by the helical nature of both filaments, all myosin heads in the fiber can find actins with which they can interact. This result implies that a large degree of flexibility must exist in the myosin bond and possibly also in the actomyosin bond.

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Cell-Free Synthesis of Cartilage Proteins: Partial Identification of Proteoglycan Core and Link Proteins†

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ABSTRACT: A poly(adenylic acid)-enriched RNA fraction isolated from calf articular cartilage was translated in cartilage and wheat germ cell-free systems. The radioactive translation products were assayed for the presence of two cartilage proteins: proteoglycan core and glycoprotein link. This was accomplished by utilizing the property both proteins have of binding to hyaluronic acid and forming an aggregate large enough to elute in the void volume of a Sepharose column. When an extract of calf cartilage, containing hyaluronic acid and link, was added to the cell-free mRNA directed products synthesized in a cartilage system and applied to a Sepharose 6B column, 5–10% of the radioactive material was recovered in the void volume of the column. Analysis of the radioactive material in this excluded fraction after separation by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels revealed five major radioactive bands with the following molecular weights: >300 000, 51 000, 47 000, 41 000, and 28 000. Similar results were obtained with cartilage mRNA directed synthesis in a wheat germ system. The largest protein migrates with a molecular weight described by many for proteoglycan core. The four lower bands have molecular weights similar to those described for proteoglycan links. The 41 000 and 28 000 proteins form a precipitate with antibody to homogeneous link.

Articular cartilage consists of chondrocytes dispersed in a hyaline matrix formed by type II collagen and at least two glycosylated proteins noncovalently bound to hyaluronic acid (Rosenberg, 1978; Mankin, 1970; Hascall, 1977). One of the glycoproteins (proteoglycan link) of molecular weight 45 000–50 000 is believed to stabilize the interaction between hyaluronic acid and the second glycoprotein, referred to here as proteoglycan subunit [M_r (0.5–4.0) × 10^6] (Bonnet et al., 1978; Baker & Caterson, 1978, 1977; Keiser, 1975; Oegema et al., 1977; Caterson & Baker, 1977; Swann et al., 1976). The protein part of the subunit, known as core [M_r (1.8–2.0) × 10^5], constitutes only 10% of the proteoglycan with the bulk of the molecule consisting of the sulfated glycosaminoglycans chondroitin sulfate and keratan sulfate. The chondroitin sulfate chains are covalently bound to serine and threonine residues of core and account for the majority of the molecule's polysaccharide component. The remaining carbohydrate is keratan sulfate bound to one of three amino acids: glutamic acid, serine, or threonine (Sweet et al., 1978; Brandt et al., 1973; Bayliss & Ali, 1978; Hardingham & Muir, 1972).

Core protein is considered by many to contain three distinct regions: a hyaluronate binding area [M_r (6–7) × 10^4] of constant amino acid composition, an area adjacent to this which is rich in keratan sulfate, and a polysaccharide attachment region of variable length and amino acid composition to which is bound the majority of chondroitin sulfate chains (Serafini-Fracassini & Smith, 1974). There is evidence that the hyaluronate binding area of the proteoglycan contains residues that form an ionic bond with the carboxyl groups of hyaluronic acid (Hardingham et al., 1976; Lindahl & Hook,

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1978). Core protein with its attached glycosaminoglycans has been shown to be polydisperse by electron microscopy, sieve chromatography, and ultracentrifugation (Sweet et al., 1978; Brandt et al., 1973; Hardingham et al., 1976). This polydispersity is believed by some to be due to the variable length of the chondroitin sulfate attachment region of core. There are others, however, who support a theory attributing size heterogeneity of the subunit to differences in the size of the attached chondroitin sulfate molecules [see Hardingham et al. (1976) for the proposed theories].

The results we have obtained with cell-free translation of cartilage mRNA show a single protein of high molecular weight which we tentatively identify as core. In addition, we present evidence for the synthesis of four proteins of relatively low molecular weights similar to those reported for the glycoprotein link. Further evidence in support of our belief that some of these proteins are proteoglycan core and link protein is inferred from results demonstrating the capacity of the newly synthesized peptides to form a complex of high molecular weight upon addition of a cartilage link fraction and the fact that two of the four lower molecular weight proteins react with antibody raised against a highly purified link preparation.

Experimental Procedures

Materials. Ham's F-12, glutamine, and fetal calf serum were from Grand Island Biological Co., Long Island, NY, collagenase type II used for cell isolation was obtained from Sigma Chemical Co., highly purified collagenase free of protease activity was obtained from Advanced BioFactures Corp., Lynbrook, NY, [^{35}S]methionine (sp act. 500–600 Ci/mmol) and [^{14}C]cytochrome were obtained from New England Nuclear, oligo(dT)–cellulose type T-2 was from Collaborative Research, Waltham, MA, and micrococcal nuclease (sp act. 100–200 units/mg) was from Sigma. Goat antirabbit antisera was obtained from Farmstead Antisera, Reheboth, MA.

Isolation of Chondrocytes for Preparation of Cell-Free Translation System and Messenger RNA. Calf articular chondrocytes isolated from cartilage by a modification of the method of Green (1971) were used for preparation of a cell-free protein synthesizing system. The ankle joints of 7–10-day-old calves obtained from a local slaughter house were washed thoroughly with soap and water and treated with the antiseptic Betadine. The joints were placed in a sterile hood and opened aseptically, and the articular cartilage (about 16 g per joint) was resected and placed in sterile phosphate-buffered saline. The resected cartilage (in 1-cm slices) was washed 5 times with 5 volumes of sterile phosphate-buffered saline. After the final wash the slices were transferred to a sterile bottle containing 4 volumes of sterile Ham's F-12 medium supplemented with 10% fetal calf serum, 150 units of penicillin, and 50 μg of streptomycin per mL of medium. To this solution was added collagenase (clostridial, Sigma, Type II) to a final concentration of 0.075%. The final solution containing cartilage slices was stirred on a magnetic stirrer at 37 °C for 15–18 h. During this procedure 80–90% of the cartilage was digested. The undigested material was removed by passing the suspension through pads of two-ply sterile gauze. The cells were harvested by centrifugation at 600*g* for 15 min and washed by suspending the pellet in 50 volumes of sterile phosphate-buffered saline. The cells were repelleted and washed one additional time.

The washed chondrocytes can be used immediately for message isolation. In some experiments cells were transferred to a sterile flask containing Ham's F-12 medium [(5-10) × 10^8 cells/L] supplemented with 15% fetal calf serum, ascorbate (50 μg/mL), glutamine (4 M), and the two antibiotics at half the concentration used for cell isolation. The cells were rested in this solution with constant stirring for 24 h at 37 °C before harvesting by centrifugation at 600*g* for 10 min. The pellets were pooled and washed 3 times with 50 volumes of saline, and the final pellet was used for preparation of the cartilage-derived cell-free system and messenger extraction. The cells were examined for viability by the trypan blue exclusion method. A typical preparation yielded cells which were greater than 99% viable. The preparation was discarded if microscopic examination showed bacterial or yeast contamination. The yield of chondrocytes varied between 0.7 and 1.0 mL of packed cells per joint (400 × 10^6 cells).

Preparation of Cell-Free Translation Systems. The following procedures were carried out at 4 °C. To 2 mL of packed cartilage cells prepared as described above was added 5 mL of the following hypotonic buffer: 10 mM KCl, 1.5 mM $Mg(OAc)_2$, 20 mM Hepes,[1] pH 7.6, and 10 mM 2-mercaptoethanol. The cells were suspended by vortexing and allowed to swell for 15 min. The swollen cells were disrupted with 25 strokes of an all-glass, motor-driven Kontes homogenizer, set at 4000 rpm. The homogenate was clarified by centrifugation at 1000*g* for 10 min; the supernatant was removed and recentrifuged at 30000*g* for 20 min. The resulting pellet of microsomes was set aside to be used for mRNA extraction. The supernatant (S-30), which contains all the protein synthetic machinery required for translation of messenger RNA, was dialyzed for 12 h against 100 volumes of a buffer containing 120 mM KCl, 2.5 mM $Mg(OAc)_2$, 20 mM Hepes, pH 7.6, 10 mM 2-mercaptoethanol, and 50% glycerol and stored in 100-μL aliquots at −40 °C. A typical preparation had a protein concentration of 12 mg/mL as determined by the procedure of Bradford (1976). There was no appreciable loss of activity after storage for over 10 months.

The cartilage cell-free system contained saturating quantities of endogenous mRNA, masking any stimulation of translation by added message. To deplete the S-30 of mRNA, we incubated the system with micrococcal nuclease according to the procedure described by Pelham & Jackson (1976) for inactivating mRNA in lysates prepared from reticulocytes.

The wheat germ system was prepared according to the procedure of Roberts & Patterson (1973) except that the preincubation step to run ribosomes off endogenous message was omitted. Raw wheat germ was generously supplied by A. Mailhot of General Mills, Inc., Milwaukee, WI.

Messenger RNA Extraction and Purification from Chondrocytes. This procedure was essentially that of Marcu et al. (1978) with some modifications. The microsomal pellet (30000*g*) obtained during the preparation of the cartilage S-30 translation system was used as the starting material for message purification. Unless otherwise stated, this pellet was from cells rested for 24 h. The following steps were carried out at room temperature unless otherwise stated. All vessels and solutions were autoclaved prior to use.

The microsomal pellet was suspended in 6 mL of a buffer containing 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, and 1.5% $NaDodSO_4$. The same buffer was used to saturate the phenol. The dissolved pellet was vigorously shaken for 2 min with an equal volume of a 1:1 buffer containing a phenol–chloroform solution, and the phases were separated by centrifugation at low speed. The aqueous layer was removed and extracted 2 more times. The phenol–chloroform

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Cl_3AcOH, trichloroacetic acid.

layer from the first extraction was reextracted with buffer. The aqueous layers were pooled and RNA was precipitated by the addition of 2.5 volumes of 95% ethanol. After the mixture had been kept at −40 °C for 18 h, the precipitate was collected by centrifugation at 10000*g* for 15 min at 4 °C. The supernatant was discarded and the pellet was lyophilized to dryness. The yield of RNA was 5–6 mg/96 g of cartilage.

Affinity Chromatography on Oligo(dT)–Cellulose. The lyophilized RNA (5–6 mg) from the previous step was dissolved in 2–3 mL of a buffer containing 500 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1% $NaDodSO_4$. This solution was heated for 4 min at 70 °C, immersed in ice, and applied to an oligo(dT)–cellulose column equilibrated with the same buffer. The column bed volume was 10 mL and the flow rate was 1 mL/min. The column was washed with starting buffer until the eluant had an absorbance at 260 nm of less than 0.01. The bound material was eluted from the column with a buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1% $NaDodSO_4$. The peak 260 nm absorbing material (RNA) was pooled, and the RNA was precipitated by adjusting the pooled fraction to 100 mM in NaCl and adding 2.5 volumes of 95% ethanol. Precipitation was allowed to proceed for 18 h at −40 °C. The precipitate was collected by centrifugation at 20000*g* for 20 min, the supernatant was decanted, and the pellet was washed several times (5–6) with cold 95% ethanol to remove excess salt. After the final wash, the pellet was lyophilized to dryness and then redissolved in 0.15–0.20 mL of distilled water and stored at −40 °C. The yield of poly(A)-enriched RNA was 60–70 μg/96 g of cartilage.

Translation of Cartilage mRNA. The reaction mixture contained, in a volume of 25 μL, 2 mM $Mg(OAc)_2$, 50 mM KOAc, 50 mM KCl, 25 mM Hepes, pH 7.6, 0.1 mM spermine (wheat germ system requires 0.35 mM), 1 mM dithiothreitol, 1 mM 2-mercaptoethanol, 1 mM ATP, 0.3 mM GTP, 0.7 mM CTP, 16 mM creatine phosphate, 0.5 μg of creatine kinase, 19 amino acids at 65 μM each, 12 μCi of [^{35}S]methionine, 0.8 μg of oligo(dT)-purified mRNA where indicated, and 10 μL of either the cartilage or the wheat germ S-30. The tubes were incubated for 2 h at 25 °C. Unless stated otherwise, the reaction was terminated by the addition of 2 mL of 10% Cl_3AcOH. The tubes were heated at 90 °C for 15 min and cooled to 4 °C, and their contents were filtered onto nitrocellulose membranes. The membranes were washed 3 times with 2 mL of cold 10% Cl_3AcOH, placed in a vial containing 10 mL of Bray's solution, and counted in a scintillation counter.

Collagenase Digestion of Cell-Free Translation Products. After completion of the translation incubation, the reaction mixtures were made 13 mM with $CaCl_2$, 0.1 μg of highly purified clostridial collagenase was added, and the samples were incubated for 3 h at 37 °C. Reactions were terminated and the charged aminoacyl-tRNAs were deacylated to remove [^{35}S]Met-tRNA by adjusting the reaction mixture to pH 10 with 0.1 N NaOH and incubating for 10 min at 37 °C. The protein was precipitated with 2 mL of ice-cold 10% Cl_3AcOH containing 0.5% tannic acid, and the samples were filtered and prepared for liquid scintillation counting as described above for the cell-free translation assay.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Products from the cell-free protein synthesis reactions were identified by $NaDodSO_4$–polyacrylamide gel electrophoresis according to the procedure of Weber & Osborn (1969). The gels (0.7 × 11 cm) contained 5% acrylamide, 0.1% $NaDodSO_4$ and 0.1 M sodium phosphate, pH 7.0. Immediately after the translation incubation, the entire reaction mixture was dissolved in 70 μL of a buffer containing 1% 2-mercaptoethanol, 1% $NaDodSO_4$, 50% glycerol, and 10 mM sodium phosphate, pH 7.0, and heated for 3 min at 90 °C. It was then layered onto the gel surface and subjected to electrophoresis at 6 mA/gel for 5 h.

Autoradiographic Analysis of Polyacrylamide Gels. After electrophoresis the gels were removed from the tubes and sliced in half longitudinally, and half was treated for fluorography according to the procedure of Laskey & Mills (1975). The dried gels were placed in contact with Kodak X-ray (XR-1) film and allowed to develop from 3 to 24 days at −40 °C.

Sepharose 6B Column Chromatography of Cartilage mRNA Directed Translation Products. At the completion of the translation reactions 25 μL of 8 M guanidinium chloride was added to each reaction mixture, and the tubes were maintained at 37 °C for 15 min. The contents of the reaction tubes were transferred to 1-mL plastic conical centrifuge tubes and centrifuged at 17000*g* for 10 min to remove insoluble material. The supernatants were removed and assayed for the presence of radioactive components capable of forming high molecular weight aggregates in the presence of a proteoglycan link fraction. The link fraction, known as A1-D4,[2] was prepared by the procedure of Hascall & Heinegard (1974). It consists of cartilage structural components from a calf articular cartilage salt extract present in the upper one-fourth of a cesium chloride–guanidinium chloride dissociative buoyant density gradient.

Nine micrograms of fraction A1-D4 was added to the supernatants, which were then diluted to 250 μL with 0.5 M NaOAc, pH 7.0 (aggregation conditions), incubated for 10 min at 37 °C, and placed on ice. Nine micrograms of bovine serum albumin was substituted for fraction A1-D4 in control tubes. Both fraction A1-D4 and albumin were in 0.25 M NaOAc, pH 7.0. The cooled samples were applied to a Sepharose 6B column (1 × 30 cm; bed volume 23 mL; flow rate 30 mL/h). Fractions (0.6 mL) were collected and assayed for the presence of radioactivity by counting 200-μL aliquots dissolved in 5 mL of Bray's solution in a scintillation counter. The column's exclusion volume, fractions 12–14, was determined with blue dextran 2000, and the included volume, fractions 23–36, was determined with β-galactosidase (M_r 532000). Determination of the number of radioactive proteins eluting in the void volume and their apparent molecular weights was accomplished by autoradiographic analysis of fractions 12–14 after electrophoresis on $NaDodSO_4$–polyacrylamide gels. These fractions were prepared for electrophoresis in the following way. Fractions 12–14 were pooled, dialyzed for 6 h against distilled water, and lyophilized to dryness. The dry residue was dissolved in 300 μL of a buffer containing 1% $NaDodSO_4$, 1% 2-mercaptoethanol, and 10 mM sodium phosphate, pH 7.0, heated for 3 min at 90 °C, and concentrated fourfold by dialysis against this buffer containing 50% glycerol. The samples were applied to 5% polyacrylamide gels and subjected to electrophoresis for 5 h. Autoradiographs were made of the sliced gels as described above.

Immunoprecipitation of Sepharose 6B Excluded Cell-Free Products: Antilink Antiserum Raised in Rabbits against Homogeneous Link Prepared by a Technique Devised in This Laboratory (Unpublished Experiments). The antiserum was monospecific when analyzed by Ouchterlony immunodiffusion. The IgG fraction was concentrated to 20–25 mg/mL by ammonium sulfate fractionation.

[2] The nomenclature A1-D defines the proteoglycan fractions as described by Hascall & Heinegard (1974).

Table I: Cell-Free Translation of Cartilage RNA[a]

system	Cl_3AcOH-precipitable products (cpm)
wheat germ	
complete	193 000
mRNA omitted	58 000
cartilage	
complete	225 000
mRNA omitted	230 000
cartilage (nuclease treated)	
complete	150 000
mRNA omitted	40 000

[a] The translation assay was as described under Experimental Procedures. Incorporation represents Millipore filter retention of hot Cl_3AcOH-precipitable counts per minute of [^{35}S]methionine present in the 25-μL reaction mixtures after incubation for 2 h at 25 °C.

For immunoprecipitation of the [^{35}S]methionine-labeled cell-free products, the following procedure, based on a modification of that of Parry & Hawkes (1978), was carried out. The material present in the excluded portion of the Sepharose 6B column (fractions 12–14) after applying the cartilage reaction mixtures (see Experimental Procedures) was centrifuged for 15 min at 17000g to remove insoluble material. The supernatant was carefully removed and adjusted to the following component concentrations (buffer A): 500 mM NaCl, 1 mM PMSF, 1% Triton X-100, and 1% deoxycholate. To each sample was added 1 μg of carrier link protein and either 1 μL of antiserum or 1 μL of normal rabbit serum. The tubes were incubated for an initial 30 min at 37 °C and then for 2 h at room temperature. Three hundred microliters of goat antirabbit antiserum in buffer A was delivered to each sample, followed by incubation for 2 h at 37 °C and another incubation for the same amount of time at room temperature. The immunoprecipitate was collected by centrifugation for 10 min at 6000g. The supernatant was decanted and the pellet was resuspended in 2 mL of buffer A. Centrifugation and pellet resuspension were repeated 3 additional times. The final pellet was dissolved in 500 μL of buffer containing 1% $NaDodSO_4$, 1% 2-mercaptoethanol, and 10 mM sodium phosphate, pH 7.0, and heated at 90 °C for 3 min. The dissolved pellet was concentrated fourfold by dialysis against buffer containing 1% $NaDodSO_4$, 1% 2-mercaptoethanol, 10 mM sodium phosphate, pH 7.0, and 50% glycerol and applied to the surface of a 5% $NaDodSO_4$–polyacrylamide gel. Electrophoresis and autoradiography were carried out as described above.

Results

Previous work by Wiebkin & Muir (1977) has demonstrated the ability of chondrocytes to synthesize proteoglycans in cell culture. In experiments not reported here, we demonstrated that calf articular chondrocytes suspended in medium for 24 h incorporate $Na^{35}SO_4$ into molecules large enough to elute near the void volume of a Sepharose 2B column. The cultured cells are also capable of producing matrix that stains metachromatically with toluidine blue. These results, taken together, indicate that the chondrocytes from which we prepared mRNA were capable of synthesizing cartilage proteins.

Table I shows the mRNA-dependent incorporation of [^{35}S]methionine into Cl_3AcOH-precipitable material in the two translating systems. The amount of oligo(dT)-purified mRNA required to saturate the wheat germ system is between 0.6 and 0.8 μg as depicted in the mRNA saturation curve of Figure 1. A similar profile (not shown) is obtained with the nuclease-treated cartilage system.

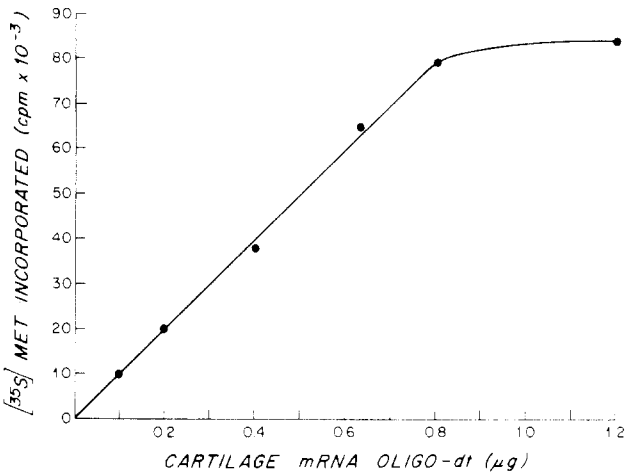

FIGURE 1: Cartilage mRNA at the oligo(dT) state of purification was translated in the wheat germ system as described under Experimental Procedures. Radioactive incorporation represents hot Cl_3AcOH precipitable counts per minute retained on a Millipore filter. Reaction volumes were 25 μL; incubations were for 2 h at 25 °C.

Table II: Effect of Collagenase on Radioactive Products[a]

expt	system	treatment after translation	[^{35}S]Met (cpm)	decrease (%)
1	wheat germ	none	184 000	
		collagenase	138 000	25
	cartilage	none	58 000	
		collagenase	40 000	31
2	wheat germ	none	200 000	
		collagenase	202 000	<1
	cartilage	none	189 000	
		collagenase	190 000	<1

[a] The cell-free translation assay as described under Experimental Procedures was used except that prior to precipitation with cold Cl_3AcOH and tannic acid the reaction mixtures were incubated with 10 μL of 0.1 N NaOH and prepared for liquid scintillation counting. RNA (0.8 μg) added to the tubes in experiment 1 was prepared from freshly isolated chondrocytes. The RNA used in experiment 2 (also 0.8 μg/tube) was isolated from chondrocytes which had been rested for 24 h.

Since 60% of the cartilage matrix is collagen, it was reasonable to expect that collagen would represent a significant percentage of the radioactive peptides among the translation products. Support for this premise can be seen from the data in experiment 1 of Table II. Between 20 and 30% of the protein synthesized in the system is susceptible to digestion by protease-free clostridial collagenase. This is in close agreement with the results obtained by Schwarz & Bissell (1977), Schwartz et al. (1976), and Dehm & Prockop (1971), who demonstrated that 25–30% of total protein synthesis in freshly isolated avian tendon cells is devoted to collagen. Interestingly, these results were obtained only with cartilage mRNA prepared from freshly isolated chondrocytes. Experiment 2 of Table II shows that there was little or no collagen synthesized when the assay was carried out with mRNA isolated from chondrocytes after a 24-h rest period in medium (see Experimental Procedures). Schwarz & Bissel (1977) showed that serum concentrations in excess of 0.5% dramatically inhibit collagen synthesis in primary tendon cells, while total protein synthesis was unaffected. The results reported here suggest that the resting of chondrocytes in medium containing 15% serum inhibits the production of active collagen mRNA.

Proteoglycan subunit and link have an affinity for hyaluronic acid, and, under appropriate conditions, a mixture of these

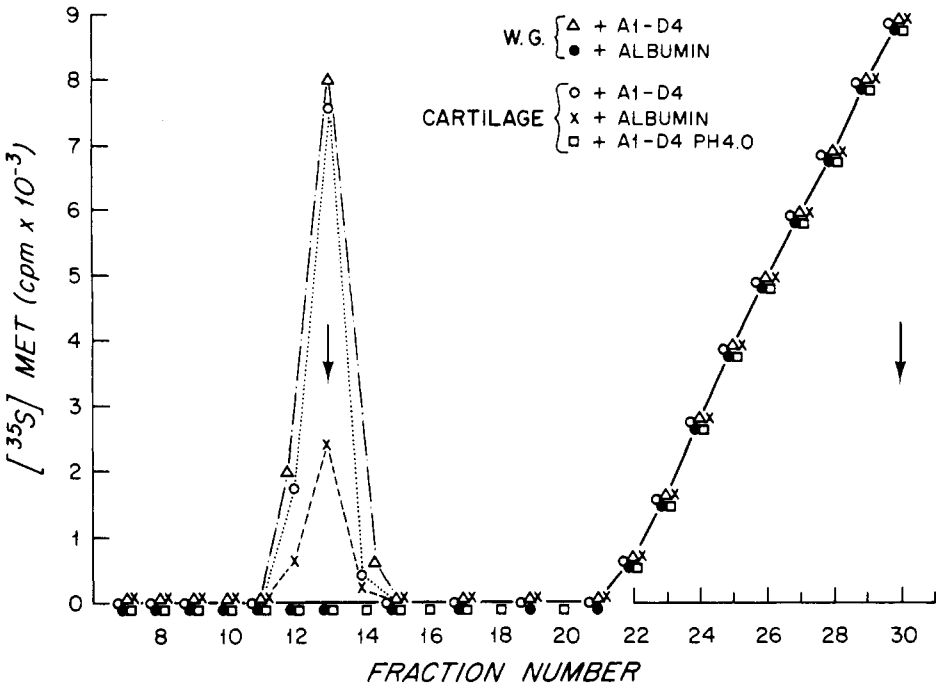

FIGURE 2: Elution profile from a Sepharose 6B column of protein synthesized in cell-free systems directed by cartilage mRNA. The conditions for the translation incubations and details of the preparation of the reaction mixtures for the column are described under Experimental Procedures. (△) Cartilage mRNA directed wheat germ system translation products incubated with 9 μg of cartilage fraction A1-D4 and (●) with 9 μg of albumin. (○) Cartilage mRNA directed cartilage system translation products incubated with 9 μg of fraction A1-D4 and (×) with 9 μg of albumin. (□) Cartilage mRNA directed cartilage system translation products incubated with fraction A1-D4 but with the pH of the column elution buffer lowered from 7.0 to 4.0.

three substances forms a large aggregate. This interaction was exploited as a means of identifying proteoglycan corelike and linklike proteins among the peptides synthesized in the two cell-free systems. The results are shown in Figure 2. When, following the translation incubation, the reaction mixtures were passed through a Sepharose 6B column, most of the radioactive proteins synthesized in the cartilage system and all of the counts per minute in the wheat germ system eluted from the column in the included volume, begining at tube 22. When, however, a fraction isolated from whole calf cartilage (fraction A1-D4, see Experimental Procedures) containing link, hyaluronic acid, and low molecular weight proteoglycans was added to the translated products, 5–10% of the total Cl_3AcOH-precipitable radioactivity was shifted to the excluded portion of the eluate (fractions 12–14). As much as a 40-fold excess (360 μg) of A1-D4 was incubated with the translated products to determine if the effect of this extract was due to a nonspecific interaction, such as the presence of a "sticky" protein. The results, shown in Table III, demonstrated no change in the amount of radioactive material eluting in the excluded volume when compared to the results obtained with 9 μg of this fraction. The appearance of a small amount of radioactivity in the column void volume of Figure 2, when the cartilage system was applied without incubation with fraction A1-D4, was probably due to the presence in the cartilage translation system of trace amounts of aggregating components. In support of this explanation are results showing a complete absence of excluded radioactivity when the cartilage translation products, after incubation with A1-D4, are eluted with buffer at pH 4.0. As shown by Hardingham et al. (1976), aggregation is prevented at this pH.

The autoradiographs of the excluded material after $NaDodSO_4$–polyacrylamide gel electrophoresis are shown in Figure 3. This figure suggests that five proteins synthesized in both cell-free systems constitute the bulk of the radioactive material that elutes in the void volume of the column in the

Table III: Sepharose 6B Column Chromatography[a]

fraction A1-D4 (μg)	[35S] Met excluded fractions (cpm)
0	
3	1500
6	2600
9	9400
90	9100
360	9200

[a] Radioactive products synthesized in the wheat germ S-30 system programmed with 0.8 μg of cartilage mRNA were prepared for Sepharose 6B column chromatography as described under Experimental Procedures. Products (90 000 Cl_3AcOH-precipitable cpm for each run) were incubated in the presence of quantities of fraction A1-D4 as indicated above and applied to the column.

presence of proteoglycan subunit, link fraction, and hyaluronic acid. Parts B and C of Figure 3 depict those proteins synthesized in the wheat germ and cartilage cell-free systems, respectively. The apparent molecular weights of these bands are >300 000, 51 000, 47 000, 41 000 and 28 000. As expected, autoradiographs of the excluded material when A1-D4 was replaced by albumin did not show the presence of these five proteins.

Incubation of the Sepharose 6B excluded cell-free products with antibody to link protein and autoradiographic analysis of the resultant precipitate on $NaDodSO_4$–polyacrylamide gels show two major proteins, with molecular weights of 40 000–42 000 and 28 000–30 000. The two major radioactive protein bands present in the excluded fractions of the Sepharose 6B column, with molecular weights of 51 000 and 47 000, were not precipitated by this antibody.

Discussion

This study describes a procedure for the preparation of a cartilage-derived cell-free protein synthesis system capable of

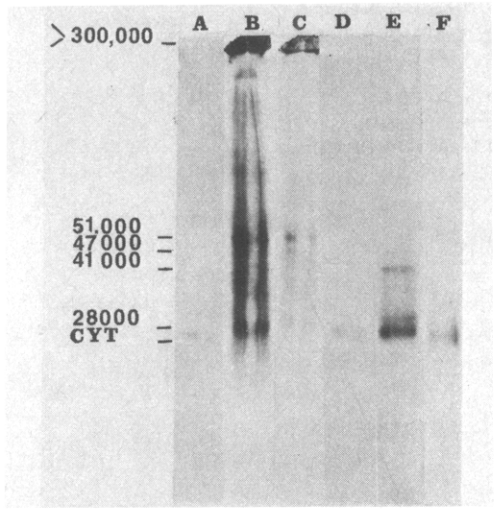

FIGURE 3: Autoradiograms of cell-free translation products. Translation reactions were performed as described under Experimental Procedures. The samples, each containing 90 000 Cl_3AcOH-precipitable cpm of [^{35}S]Met, were prepared and applied to a Sepharose 6B column as described under Experimental Procedures, and fractions 12–14 were pooled. Samples to be applied to gels A–D were prepared for $NaDodSO_4$–polyacrylamide gel electrophoresis. Samples to be applied to gels E and F were first incubated with antibody to link (E) or normal rabbit serum (F), and the immunoprecipitates were applied to the gel surfaces and electrophoresed. Autoradiograms were made of the gels as described under Experimental Procedures. (A) Cartilage system with 9 μg of albumin; (B) cartilage system with 9 μg of fraction A1-D4; (C) cartilage mRNA directed wheat germ system with 9 μg of fraction A1-D4; (D) the same as (C) but with 9 μg of albumin substituted for fraction A1-D4. (E) Immunoprecipitate formed with link antibody added to cartilage cell-free products; (F) immunoprecipitate formed with normal rabbit serum. Molecular weights were determined by comparison with the following standards: myosin, 200 000; bovine serum albumin, 68 000; ovalbumin, 45 000; myoglobin, 17 800. An internal standard ([^{14}C]cytochrome, 12 300) was mixed with each sample before application to the gel surface.

translating mRNA. In addition, we have isolated and translated poly(A)-containing mRNA from chondrocytes in cell-free systems prepared from cartilage and wheat germ. We found the conditions of translation required for optimal protein synthesis to be similar for both cell-free systems. The cartilage S-30, like the reticulocyte-derived systems, exhibits a wide temperature range (22–37 °C) for protein synthesis whereas the wheat germ system is virtually inactive at 37 °C (results not shown).

Our results demonstrate that freshly isolated chondrocytes contain significant quantities of mRNA which codes for proteins that are digested by clostridial collagenase. We believe the digestible proteins are collagen since the collagenase preparation was free of nonspecific proteases. These results contrast with those obtained when mRNA isolated from rested chondrocytes was translated and the products were treated with collagenase. This experiment showed that the radioactive products are insensitive to digestion by collagenase.

The reason for these findings is unknown, but one explanation would attribute this effect to the exposure of the cartilage to collagenase during chondrocyte isolation. Removal of the cartilage matrix may cause a stimulation of collagen mRNA synthesis which after a 24-h rest period regresses to negligible levels. This, however, is unlikely in light of data (unpublished results) obtained in this laboratory showing 30% of the protein synthesized by fresh cartilage slices in organ culture is collagenous. Another, and perhaps more reasonable, explanation for these results is based on the experiments of Schwarz & Bissel (1977), who have shown that serum in concentrations greater than 0.5% inhibits collagen synthesis in cultures of primary avian tendon fibroblasts. The procedure we used involved resting the chondrocytes in 15% fetal calf serum before mRNA isolation, and this high serum level may shut off collagen synthesis during the 24-h period. We do know that chondrocytes suspended in medium for this length of time actively incorporate sulfate and secrete sulfated products large enough to elute near the void volume of a Sepharose 2B column. This secreted material stains metachromatically with toluidine blue, indicating active proteoglycan synthesis (or at least the presence of proteoglycan).

These results agree with those of Dondi & Muir (1976), who demonstrated the absence of coordinate or coupled synthesis between proteoglycan and collagen. They showed that collagen synthesis was unaffected when production of completed proteoglycan molecules was inhibited.

There have been many reports describing the interaction of proteoglycan subunit and glycoprotein link with hyaluronic acid to form a high moleculer weight aggregate. The evidence for this phenomenon has been obtained with a variety of techniques, such as buoyant density centrifugation, sieve chromatography, viscometry, and electron microscopy (Sweet et al., 1978; Brandt et al., 1973; Hardingham et al., 1976). The most dramatic evidence for the existence of the aggregate is the electron micrographs of Rosenberg et al. (1975) and Heinegard et al. (1978). The latter group has estimated that 30 proteoglycan subunits can bind to a single hyaluronic acid molecule of molecular weight 0.5×10^6 to yield an aggregate with a molecular weight of 70×10^6.

Hardingham et al. (1976) have shown that removal of chondroitin sulfate residues from proteoglycan subunit by digestion with chondroitinase ABC had no effect on the hyaluronic acid–proteoglycan interaction. These investigators also demonstrated the importance of the hydrogen ion concentration in aggregation. If the pH of the buffer containing the aggregate components is lowered to 4.0, the aggregate dissociates. They propose that among the requirements for aggregate formation are negatively charged groups on hyaluronate and positively charged basic amino acid residues clustered on the proteoglycan core.

Our results showing the formation of high molecular weight aggregates with proteins synthesized in cell-free systems are of interest for several reasons. From what is known about the mechanism of glycosylation, it is unlikely that the protein made in either our cartilage or our wheat germ system contains carbohydrate residues. Therefore, our results are in agreement with the idea that only the peptide backbone of core and link proteins is required for aggregate formation.

There is at present controversy concerning the role link protein plays in aggregate formation and the number of link proteins. Most agree that link protein exerts a stabilizing effect on the proteoglycan–hyaluronic acid interaction by binding to both these components. Caterson & Baker (1977) have data demonstrating the binding of link to proteoglycan in the absence of hyaluronate. They postulate that this interaction may alter the proteoglycan conformation to favor binding to hyaluronate. They did not, however, rule out possible contamination of the proteoglycan preparation with small amounts of hyaluronate. This group has also reported on other proteins which they believe to be links, one with a molecular weight on $NaDodSO_4$ gels of 47 000 and a very faint band of slightly lower molecular weight. Although 51 000 and 47 000 molecular weight species have been reported by several groups, the faint band has been described by only a few. These investigators believe that it is a degradation product of one of the other two.

Our results demonstrate four radioactive bands in the excluded portion of the Sepharose 6B column which migrate with similar molecular weights (51 000, 47 000, 41 000, and 30 000) to those described above. It is interesting that only the two lower molecular weight proteins were precipitated by antibody to link protein. The 51 000 and possibly the 47 000 molecular weight bands could, in fact, represent the hyaluronate binding area of the proteoglycan subunit. There has been speculation that this area of the proteoglycan is synthesized as a separate entity and later joined to the subunit (DeLuca et al., 1978).

A recent publication by Upholt et al. (1979) presents evidence for the cell-free synthesis of a chick limb bud cartilage protein of $M_r > 300\,000$ precipitable by antibody to the proteoglycan subunit. These data support our contention that the protein of $M_r > 300\,000$ synthesized in our system represents nascent core proteins.

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