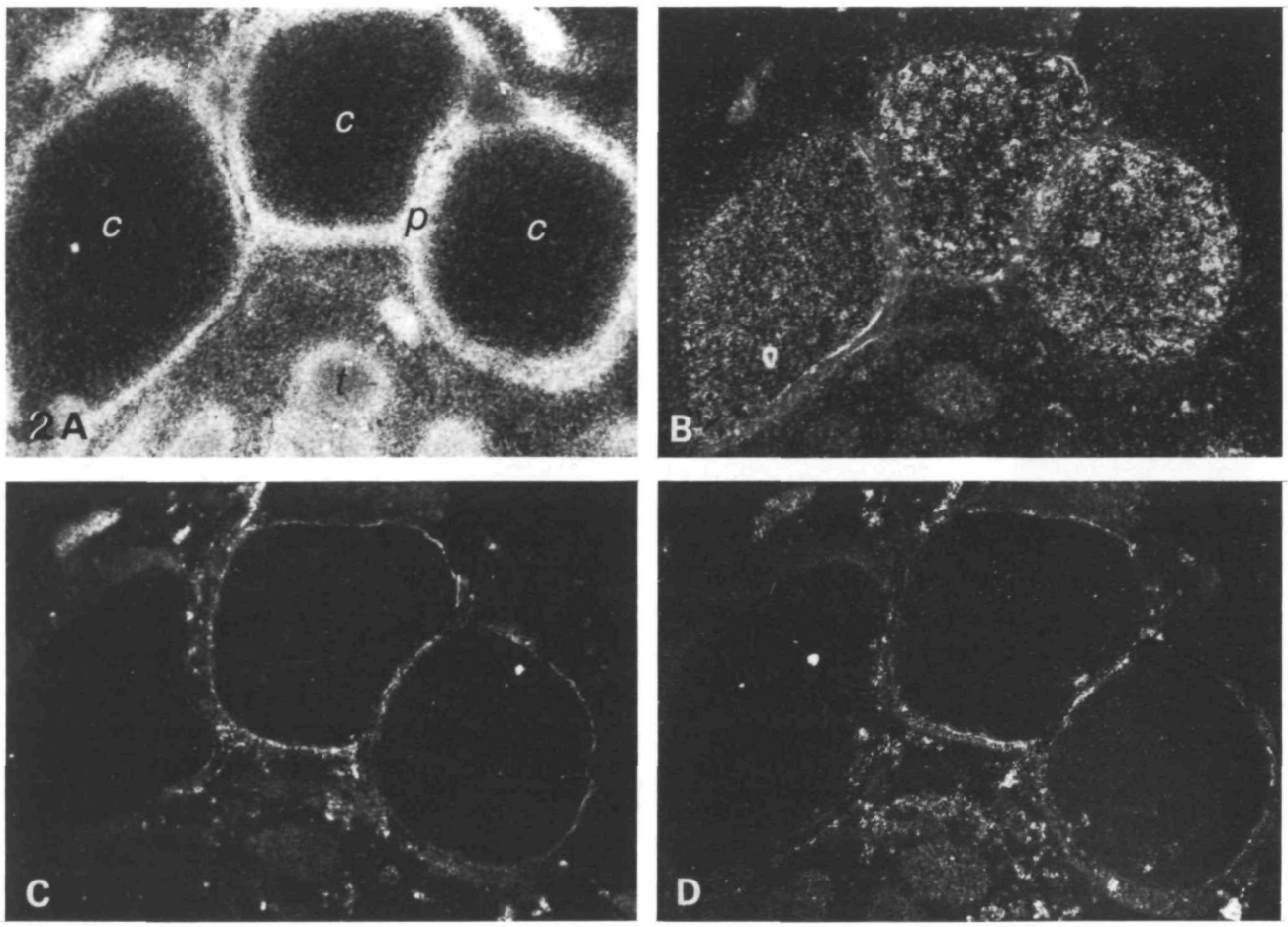


**Fig. 2.** Localization *in situ* of collagen mRNAs in 10-day-old chick embryo leg. Serial sections were hybridized with $^{32}$P-labelled antisense (cRNA) or sense (mRNA) transcripts generated from the collagen gene – pGEM 1 recombinant plasmids. Photographic emulsion was exposed for 7 days. Probes are: (A) type I collagen antisense RNA; (B) type II collagen antisense RNA; (C) type I collagen sense RNA; (D) type II collagen sense RNA. All sections are illuminated under dark field. *c*, cartilage; *t*, tendons; *p*, perichondrium. Bar, 100 $\mu$m.

as specificity controls for each other. However, as an additional control, sections were probed with sense probes derived from the type I and from the type II collagen constructs. No hybridization was observed with either probe. This was true for all limb-bud experiments.

### *Localization of collagen mRNAs in developing limb buds*

Transverse sections through a stage-18 chick embryo at the level of the wing buds were probed (Fig. 3A–C). The notochord hybridized very strongly with the type II collagen antisense probe while some immediately adjacent tissues exhibited a weaker signal (Fig. 3C). No hybridization above background was evident in the limb buds at this stage. An adjacent section probed with the type I collagen antisense probe showed a low level of hybridization in almost all tissues including the limb buds (Fig. 3B). The neural tube did not hybridize significantly to either collagen probe (Fig. 3B,C).

As the limb bud develops, changes in the localization of collagen mRNAs were apparent in longitudinal sections (Fig. 3). In stage-24 limb buds (4·5 days) a core region of hybridization with the type II collagen probe was discernible (Fig. 3F) which was not apparent at stage 23 (data not shown). In addition, there were low levels of hybridization to the surrounding nonchondrogenic tissue. The type I collagen probe hybridized at low levels over the whole section at both stages (Fig. 3E and data not shown). At later stages, signal density increased in the core region of limb buds probed with the type II collagen probe (Fig. 3I,L) while the low-level hybridization to the surrounding nonchondrogenic tissue progressively disappeared. Hybridization with the type I collagen probe appeared to be greater in the ectoderm and the mesenchyme surrounding the prospective cartilage than in the core (Fig. 3H,K).

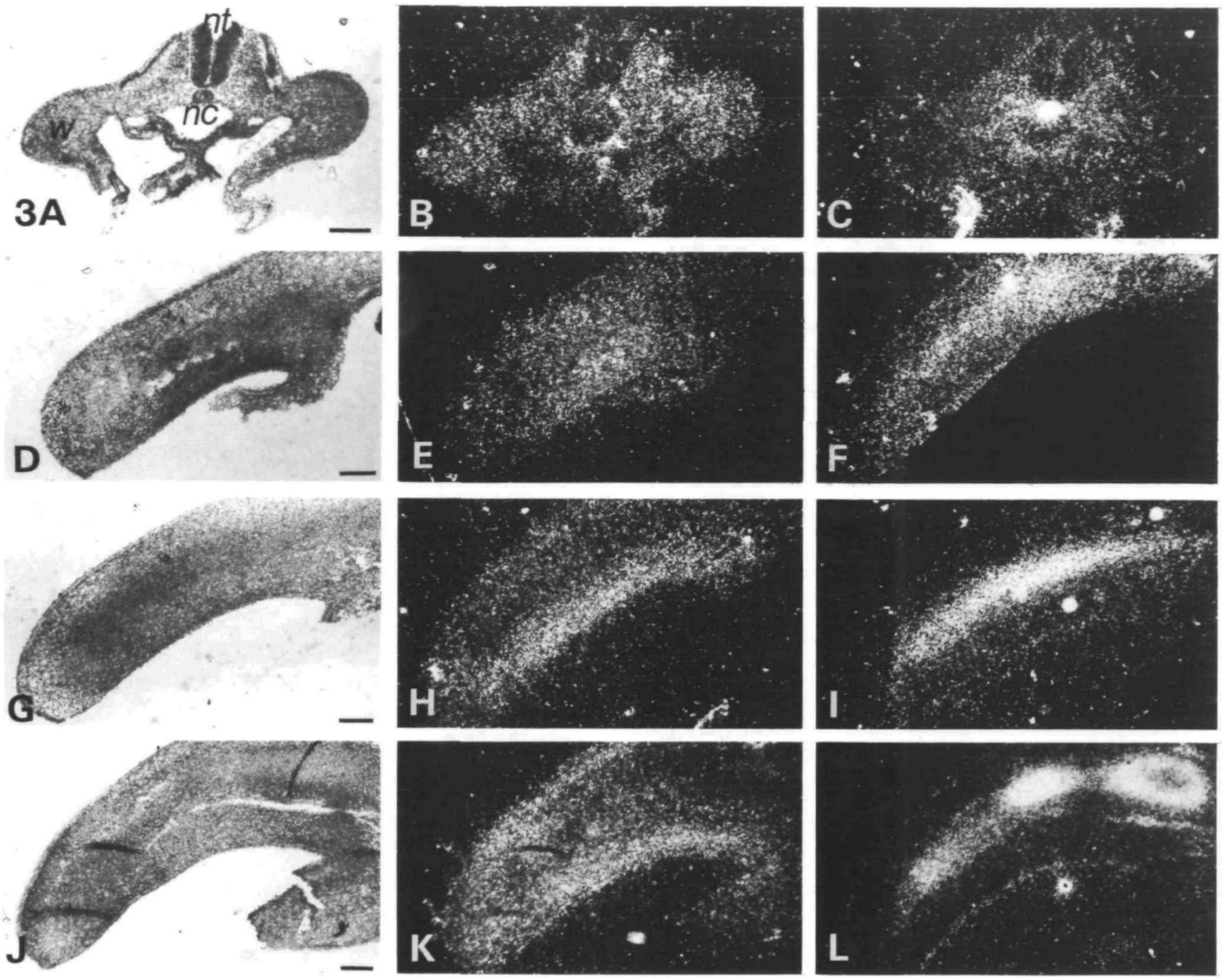


**Fig. 3.** Localization *in situ* of collagen mRNAs in the developing chick embryo limb bud. Serial sections taken from buds at various stages were hybridized as in Fig. 2. For each stage, there are shown an unprobed bright-field view (A,D,G,J) and two dark-field views of sections probed with type I (B,E,H,K) and type II (C,F,I,L) collagen-labelled antisense RNAs, respectively. (A–C) Transverse sections of a stage-18 embryo at the wing level. The following are longitudinal sections: (D–F) Stage-24 wing buds; (G–I) stage-25 wing buds; (J–L) stage-27 wing buds. The exposure time was 7 days. *w*, wing bud; *nt*, neural tube; *nc*, notochord. Scale bars, 100 $\mu$m.

In order to make a more detailed and semiquantitative analysis, transverse sections of stage-24, -25, -27 and -29 wing buds were cut and sections from two different proximodistal levels selected for probing. Sections 200–500 $\mu$m away from the bud tip were used for the distal level, to fall within the region of the digits. Sections for the more proximal level of ulna and radius were taken 900–1400 $\mu$m away from the tip, depending upon the stage. Following *in situ* hybridization, the number of grains per cell above background was determined and this was used as an index of the levels of type I and type II collagen probe hybridization (Fig. 4). The most-significant event occurs in the core of the proximal region, where the hybridization of the type II collagen probe increased with development of the limb bud. In Fig. 5, two regions of increased signal, which correspond to the radius and ulna of the limb bud, could be seen. In the periphery, levels of type II collagen mRNA were very much lower and dropped slightly during development.

In contrast to the hybridization pattern observed for the type II collagen probe, that of the type I collagen probe remained more or less uniform at all stages, in terms of grains per cell, and was present over the cartilage. The bright-field sections shown in Fig. 3D,G,J appear to show an increased cell density in the core of the proximal wing bud, whilst the hybridization signal with the type I collagen probe was greater in the periphery than in the core at stages

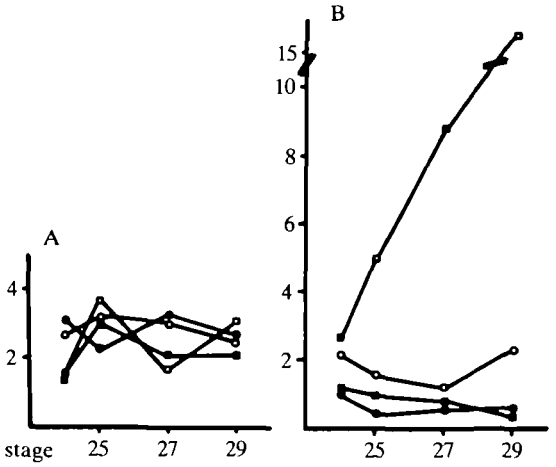


**Fig. 4.** Quantification of accumulation of collagen mRNAs in the developing chick limb bud using *in situ* hybridization. Transverse sections were taken from the progress zone and the prospective ulna/radius region of limb buds of various stages and hybridized as before. Following 8 days exposure, silver grains over the central regions and over the cells directly beneath the ectoderm were counted. Silver grains per cell were plotted against stage. Each point represents the average number of grains per cell in a 1604 $\mu m^2$ are determined from two to four serial sections. Probes: (A) type I collagen antisense RNA; (B) type II collagen antisense RNA. □, ulna/radius core; ■, ulna/radius periphery; ○, progress zone core; ●, progress zone periphery.

25 and 27 (Fig. 3G,J). However, the impression given by low power haematoxylin and eosin sections of high cell density in the core of the wing bud is misleading and is probably due to increased pseudopodial processes and to the presence of extracellular matrix (Gould *et al.* 1972). Thus, the number of grains per cell was approximately the same in the periphery and the core at these stages (Fig. 4A). Surprisingly, even in the 10-day leg tendon there was only a fivefold increase in the number of grains per cell seen with the type I collagen probe over that in the early limb bud, and a similar level of grains per cell to that in the tendon was found in the cartilage (data not shown).

## Discussion

We have used antisense RNA probes to investigate the localization of type I and type II collagen mRNAs in the developing chick limb bud. Our probes are specific as judged by Northern blot analysis. The results show that while the distribution of mRNA for type II collagen corresponds well with the pattern of protein synthesis, there is much less correspondence between the patterns of expression of type I collagen mRNA and protein. This suggests different mechanisms of control for the synthesis of these two proteins during limb development.

At stage 18, we could detect no mRNA for type II collagen in the limb bud although the notochord, known to synthesize type II collagen protein, gave a very strong signal. The earliest we could detect type II collagen mRNA in the limb bud was at stage 23–24. From this time onward in the proximal region of the bud there was a progressive spatial localization, the mRNA levels increasing within the core and dropping in the periphery. By contrast, the progress zone maintained a low level of expression in both the core and the periphery. Thus, the increase in type II collagen mRNA levels corresponds well with the increased synthesis of type II collagen by the developing cartilage cells as studied with fluorescent antibodies (Von der Mark *et al.* 1976*a*,*b*; Dessau *et al.* 1980). This suggests that control of type II collagen protein synthesis is primarily at the level of transcription and/or mRNA accumulation. The presence of low levels of type II collagen mRNA in the nonchondrogenic periphery of stage-24 wing buds, where type II collagen protein is immunologically undetectable, might reflect an element of control at the level of RNA processing or of translation in these cells.

The situation for type I collagen is quite different. Type I collagen mRNA could already be detected at low levels by stage 18. During the development of the limb, the levels of type I collagen mRNA remained more or less constant. However, the distribution of type I collagen mRNA does not entirely correspond with the pattern of type I collagen protein synthesis. Thus, type I collagen protein is present throughout the bud up to stage 25 (Dessau *et al.* 1980) but is, thereafter, progressively lost from the cartilage matrix in the core of the bud, until from stage 28 onwards it is undetectable (Von der Mark *et al.* 1976*b*; Dessau *et al.* 1980). In contrast, we find no significant decrease in the levels of type I collagen mRNA in the core of the wing bud between stages 24 and 29 (Figs 3E,H,K, 4A). Indeed, on a cellular basis the levels of mRNA here are similar to those of the surrounding mesenchyme which does synthesize type I collagen protein. This is so even at 10 days, for there are significant levels of type I collagen mRNA in the mature cartilage of the leg. These surprising results suggest that the control of expression of type I collagen protein may be at the level of translation or, since *in situ* hybridization will detect both mature mRNA and precursor forms, of RNA processing. This agrees with the RNA blotting data of Kosher *et al.* (1986), who detected substantial levels of type I collagen mRNA in 7-day limb cartilage and 12-day sternal cartilage, drawing attention to the possible

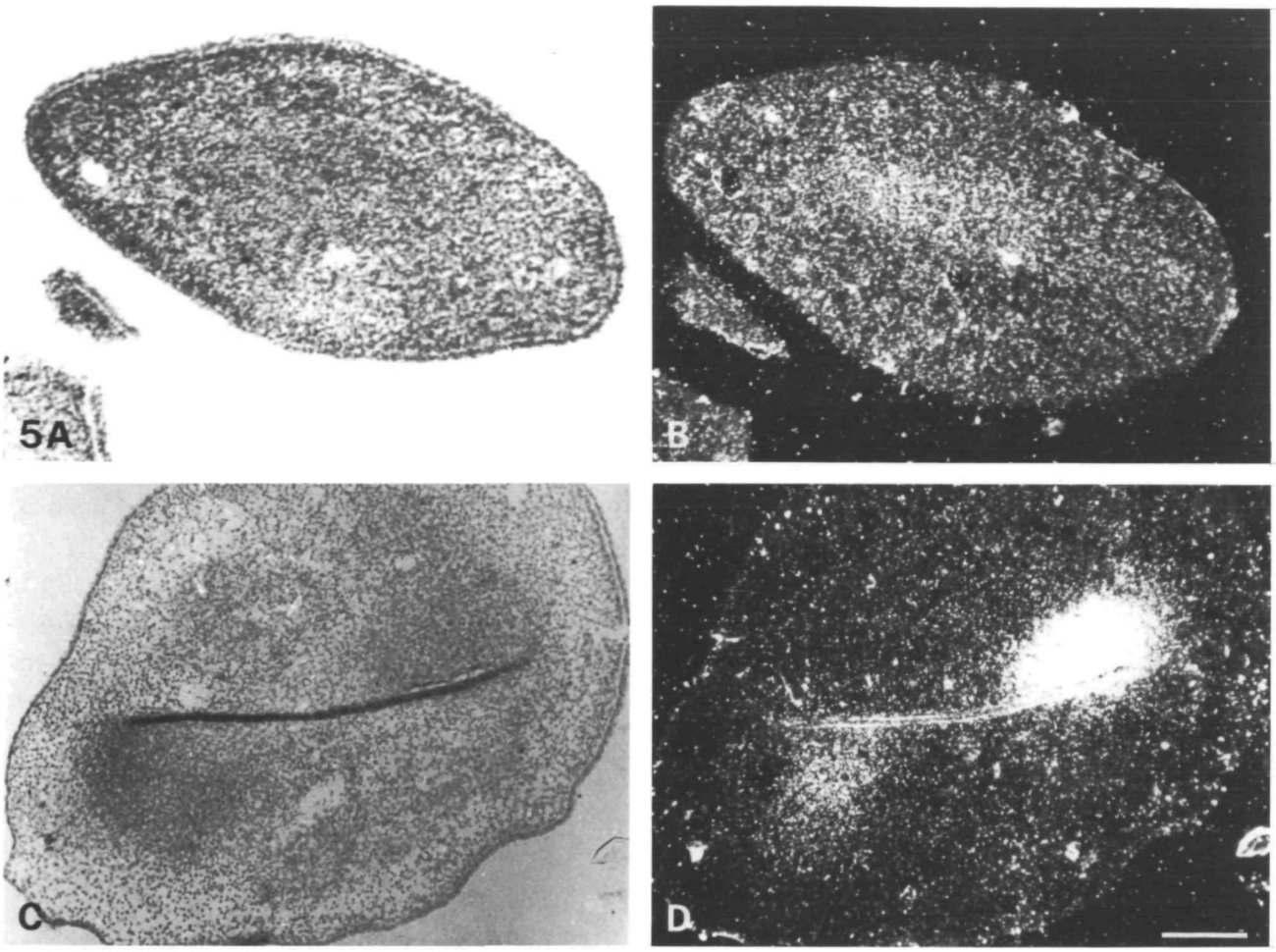


**Fig. 5.** Localization *in situ* of type II collagen mRNA in transverse sections of the ulna and radius region of the developing chick wing bud. (A,B) A section 1000 $\mu$m from the tip of a stage-24 wind bud; (C,D) a section 1500 $\mu$m from the tip of a stage-29 wing bud; (A,C) bright-field illumination; (B,D) dark-field illumination. Bar, 100 $\mu$m.

importance of translational control of type I collagen synthesis in these tissues.

Kosher *et al.* (1986), using RNA–blot hybridization analysis, found low but detectable levels of type II collagen mRNA at stage 18. We were unable to detect type II collagen mRNA at this stage, leaving open the question as to whether post-transcriptional mechanisms control type II collagen synthesis at this early stage. Further support for control of type II collagen expression at the level of transcription and mRNA accumulation comes from our recent results in which the inhibition of cartilage formation in micromass culture by ectoderm, described by Solursh (1984), is associated with the absence of mRNA for type II collagen (B. Gregg & C. Devlin, unpublished data).

Drawing these results together, it is clear that the laying down of type I and type II collagen in the extracellular matrix during limb bud development involves the complex regulation of expression of the corresponding genes, not only at the level of transcription and/or mRNA accumulation but also, in the case of type I collagen at least, at the level of RNA processing and/or translation.

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