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Dynamic Expression of a Native Chondroitin Sulfate Epitope Reveals Microheterogeneity of Extracellular Matrix Organization in the Embryonic Chick Heart

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ABSTRACT

TC2 is a novel monoclonal antibody produced by in vitro immunization of splenocytes with a peanut agglutinin-positive fraction from extracts of prechondrogenic micromass cultures of chick limb mesenchyme. ELISA results demonstrated TC2 reactivity with a native epitope on a glycosaminoglycan (GAG) enriched in chondroitin-4-sulfate and with multiple intact proteoglycans, but not with other GAGs tested. TC2 immunohistochemical reactivity was abolished by pretreatment of sections with chondroitinase AC or preadsorption with chondroitin-4-sulfate GAG. Strong TC2 localization occurred throughout the developing heart at stage 9. As looping ensued, a graded reactivity was observed from lowest in the atrium to highest in the conotruncus that correlated well with versican localization. The superior atrioventricular cushion stained preferentially with TC2 as compared to the inferior cushion at stages 16–18. At these later stages TC2 patterns did not agree completely with anti-versican reactivity. By stage 23 there was a marked reduction in TC2 localization in the heart, however, strong reactivity remained at certain sites, including the conotruncus and in subcompartments of both atrioventricular cushions. A heterogeneous distribution of other native chondroitin sulfate glycosaminoglycan epitopes recognized by monoclonal antibodies d1C4 and CS-56 was observed as well. The distribution of the TC2 epitope usually did not overlap with d1C4 or CS-56 localization at the stages examined. Overall, the spatiotemporal characteristics of TC2 reactivity in the developing chick heart appear to correlate with subdomains of the endocardial cushions as well as with trabecular and atrial septal formation. Anat Rec 254:181–195, 1999.

Key words: atrioventricular cushions; chondroitin sulfate proteoglycan; extracellular matrix; glycosaminoglycan; heart development; immunohistochemistry; in vitro immunization; monoclonal antibodies

The embryonic tubular heart is comprised of two concentric epithelial layers, the myocardium and endocardium, separated by an extensive extracellular matrix (ECM) termed the cardiac jelly (Davis, 1924). The cardiac jelly contains numerous basement membrane components produced by the endocardium and myocardium (Kitten et al., 1987) as well as foregut endoderm (Hurle and Ojeda,

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1977). One class of molecules well represented in this specialized ECM are proteoglycans which are involved in numerous morphological processes, including proliferation, migration, tissue remodeling, and migration (Rouslahti, 1988; Kjellen and Lindahl, 1991). The presence of chondroitin sulfate proteoglycans in the cardiac jelly of the atrioventricular cushions has been demonstrated biochemically (Funderburg and Markwald, 1986) and the large chondroitin sulfate proteoglycans, versican and aggrecan, have been localized immunohistochemically in the developing chick heart (Zanin et al., 1998). That proteoglycans play an essential role in development of the heart has been demonstrated by the identification of an insertional transgene mutation in the versican gene in the heart defect mouse (*hdf;* Mjaatvedt et al., 1998). The loss of versican expression in the homozygous *hdf* mutant prevented normal conotruncus formation, interfered with continued differentiation of the right ventricular segment, and prevented endocardial cushion formation, emphasizing the importance of this chondroitin sulfate proteoglycan in early heart development.

Many of the functions of proteoglycans can be attributed to their associated glycosaminoglycan (GAG) chains (Rouslahti, 1988; Kjellen and Lindahl, 1991). While the presence of different classes of sulfated GAGs in the embryonic heart has been well described using biochemical and histochemical methodologies (Ortiz, 1958; Manasek, 1970, Markwald and Adams-Smith, 1972; Manasek et al., 1973; Markwald et al., 1978), few studies have evaluated the microheterogeneity of chondroitin sulfate GAG chains during this critical period of cardiac development. Funderburg et al. (1991) reported differences in the immunolocalization of two chondroitin sulfate glycosaminoglycan epitopes in atrioventricular cushion tissue. In that study, CS-56 (recognizing an epitope on both chondroitin-4- and chondroitin-6-sulfate GAG chains; Avnur and Geiger, 1984) stained the premigratory cardiac jelly as well as migrating atrioventricular cushion mesenchyme, while staining with M0-225 (recognizing a disulfated chondroitin sulfate type D epitope; Yamagata et al., 1987) was restricted to cushion mesenchyme. We have shown previously that immunostaining with d1C4, which reacts with an unidentified native epitope on GAG chains enriched in chondroitin-6-sulfate (Capehart et al. 1997), was essentially absent from the premigratory matrix of the atrioventricular cushions. Expression of the d1C4 epitope increased dramatically in the matrix surrounding migrating atrioventricular cushion mesenchyme and was differentially expressed by mesenchyme in the outflow tract cushions.

In the present study we report the in vitro production and initial characterization of a novel monoclonal antibody (Mab TC2) that preferentially recognizes a native epitope on GAG chains enriched in chondroitin-4-sulfate produced by the myocardium. Immunofluorescence microscopy was used to demonstrate a preferential localization of TC2 reactivity in the cardiac jelly of the straight tube heart. Staining intensity was accentuated markedly by pretreatment with Streptomyces hyaluronidase. During subsequent stages of development TC2 reactivity diminished in general, however, staining persisted in selected regions exclusive of other native chondroitin sulfate GAG epitopes. These observations show a dynamic heterogeneity of chondroitin sulfate GAG expression in the preseptation chick heart that has not been appreciated previously. The selective persistence of TC2 reactivity at multiple sites of cardiac remodeling suggests that the TC2 antigen may be involved in the maintenance of morphological plasticity in those regions.

MATERIALS AND METHODS

Production of Mab TC2 In Vitro

Six 0.5 ml high density micromass cultures derived from stages 22–23 (Hamburger and Hamilton, 1951) chick limb buds were prepared as described previously (Ahrens et al., 1977; Capehart et al., 1997). After 18 hr of incubation, cultures were washed with phosphate buffered saline (PBS) and extracted for 30 min on ice in 0.5 ml RIPA buffer (Harlow and Lane, 1988) containing protease inhibitors. Pooled extracts were clarified by centrifugation, supernatants precleared with strepavidin-paramagnetic beads (Promega, Madison, WI) and incubated on a rotary shaker with 0.1 mg/ml biotinylated peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA) 1 hr at 4°C followed by incubation for an additional 1 hr with 0.3 ml strepavidin-paramagnetic bead suspension. Beads were washed thoroughly with RIPA buffer and eluted 15 min at 80°C in SDS-PAGE sample buffer (Laemmli, 1970). Eluates were electrophoresed on 10% polyacrylamide gels and electroblotted to nitrocellulose (Schleicher & Schnuell, Keene, NH).

Preliminary experiments had previously revealed several unidentified PNA-positive components in PNA lectin precipitates from RIPA extracts of prechondrogenic micromass cultures of chick limb mesenchyme at approximately 50, 90, 120, and 200 kD. The 200 kD PNA-binding component(s) was prepared as immunogen for in vitro immunization on nitrocellulose (Gratecos et al., 1987; Sheng et al., 1987) by excision of a strip containing the 200 kD region of the membrane following verification of the presence of the immunogen by lectin blotting of a small side strip with biotinylated PNA. The immunogen-bearing nitrocellulose was minced and sonicated into smaller fragments in 0.5 ml serum free RPMI 1640 medium (Gibco, Grand Island, NY). Subsequent in vitro immunization of mouse lymphocytes in 25 cm^2 flasks (Corning Glass Works, Corning, NY) with the nitrocellulose-bound immunogen, myeloma fusion, and culture of hybridomas were performed as described previously (Capehart et al., 1997). Hybridoma supernatants were screened by indirect immunofluorescence on frozen sections of stage 24 chick limb buds. One of these, TC2, is the focus of this report. The TC2 antibody is of the IgM class and the hybridoma was subcloned twice by limiting dilution (Campbell, 1984).

Immunohistochemistry

Chick embryos were cryopreserved by freeze-substitution into ethanol (Kitten et al., 1987), embedded in paraffin, and sectioned at 5 μm. Deparaffinized sections were rehydrated through graded ethanols to PBS and blocked 1 hr at room temperature with PBS containing 3% bovine serum albumin (BSA; Sigma) and 1% normal goat serum (NGS; Cappel, Malvern, PA). Sections were incubated with TC2 hybridoma supernatants diluted 1/20 in blocking buffer overnight at 4°C. Additional antibodies were utilized as follows: Mab d1C4 IgM, which recognizes an unidentified native epitope on glycosaminoglycan chains enriched in chondroitin-6-sulfate (Capehart et al., 1997), was used as an undiluted hybridoma supernatant; Mab CS-56 IgM ascites (Sigma), which reacts with an unidenti-

fied native epitope on both chondroitin-4- and chondroitin-6-sulfate chains (Avnur and Geiger, 1984), was used at 1:4,000; rabbit polyclonal anti-versican IgG (Zanin et al., 1998), which was made against a peptide encoded in the chondroitin sulfate attachment domain α (CS-α) of chicken versican (Zako et al., 1997), was diluted 1:250. Following primary antibody incubations, specimens were washed four times with PBS and incubated 2 hr at room temperature with the appropriate fluorescein- or rhodamine-conjugated anti-mouse IgM and anti-rabbit IgG secondary antibodies (Cappel) diluted 1:100 in blocking buffer. All samples were washed five times with PBS and postfixed in 80% and 50% ethanols (5 min each). Sections were equilibrated in PBS and mounted in 10% PBS-90% glycerol containing 100 mg/ml 1,4-diazabicyclo(2,2,2)octane (DABCO; Sigma; Johnson et al., 1982).

Chick embryos were prepared for whole mount immunofluorescence labeling by fixation overnight at −20°C in 80% methanol-20% dimethylsulfoxide (DMSO; Dent and Klymkowsky, 1987). Samples were rehydrated to PBS and blocked with PBS-3%BSA-1%NGS overnight at 4°C. Embryos were incubated with undiluted hybridoma TC2 supernatants containing 1% DMSO overnight at 4°C and washed thoroughly with PBS containing 1% BSA. Samples were incubated with rhodamine-conjugated anti-mouse IgM diluted 1:100 in PBS-1% BSA-1% DMSO overnight at 4°C, washed three times with PBS-1%BSA, twice with PBS alone, and postfixed with 80% and 50% ethanols (30 min each). Embryos were equilibrated in PBS and mounted in DABCO medium.

Controls for immunohistochemistry included omission of the primary antibody or use of an irrelevant IgM for the primary antibody incubation. Specificity of TC2 immunoreactivity was also tested by inclusion of 50 μg/ml chondroitin-4-sulfate GAG (bovine trachea, Sigma) or chondroitin-6-sulfate GAG (shark cartilage, Seikagaku, Ijamsville, MD) with the primary antibody incubation. Specimens were viewed with a Zeiss Axioskop equipped with epifluorescence optics or a Bio-Rad Model MRC-1000 confocal microscopy system using standard filter and collection functions for fluorescein and rhodamine.

Characterization of the TC2 Epitope

Deparaffinized embryonic chick sections were treated for 2 hr at 37°C with 0.1 U/ml chondroitinase ABC (Sigma) or 0.2 U/ml chondroitinase AC (Sigma) in 50 mM Tris-Cl, 60 mM sodium acetate, pH 8 (Linhardt, 1994), containing 1 mM phenylmethylsulfonylfluoride, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM ethylenediaminetetraacetic acid. For hyaluronidase digestion, tissue sections were incubated 2 hr at 37°C with 0.1% testicular hyaluronidase (Sigma) or 10 U/ml Streptomyces hyaluronidase (Sigma) in 30 mM sodium acetate, pH 5.2 containing 125 mM sodium chloride (Linhardt, 1994) and protease inhibitors as above. Control sections were treated with the appropriate buffers containing protease inhibitors. Following enzyme treatments, samples were washed thoroughly with PBS before blocking and immunostaining steps. Periodate oxidation of tissue sections was performed using 10 mM periodic acid in 50 mM sodium acetate, pH 4.5, for 1 hr in the dark (Woodward et al., 1985). Samples were washed with PBS and incubated in 50 mM sodium borohydride-PBS for 30 min. Control sections were incubated in 50 mM sodium acetate alone followed by reduction with 50 mM sodium borohydride. Specimens were washed thoroughly with PBS before blocking and immunostaining.

Microtiter plates (Immulon 4; Dynex Technologies, Chantilly, VA) were treated with 1 μg/ml poly-L-lysine (Capehart et al., 1997), washed with PBS, and incubated overnight at 4°C with 0–100 μg/ml chondroitin-4-sulfate GAG (bovine trachea; Sigma) or chondroitin-6-sulfate GAG (shark cartilage; Sigma). In other experiments, poly-L-lysine-coated plates were treated as above with various GAGs at 60 μg/ml, i.e., chondroitin-4-sulfate (bovine trachea, Sigma), chondroitin-6-sulfate (shark cartilage; Seikagaku), chondroitin (from chondroitin-4-sulfate; Seikagaku), dermatan sulfate (hog skin; Seikagaku), heparan sulfate (bovine kidney, Seikagaku), or keratan sulfate (bovine cornea; Seikagaku) GAGs. In selected experiments, 100 μg/ml chondroitin-4-sulfate GAG and keratan sulfate GAG were treated with 0.2 U/ml chondroitinase ABC (Sigma) 4 hr 37°C prior to coating ELISA plates. TC2 reactivity was also evaluated with intact proteoglycans. In this instance, poly-L-lysine-coated plates were incubated 2 hr room temperature with 1 μg/ml immunoaffinity-purified aggrecan, neurocan, phosphacan, and versican proteoglycans isolated from embryonic chick brain (Zanin et al., 1998). For all analyses, wells were washed three times with PBS, treated for 1–2 hr with 1% BSA-PBS, washed with PBS again, then incubated with undiluted TC2 or control IgM hybridoma supernatants 1–2 hr at room temperature or overnight at 4°C. After four washes with PBS, plates were incubated with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgM (Sigma) in PBS-1% BSA 1–2 hr room temperature. Wells were washed five times with PBS before visualization of reactivity with 1 mg/ml p-nitrophenyl phosphate (Sigma) in 10 mM diethanolamine, pH 9.5, containing 0.5 mM magnesium chloride. Absorbance was measured at 405 nm using a Bio-Tek Microplate Autoreader (Bio-Tek Instruments, Winooski, VT).

RESULTS

In vitro immunization of mouse lymphocytes was employed to generate Mabs to an unidentified ~200 kD component found in PNA affinity eluates from extracts of prechondrogenic micromass cultures of chick limb mesenchyme. Screening of hybridomas against stage 24 chick limb sections showed strong immunoreactivity of the TC2 antibody distributed in a particulate pattern primarily in the ECM of developing chondrogenic tissues in the pectoral region and proximal wing (Fig. 1). Because of the extracellular distribution of the TC2 antigen in chondrogenic regions, TC2 reactivity on chick limb tissue sections was tested for sensitivity to testicular hyaluronidase (degrades hyaluronic acid and chondroitin sulfate GAGs), Streptomyces hyaluronidase (degrades only hyaluronic acid), and periodate oxidation. Results showed that TC2 reactivity was abolished by testicular hyaluronidase pretreatment, enhanced by Streptomyces hyaluronidase, and was insensitive to periodate oxidation (data not shown). These observations suggested that the epitope was a nonterminal carbohydrate structure within a chondroitin sulfate GAG chain or was a terminal carbohydrate sequence resistant to periodate treatment (Caterson et al., 1990).

ELISA analysis demonstrated a preferential concentration dependent reactivity of TC2 with chondroitin-4-sulfate GAG from bovine trachea and little or no reactivity with chondroitin-6-sulfate GAG from shark cartilage (Fig.

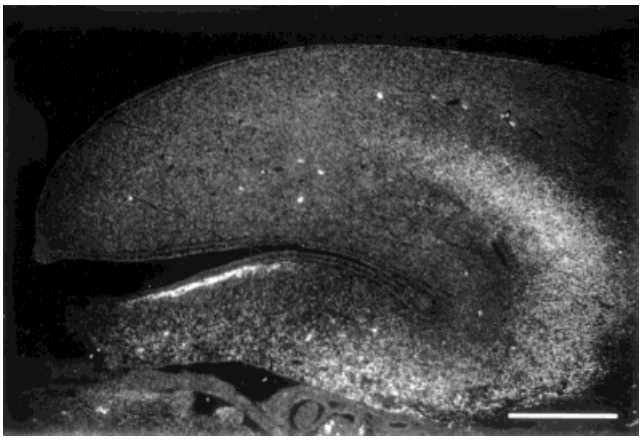

Fig. 1. TC2 immunostaining in the stage 24 chick wing bud. TC2 reactivity was localized in the developing chondrogenic core of the proximal wing and extended into the pectoral region. Note intense staining in a segment of the basement membrane of the lateral body wall inferior to the axilla. Scale bar = 250 μm.

2A). Using the same experimental conditions, Mab d1C4 was demonstrated previously to react strongly with this same chondroitin-6-sulfate GAG preparation (Capehart et al., 1997). Chondroitin-4-sulfate GAGs isolated from whale cartilage (Seikagaku) and sturgeon notochord (Seikagaku) had reduced and no reactivity with TC2, respectively, in comparison to preparations from bovine trachea (data not shown). Little TC2 reactivity was seen with a different commercial source of shark cartilage chondroitin-6-sulfate GAG or with unsulfated chondroitin (prepared from chondroitin-4-sulfate GAG), dermatan sulfate (hog skin), and heparan sulfate (bovine kidney) GAGs (Fig. 2B). Some reactivity of TC2 with keratan sulfate (bovine cornea) GAG was noted. However, when the keratan sulfate GAG preparation was treated with chondroitinase ABC, TC2 reactivity was lost (Fig. 2C). As keratan sulfate is not digested by chondroitinase ABC (Yamagata et al., 1968), this result suggested probable contamination of this particular preparation with a chondroitin sulfate GAG. TC2 also reacted with several immunoaffinity-purified intact proteoglycans isolated from embryonic chick brain, notably aggrecan and versican (Fig. 2D).

Immunohistochemical observation of younger chick embryos revealed strong TC2 reactivity preferentially in the developing heart. In whole mount preparations at stage 9, intense TC2 staining extended from the posterior heart tubes through the future right ventricle (Fig. 3). At stage 10, TC2 stained most intensely in the cardiac jelly along the basal surface of the myocardium with a decreasing gradient of reactivity extending toward the endocardium (Fig. 4A). Little or no TC2 reactivity was observed in the cardiac matrix in the vicinity of the pharyngeal endoderm. TC2 staining was also noted between the notochord and neural tube. As seen previously in the limb, pretreatment of sections with Streptomyces hyaluronidase enhanced TC2 immunoreactivity in the cardiac matrix (Fig. 4B) indicating that the TC2 epitope was masked in part by hyaluronic acid in the cardiac jelly. Pretreatment of sections with either chondroitinase ABC or AC revealed sensitivity of the TC2 epitope (Fig. 4C), further suggesting that the antigen was a chondroitin sulfate. Immunoreactivity of TC2 could be competed by preincubation of the antibody with excess chondroitin-4-sulfate GAG (Fig. 4D), whereas preincubation of the antibody with the same concentration of chondroitin-6-sulfate GAG was ineffective (Fig. 4E). As seen in Figure 4F, staining of the cardiac jelly with an anti-versican peptide IgG correlated well with TC2 reactivity in the stage 10 heart. Anti-versican immunohistochemical reactivity could be competed by preincubation with excess peptide immunogen (data not shown). Immunostaining of sister sections with CS-56, a Mab that recognizes a native epitope on both chondroitin-4- and chondroitin-6-sulfate GAG chains, showed reactivity that was clearly different from either TC2 or anti-versican staining (Fig. 4G). In the stage 10 heart, little CS-56 reactivity was seen within the cardiac jelly, but was associated predominantly with the endocardium close to the pharyngeal endoderm.

In the looped heart by stage 15, an increasing gradient of TC2 reactivity in the cardiac jelly was noted from the ventricular through the conotruncal segments (Fig. 5A). In the conotruncus, TC2 staining was more intense adjacent to the myocardium and decreased toward the endocardium similar to that observed in the primitive ventricle at stage 10 (Fig. 4A). There was an abrupt loss of TC2 staining noted in the transitional area between the definitive heart and the anterior splanchnic mesoderm of the aortic sac. In the atrioventricular canal, TC2 stained strongly in both atrioventricular swellings, particularly along the inner myocardium (Fig. 5B). Double labeling of sections with anti-versican revealed co-localization of the versican core protein and the TC2 epitope in the cardiac jelly of the atrioventricular canal (Fig. 5C). However, in the conotruncus anti-versican stained the entire expanse of the cardiac jelly with only a slight decrease in staining intensity closer to the endocardium.

Interestingly, TC2 stained differentially within individual segments of the stage 16 heart. At the level of the dorsal mesocardium in the stage 16 heart (Fig. 6A), little TC2 reactivity was present in the atrium or along the inner curvature closest to the atrium. However, immunoreactivity increased in the ventricle adjacent to the conus. In a slightly more cranial section, TC2 localization in the cardiac jelly extended into the left side of the atrium and increased in the ventricle and toward the conotruncus (Fig. 6B). At the level of the atrioventricular cushions, relatively weak TC2 staining was present in the matrix of the inferior atrioventricular cushion, while the matrix of the superior cushion was stained strongly (Fig. 6C). In this plane of section, TC2 reactivity appeared continuous from the atrium through the ventricle and into the conotruncus.

In the atrioventricular region of the stage 17 heart, the differential pattern of staining with TC2 became more pronounced in the cardiac jelly (Fig. 7A). In addition, staining was also now apparent within the myocardium of both cushions closest to the ventricle. In the inferior cushion, TC2 staining was restricted to the cardiac jelly and myocardium of both the atrial and ventricular extensions. Pretreatment of sections with Streptomyces hyaluronidase intensified TC2 immunoreactivity, but did not appreciably alter the differential staining pattern in either atrioventricular cushion (Fig. 7C). Double labeling of sections with anti-versican revealed a generally overlapping distribution of staining with TC2 in the superior cushion with the exception that weak anti-versican staining extended into the atrial aspect (Fig. 7D). While the

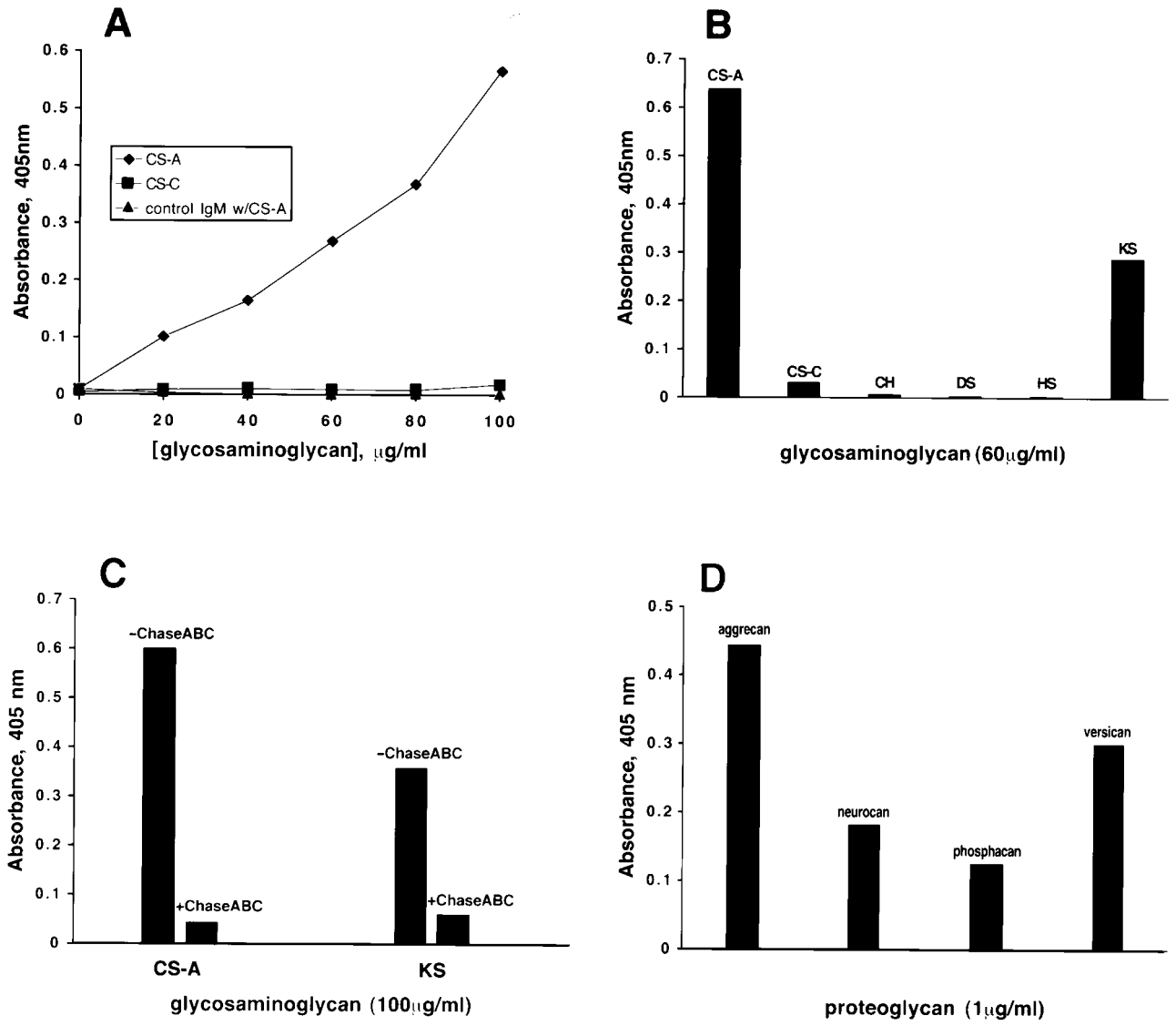

Fig. 2. ELISA analysis shows that TC2 reacts preferentially with glycosaminoglycans (GAGs) enriched in chondroitin-4-sulfate. **A:** Plates wells were treated with increasing concentrations of chondroitin-4-sulfate (CS-A) and chondroitin-6-sulfate (CS-C) GAGs. Plates were incubated with TC2 IgM or irrelevant IgM hybridoma supernatants. Note concentration dependent reactivity of TC2 with CS-A, but not CS-C. **B:** Plates were treated with 60 μg/ml CS-A, CS-C, chondroitin (CH), dermatan sulfate (DS), heparan sulfate (HS), or keratan sulfate (KS) GAGs. Note reactivity of TC2 with CS-A and KS, and lack of reactivity with other GAGs. **C:** Plates were treated with 100 μg/ml CS-A or KS GAGs without (−Chase ABC) or with (+Chase ABC) 0.2 U/ml chondroitinase ABC pretreatment. TC2 reactivity with both CS-A and KS GAGs was lost following enzyme pretreatment. **D:** Plates were treated with 1 μg protein/ml immunoaffinity purified aggrecan, neurocan, phosphacan, or versican proteoglycans isolated from embryonic chick brain tissue. The TC2 epitope was represented on each of the proteoglycans tested.

atrial and ventricular extensions of the inferior cushion stained similarly with TC2, the mid-region was only positive with the anti-versican antibody. In contrast to TC2, CS-56 reacted strongly with the outer myocardium throughout the atrioventricular region as well as with invading mesenchymal cells (Fig. 7E). Overlap of CS-56 reactivity with TC2 was seen in the atrial extension of the inferior cushion, but was also observed in the TC2-negative region leading from the atrium into the superior cushion. As seen in Figure 7F, intense TC2 reactivity was also associated with the proepicardium. In cross sections of the stage 17 heart, TC2 exhibited intense reactivity in the matrix around the forming septum primum of the atrium (Fig. 8A). Staining extended from the atrial aspect of the inferior cushion into the matrix on the left side of atrium and was intensified by pretreatment of sections with Streptomyces hyaluronidase (Fig. 8B). Little TC2 staining was observed on the right side of the atrium. Mab d1C4, which recognizes an epitope on native GAG chains enriched in chondroitin-6-sulfate, showed little reactivity with the matrix surrounding the septum primum, but stained the cardiac jelly in the remainder of the atrium as well as scattered sites within the atrial myocardium (Fig. 8C). Reactivity with d1C4 was also intensified by Strepto-

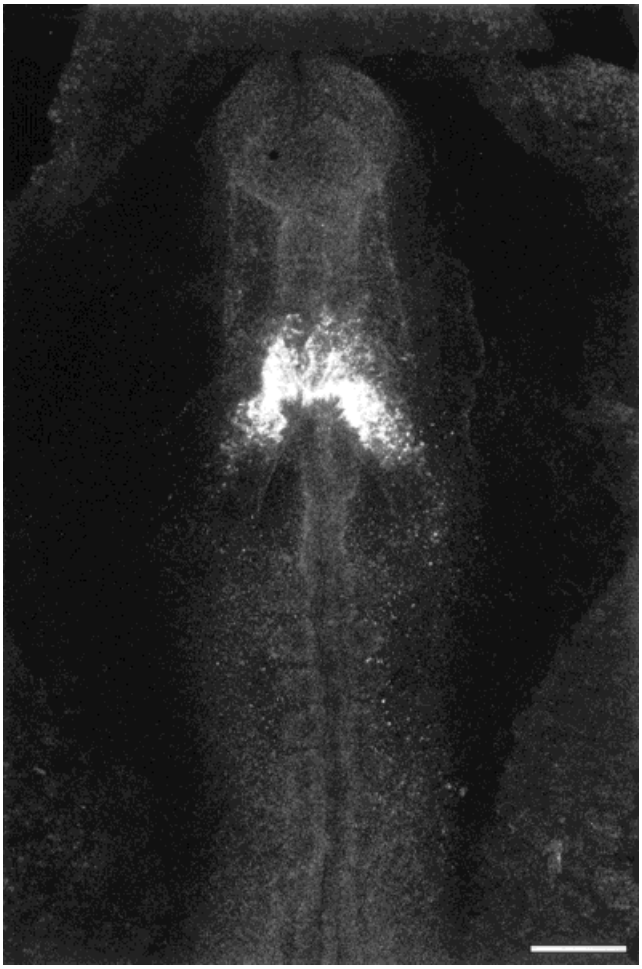

Fig. 3. The stage 9 chick heart reacts strongly with TC2. In whole mount embryo immunohistochemical preparations, TC2 localized preferentially with the developing tubular heart. TC2 staining was noted from the posterior heart tubes through the future right ventricle. Scale bar = 250 μm.

myces hyaluronidase pretreatment (Fig. 8D). CS-56 stained the cardiac jelly intensely throughout the atrium, including that of the septum primum, and numerous foci within the myocardium (Fig. 8E). Double labeling of TC2-stained

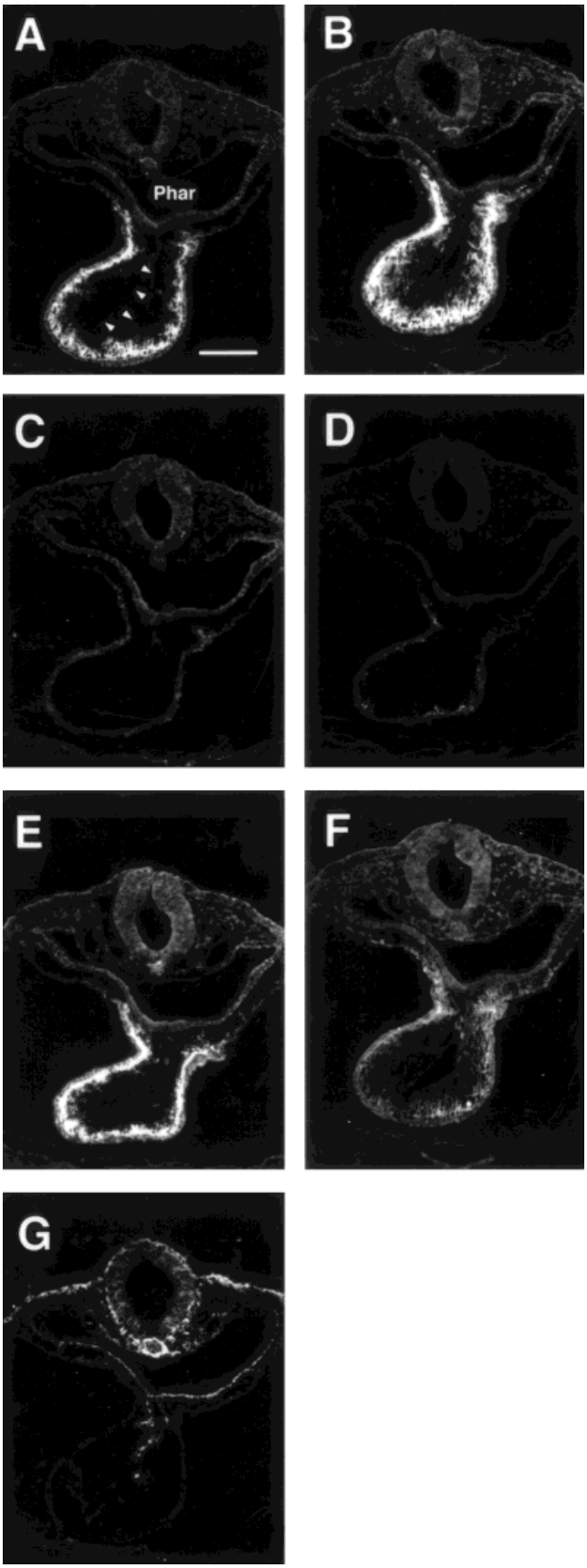

Fig. 4. TC2 detects a myocardially-derived component of cardiac jelly in the stage 10 chick heart. **A:** In the ventricle, a decreasing gradient of TC2 reactivity in the cardiac jelly extended from the myocardium toward the endocardium (denoted by arrowheads). Little TC2 reactivity was noted in the matrix in the vicinity of the ventral endoderm of the pharnyx (Phar). **B:** TC2 reactivity was enhanced in the cardiac jelly and extended further toward the endocardium following pretreatment of sections with 10 U/ml Streptomyces hyaluronidase. **C:** Pretreatment of sections with 0.2 U/ml chondroitinase AC abolished TC2 staining. **D:** Competition of TC2 immunoreactivity by addition of excess chondroitin-4-sulfate GAG (from bovine trachea, 50 μg/ml) essentially blocked immunoreactivity. **E:** Co-incubation of TC2 with chondroitin-6-sulfate GAG (from shark cartilage, 50 μg/ml) did not inhibit immunostaining. **F:** Double labeling of section in A with anti-versican (peptide immunogen) showed co-localization with TC2 in the cardiac jelly. **G:** CS-56 (a commercially available Mab to a native chondroitin sulfate GAG epitope) detected primarily endocardium in the vicinity of the pharyngeal endoderm. Little CS-56 reactivity was observed in the ventricular cardiac jelly. Scale bar = 100 μm.

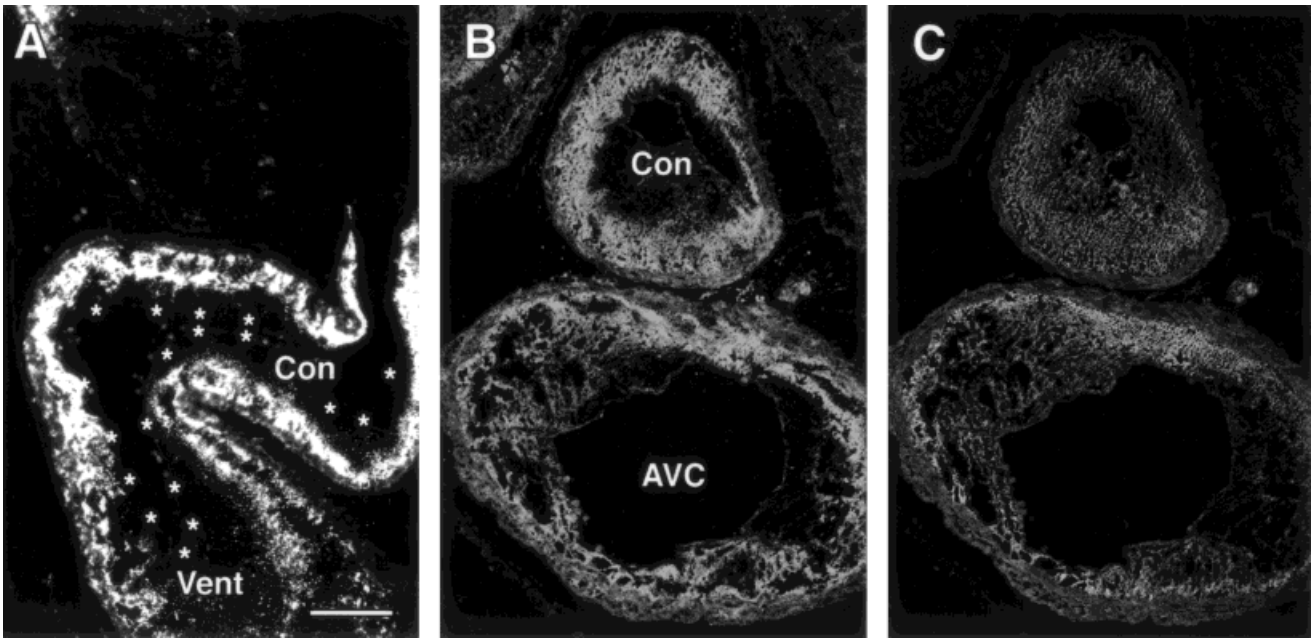

Fig. 5. In the stage 15 chick heart, TC2 reactivity is strongest in the conotruncus. A: Cross section; B,C: double-stained sagittal section. **A:** TC2 staining in the cardiac jelly increased in intensity from the ventricle (Vent) through the conotruncus (Con). In the conotruncal region, TC2 staining was most intense adjacent to the myocardium and diminished markedly toward the endocardium (outlined by asterisks). **B:** TC2 stained the cardiac jelly of the atrioventricular canal (AVC) more uniformly than in the conotruncus. **C:** Double labeling of section in B with anti-versican showed co-distribution of versican and the TC2 antigen in the AVC. In the conotruncus, anti-versican staining was also stronger adjacent to the myocardium, but reactivity diminished only slightly toward the endocardium. Scale bar = 100 μm.

sections with anti-versican showed localization of versican core protein throughout the atrial cardiac jelly with particularly intense reactivity in the matrix surrounding the septum primum (Fig. 8F).

In the stage 18 heart, the matrix of the elongating primary atrial septum stained intensely with both TC2 and anti-versican (Fig. 9A,B). Staining with both antibodies occurred in the matrix in the superior atrioventricular cushion, but more strongly adjacent to the myocardium from the atrium to the ventricle. Intense TC2 reactivity was seen in the cardiac jelly along the ventricular trabeculae, while anti-versican staining was restricted to the tips. The inferior atrioventricular cushion exhibited little TC2 reactivity in general, however, strong signal was observed adjacent to the myocardium on the ventricular side of the atrioventricular groove (Fig. 9A). In contrast to TC2, anti-versican reactivity was present in both the atrial and ventricular extensions of the inferior atrioventricular cushion. Anti-versican staining was persistent in the cardiac jelly as well.

In regions of the stage 18 superior atrioventricular cushion, TC2 reactivity was noted along the leading front of migrating mesenchyme (Fig. 9C). In contrast, d1C4 stained most intensely around migrating cushion mesenchyme in both cushions (Fig. 9D). TC2 also reacted strongly with matrix surrounding mesenchyme in the proepicardium at its origin from the sinus venosus and in its extensions toward the heart, but the epicardium covering the heart was unreactive (Fig. 9E). Streptomyces hyaluronidase pretreatment intensified TC2 staining in the proepicardium and revealed additional epitopes in the vicinity of the sinoatrial junction (Fig. 9F). Removal of hyaluronic acid also increased TC2 reactivity in the superior atrioventricular cushion, particularly within the cardiac jelly associated with endocardium along the atrial aspect of this cushion.

The overall staining intensity of TC2 in the heart was greatly reduced by stage 23, however, a restricted strong expression was retained in subcompartments of the atrioventricular cushions and in the conotruncus. TC2 reactivity in the superior atrioventricular cushion was largely confined to the cardiac jelly adjacent to the myocardium along its ventricular aspect (Fig. 10A,B). In contrast to d1C4 localization (Fig. 10C), little TC2 staining was observed in association with mesenchyme in this cushion. In the inferior atrioventricular cushion, TC2 again stained along the myocardium on the ventricular aspect, but to a lesser extent than in the superior cushion. Intense TC2 reactivity was noted, however, in the inferior atrioventricular cushion in proximity to the atrium similar to that observed at earlier stages of heart development, except that this region now contained mesenchymal cells. Only little change in TC2 staining in the atrioventricular region was observed at stage 23 following pretreatment of sections with Streptomyces hyaluronidase (data not shown). TC2 reactivity in the cardiac jelly of the ventricular trabeculae (Fig. 10A) was much reduced as compared with staining at stage 18 (Fig. 9A). In contrast to the marked reduction in TC2 immunoreactivity in the atrioventricular cushions and ventricle at stage 23, widespread TC2 staining persisted in the cardiac jelly of the conotruncus except in the matrix immediately around most mesenchymal cells (Fig. 11A). Streptomyces hyaluronidase pretreatment enhanced TC2 reactivity notably in the cardiac jelly of the outlet limb of the heart at stage 23, but the matrix around most mesenchymal cells remained unreactive (Fig. 11B).

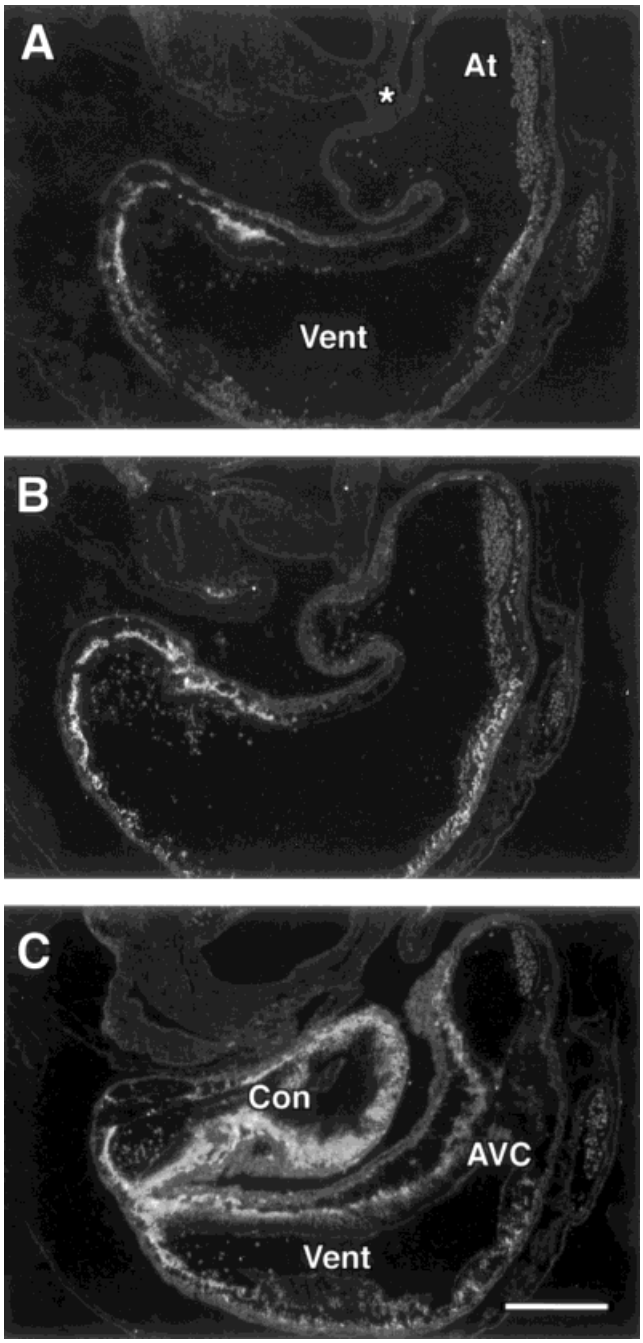

Fig. 6. Regions of the common atrium and lesser curvature of the stage 16 chick heart have little or no TC2 reactivity. **A:** At the level of the dorsal mesocardium (asterisk) in an oblique section through the heart, little TC2 reactivity was noted in either side of the common atrium (At) or the lesser curvature. TC2 reactivity increased in the ventricle (Vent) as the outlet limb of the heart was approached. Note also TC2 staining in the cardiac jelly on the left side of the atrioventricular canal. **B:** In a more cranial section, TC2 staining in the cardiac jelly extended into the left side of the atrium. The lesser curvature was still negative, but there was increased TC2 reactivity in the ventricle. **C:** More cranially, at the level of the atrioventricular canal (AVC), an increasing gradient of TC2 reactivity extended from the rightward aspect of the atrium through the conotruncus (Con). TC2 reactivity was greater in the superior atrioventricular cushion than in the inferior cushion. Scale bar = 250 μm.

DISCUSSION

The present data suggest that the TC2 epitope is a component of GAG chains enriched in chondroitin-4-sulfate that occur on a variety of proteoglycan core proteins. The backbone of chondroitin sulfate GAGs is composed of repetitive glucuronic acid-N-acetylgalactosamine disaccharide units of varied chain length, level of saccharide substitution, and, very importantly, sulfation pattern. Such structural modifications may be responsible for functional differences among individual chondroitin sulfate GAG chains and are targets for tissue-specific or developmental regulation (Seno et al., 1974; Caterson et al., 1990; Sorrell et al., 1990, 1993; Fernandez-Teran et al., 1993, Nadanaka et al., 1998). Further investigation will be required to determine whether the TC2 epitope resides on the common backbone structure of chondroitin-4-sulfate chains (glucuronic acid-N-acetylgalactosamine-4-sulfate), on a more highly sulfated sequence, or potentially on some other atypical structural component of a chondroitin sulfate GAG chain. Identification of the TC2 epitope should yield insight into candidate enzymatic activities that might be linked to cardiac morphogenetic anomalies.

Removal of hyaluronic acid from the cardiac matrix by pretreatment of sections with Streptomyces hyaluronidase often served to enhance TC2 reactivity, particularly in the younger stages examined. The presence of hyaluronate in the developing heart has been well documented (Markwald and Adams-Smith, 1972; Manasek et al., 1973; Markwald et al., 1977, 1978; Baldwin et al., 1994). It is thought that this GAG interacts with other matrix components, hydrates and expands the acellular cardiac matrices, and promotes cell migration in th eendocardial cushions. Our finding that enhancement of TC2 reactivity following pretreatment with Streptomyces hyaluronidase was greater in the pre-migratory endocardial cushions than after they were well populated with mesenchyme is consistent with the previously reported decrease in hyaluronic acid levels in the post-migratory endocardial cushion matrix (Markwald et al., 1978). Even though TC2 recognizes a native chondroitin sulfate GAG epitope (one that does not require pretreatment with chondroitinases for reactivity), the uncovering of additional binding sites for TC2 by Streptomyces hyaluronidase preincubation underscores the importance of recognizing the potential for the masking of epitopes due to matrix interactions and/or fixation effects when employing immunohistochemical methods for extracellular antigen localization.

In the present study, strong immunolocalization of the chondroitin sulfate GAG epitope recognized by TC2 was found throughout the cardiac jelly of the early straight tube heart. The decreasing gradient of staining from the myocardium to the endocardium suggested that the TC2 antigen was synthesized by the myocardium. Little TC2 reactivity was associated with the ventral foregut endoderm at these stages. By ultrastructural analysis, Hurle and Ojeda (1977) described previously a heterogenous composition of the cardiac jelly, with one region derived from the myocardium and endocardium and another synthesized by the foregut endoderm and adjacent endocardium. Interestingly, TC2 reactivity was limited to regions described by these authors as myocardial/endocardial-derived cardiac jelly and was absent from areas presumably produced by the endoderm and neighboring endocar-

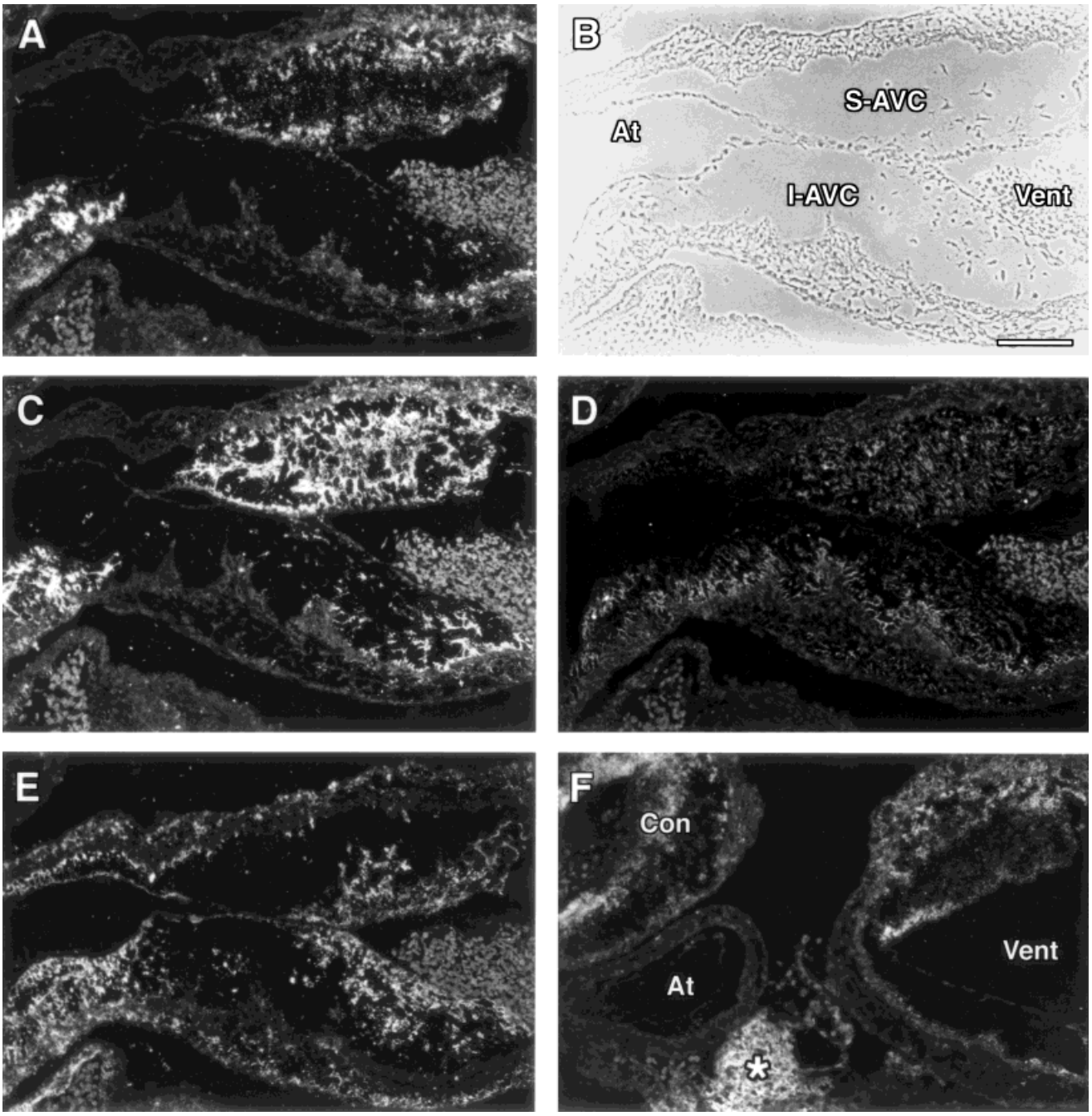

Fig. 7. TC2 immunostaining is distributed differentially in sagittal plane through the atrioventricular cushions in the stage 17 chick heart. **A:** TC2 preferentially stained the cardiac jelly of the superior atrioventricular cushion at this stage of development. TC2 reactivity was restricted to the atrial and ventricular appendages of the inferior atrioventricular cushion. **B:** Phase contrast image of A. **C:** Pretreatment with 10 U/ml Streptomyces hyaluronidase enhanced TC2 reactivity in the cardiac jelly of both atrioventricular cushions, but did not alter the basic pattern of staining. **D:** Double labeling of section in A with anti-versican showed co-distribution of TC2 antigen and versican in the superior atrioventricular cushion. Note differences between TC2 and anti-versican reactivities in the mid-region of the inferior cushion. **E:** CS-56 staining was primarily cell associated, detecting both the myocardium and migrating cushion mesenchyme. TC2 and CS-56 staining overlapped in the atrial extension of the inferior atrioventricular cushion whereas CS-56 reacted with the atrial aspect of the superior cushion that was TC2-negative. **F:** TC2 reacted strongly with the proepicardium (asterisk) in a right parasagittal section. There was a lack of TC2 reactivity at this level in the atrium and differential distribution of TC2 reactivity in the cardiac jelly of the ventricular inlet. TC2 reactivity was persistent in the matrix of the conotruncus. At, atrium; Con, conotruncus; I-AVC, inferior atrioventricular cushion; S-AVC, superior atrioventricular cushion; Vent, ventricle. Scale bar = 100 μm.

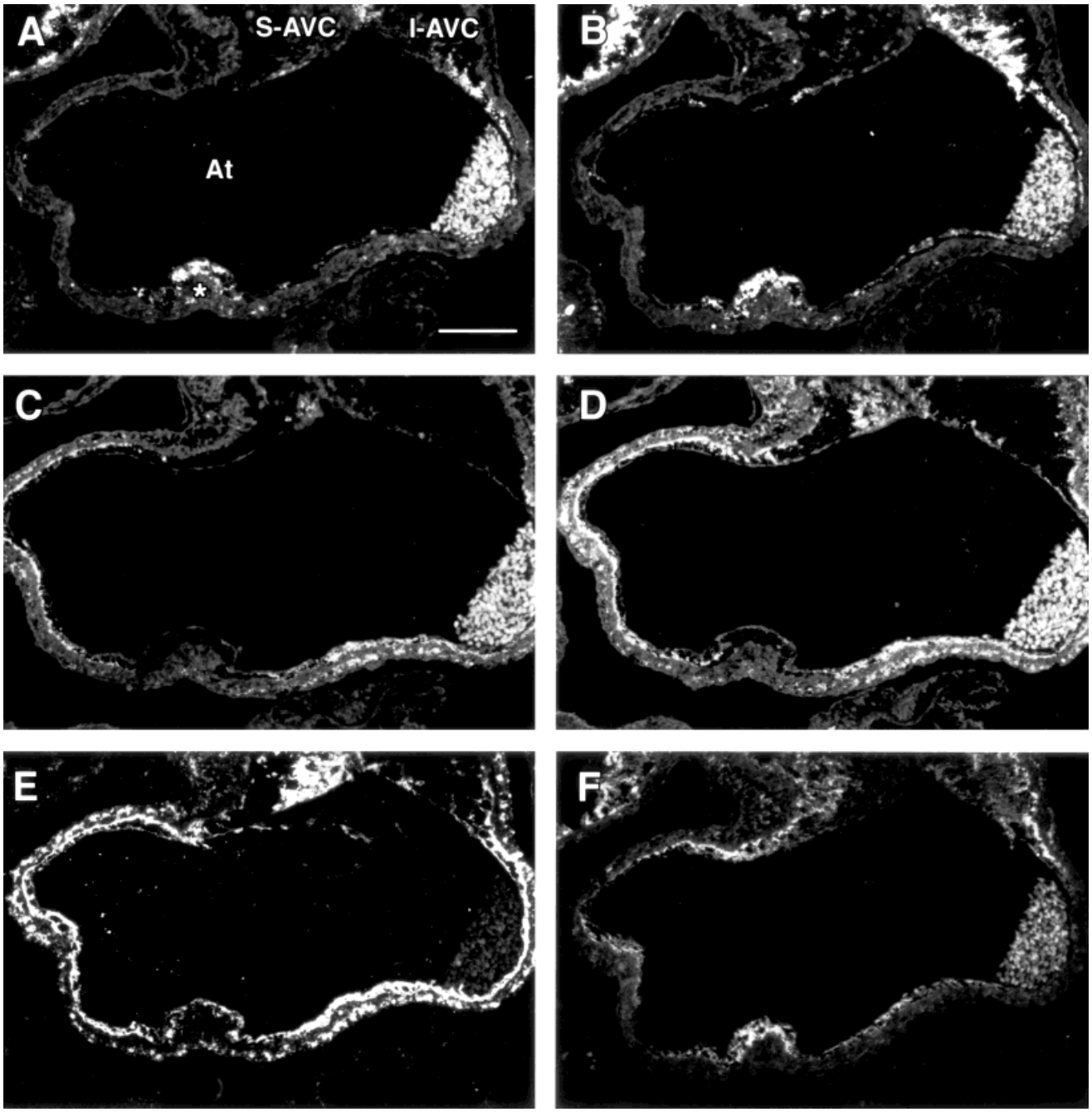

Fig. 8. TC2 immunoreactivity is localized to the matrix surrounding the forming septum primum in cross sections of the stage 17 chick atrium. **A:** TC2 preferentially stained the cardiac jelly adjacent to the septum primum (asterisk). **B:** Pretreatment with 10 U/ml Streptomyces hyaluronidase enhanced TC2 reactivity especially in the atrial extension of the inferior atrioventricular cushion. **C:** d1C4 stained scattered sites within and along the atrial myocardium. Little reactivity was associated with the matrix surrounding the septum primum. **D:** Pretreatment with 10 U/ml Streptomyces hyaluronidase resulted in little change in d1C4 reactivity in the matrix around the septum primum, however, staining was accentuated elsewhere. **E:** CS-56 stained numerous sites within the atrial myocardium and cardiac jelly. **F:** Double labeling of section in B with anti-versican showed co-localization of the TC2 antigen and versican surrounding the septum primum. In contrast to TC2, note widespread anti-versican staining in the atrial cardiac jelly. At, atrium; I-AVC, inferior atrioventricular cushion; S-AVC, superior atrioventricular cushion. Scale bar = 100 μm.

dium. Mab d1C4 represents at least a portion of the endodermal contribution (unpublished observations).

The formation of the vertebrate heart tube involves the progressive addition of primitive segments (de la Cruz, 1989), yet the precise origin of the conotruncus or outlet limb of the heart remains unclear. It has been suggested by Markwald et al. (1998) and Mjaatvedt et al. (1998) that the conotruncus arises indirectly from the right ventricle or is recruited from the anterior extracardiac splanchnic mesoderm that is subsequently incorporated into the developing heart tube. It is evident from the intense TC2 immunoreactivity observed in the conotruncus of the looped heart

that the chondroitin sulfate GAG epitope recognized by TC2 is an important constituent of the cardiac matrix during the formation of this outlet segment. The strong staining of the conotruncus is accentuated by a general reduction in TC2 reactivity in the other segments.

The atrioventricular cushions are formed by an epithelial-to-mesenchymal transformation of endocardium beginning between stages 16–18 (Moreno-Rodriguez et al., 1997). These swellings function as primitive valves (Patten et al., 1948) and contribute to the formation of mature valvuloseptal structures (Van Meirop et al., 1962). It is apparent from the distribution of the TC2 chondroitin sulfate GAG antigen that asymmetries exist in the matrices of the superior and inferior atrioventricular cushions. While TC2 reactivity was more evenly distributed through the cardiac jelly of the superior cushion, it was limited to the atrial and ventricular extensions of the inferior cushion at stages 17–18. It is uncertain why TC2 reactivity was more prevalent in the superior cushion, but the initial formation of this cushion occurs later in comparison to the inferior cushion (de la Cruz et al., 1983). Moreover, Moreno-Rodriguez et al. (1997) have demonstrated differences in the time of formation and distribution of mesenchyme along the length of each atrioventricular cushion. Perhaps the differential localization of the TC2 antigen plays a role in the temporal and spatial distribution of mesenchyme invasion or contributes to biophysical changes in the cushions that occur as cardiac development proceeds (Van Mierop et al., 1962; de la Cruz et al., 1983). As mesenchyme invaded the atrioventricular cushions initially, TC2 reactivity was diminished behind the leading front of migratory cells. As the cushions became more densely populated with mesenchyme, TC2 reactivity was reduced dramatically, being confined essentially to the cardiac jelly immediately adjacent to the myocardium, which has also been reported by Zanin et al. (1998) for versican localization. It is unclear whether the TC2 epitope was masked by other matrix components produced by the mesenchyme or the more likely possibility that the proteoglycan antigen was degraded by the migrating cells as suggested by Zanin et al. (1998). However, pretreatment of sections with Streptomyces hyaluronidase did not significantly increase TC2 staining in these densely populated cushion areas, indicating that the epitope was not masked by hyaluronic acid. Interestingly, intense reactivity persisted in the matrix of the atrial aspect of the inferior cushion even though mesenchyme cells were present.

In addition to TC2, we utilized two other antibodies that recognize native epitopes on chondroitin sulfate GAG chains to show a heterogeneous distribution of chondroitin sulfate epitopes during early cardiac development. While TC2 reacted primarily with the acellular matrix of the endocardial cushions, d1C4 exhibited little reactivity with

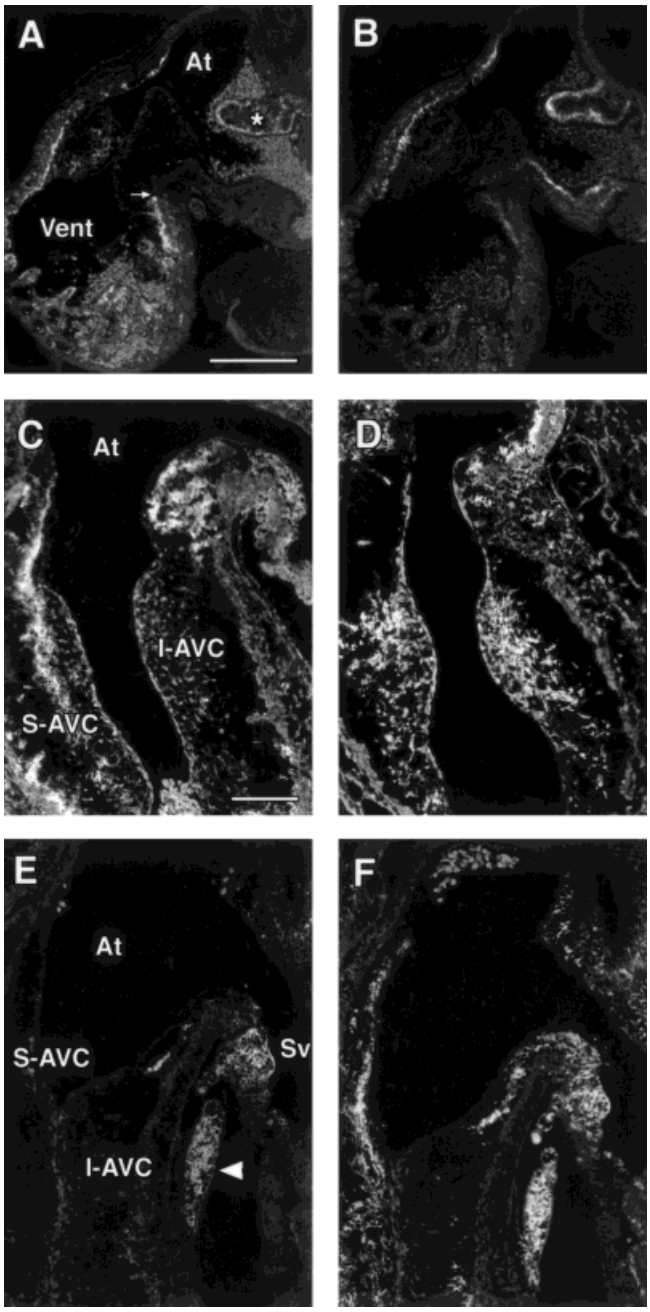

Fig. 9. TC2 immunoreactivity in the stage 18 heart. A,B: Cross sections; C–F, sagittal sections. **A:** In the atrium (At), TC2 staining was limited to the matrix surrounding the septum primum (asterisk). In the ventricle (Vent), TC2 stained the cardiac jelly primarily around the trabeculae. Note TC2 reactivity within the cardiac jelly and lining the myocardium adjacent to the superior atrioventricular cushion. TC2 also stained adjacent to the myocardium in the inferior cushion, but only along the ventricular side up to the atrioventricular groove (arrow). **B:** Versican localization co-distributed with TC2 around the septum primum and lining the myocardium adjacent to the superior atrioventricular cushion. In contrast to TC2, anti-versican staining lined the myocardium on both the atrial and ventricular aspects of the inferior atrioventricular canal. **C:** In the superior atrioventricular cushion (S-AVC), TC2 reactivity was reduced in regions populated with mesenchyme. There was little staining in the inferior atrioventricular cushion (I-AVC) except in the atrial extension of the inferior cushion as noted in previous stages. Section was pretreated with 10 U/ml Streptomyces hyaluronidase. **D:** In contrast to TC2, d1C4 stained migrating mesenchyme in both atrioventricular cushions. Both d1C4 and TC2 reactivity was observed in the cardiac jelly leading from the inferior cushion into the atrium in this Streptomyces hyaluronidase pretreated section. **E:** TC2 stained the proepicardium organ at its origin from the sinus venosus (Sv) and in its extension toward the heart (arrowhead). **F:** TC2 reactivity was enhanced in the matrix of the superior atrioventricular cushion following 10 U/ml Streptomyces hyaluronidase pretreatment. Note also increased TC2 reactivity lining the myocardium along the ventricular aspect of the inferior cushion and in the cardiac jelly of the atrial extension of the inferior cushion. Scale bars = 250 μm, A,B; 100 μm, C–F.

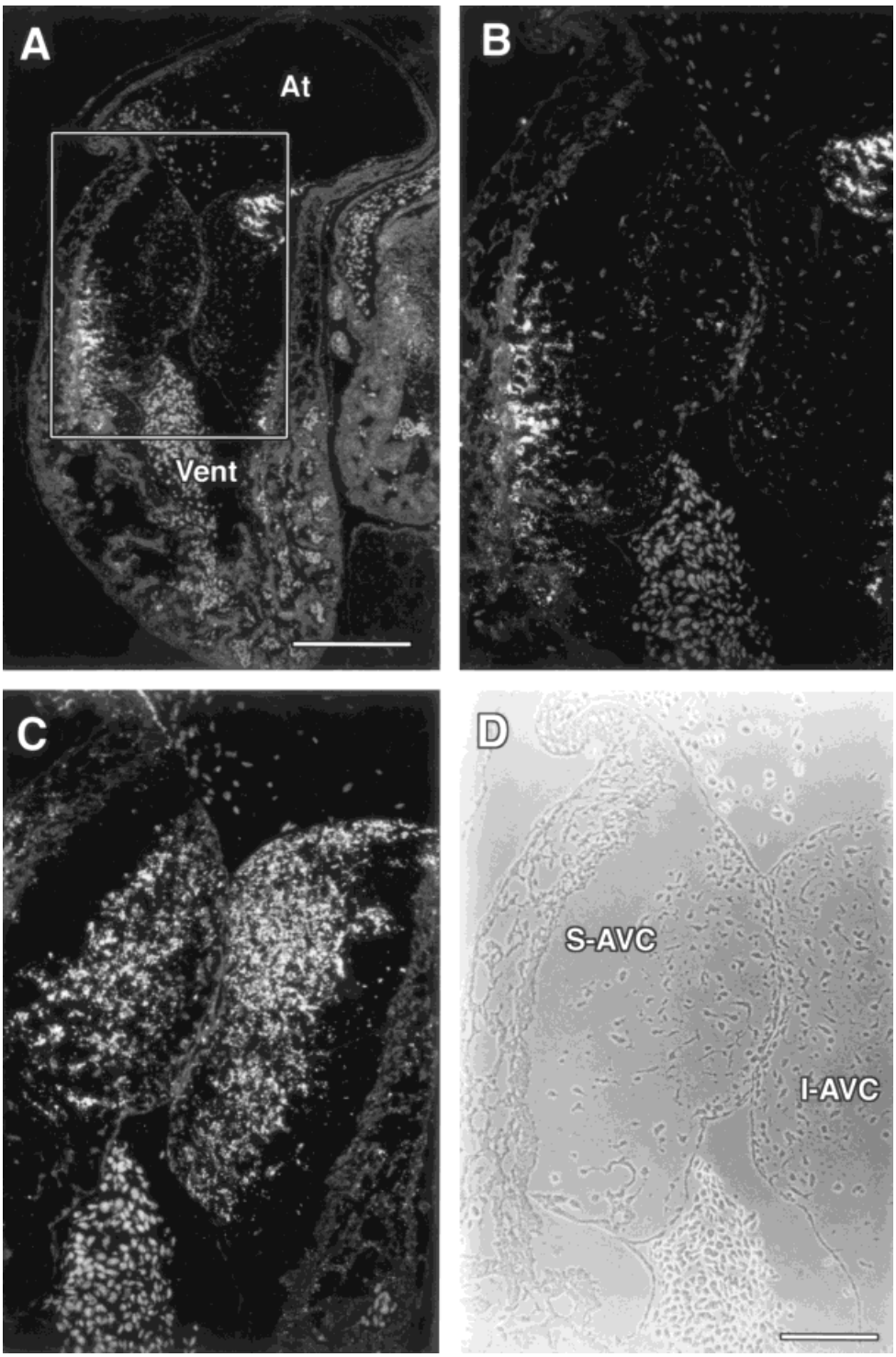

Fig. 10. Regionalized TC2 immunoreactivity persists in the atrioventricular and ventricular segments in cross sections through the chick heart at stage 23. **A:** TC2 staining was restricted primarily to the ventricular aspect of the superior atrioventricular cushion matrix and associated myocardium and to the atrial aspect of the inferior cushion. There was little or no TC2 reactivity in the matrix lining the ventricular trabeculae. **B:** Higher magnification of boxed area in A. **C:** d1C4 staining was associated primarily with cushion mesenchyme. **D:** Phase contrast image of B. At, atrium; I-AVC, inferior atrioventricular cushion; S-AVC, superior atrioventricular cushion; Vent, ventricle. Scale bars = 250 μm, A; 100 μm, B–D.

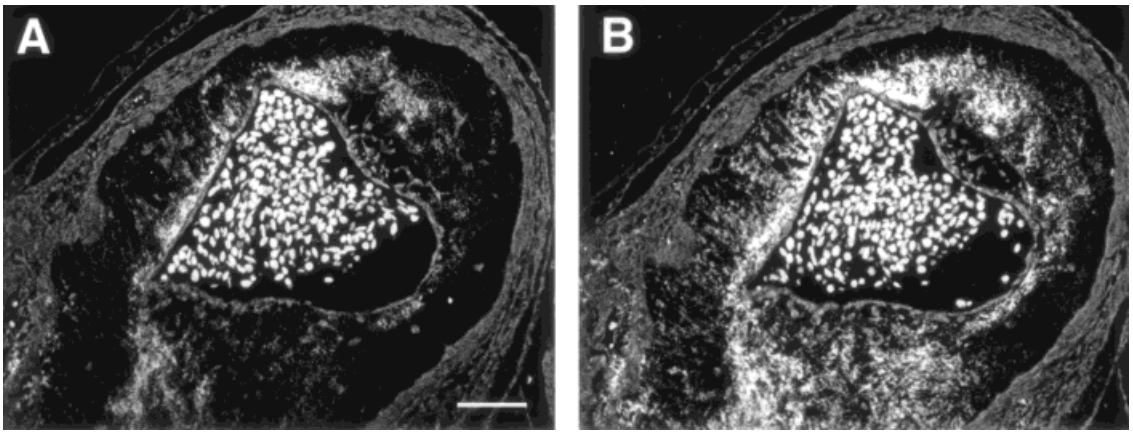

Fig. 11. Intense TC2 immunoreactivity is retained in cross sections through the conotruncus of the stage 23 chick heart. **A:** In contrast to the atrioventricular region at stage 23, widespread TC2 staining was found in the cardiac jelly of the conotruncal segment except in the matrix surrounding most mesenchymal cells. **B:** Pretreatment with 10 U/ml Streptomyces hyaluronidase enhanced TC2 reactivity in the cardiac jelly, but the matrix around most mesenchyme remained unstained. Scale bar = 100 μm.

the premigratory matrix, but intensely stained migrating mesenchyme. CS-56 showed reactivity in some areas of the acellular cushion matrix, but also stained both migrating mesenchymal cells and myocardium. Funderburg and Markwald (1986) proposed that two chondroitin sulfate proteoglycans were present in the atrioventricular cushions, a myocardially-derived species that predominated in the premigratory cardiac jelly and a mesenchymal-derived proteoglycan that served to condition the matrix and restrict the migration of trailing invasive cells. The differential immunohistochemical localization of chondroitin sulfate GAG epitopes recognized by the Mabs in the present study lends support to this hypothesis, although changes in GAG substitution on a single chondroitin sulfate proteoglycan species remains a possibility.

Contrasting patterns of reactivity using Mabs TC2, d1C4, and CS-56 were also observed in the atrium during formation of the primary atrial septum. Atrial septation in the chick heart begins with the ingrowth of the septum primum from the dorsal wall of the primitive atrium and is later accompanied by extracardiac mesenchyme from the dorsal mesocardium termed the spina vestibuli (Asami and Koizumi, 1995; Tasaka et al., 1996; Markwald et al., 1997). Rogers and Morse (1986) demonstrated histochemically the presence of sulfated GAGs in the leading edge of the developing septum primum in the rat heart and proposed a role for these components in atrial septation. The identity of these sulfated materials, however, was not determined. Of all the antibodies tested in the present study, only TC2 reactivity was restricted to the matrix surrounding the forming septum primum. While the reason for the differential expression of these chondroitin sulfate GAG epitopes around the developing primary atrial septum is unclear, it has been proposed that endocardial cells at the leading edge of the growing septum also transform into mesenchyme (Gerety and Watanabe, 1997) and, together with mesenchyme derived from the spina vestibuli, directs formation of the primary atrial septum (Markwald et al., 1998). As in the atrioventricular cushions, perhaps the presence of the TC2 antigen in the matrix surrounding the forming septum primum influences endocardial transformation.

Chondroitin sulfate GAG chains on a given proteoglycan may differ dependent upon tissue type and time of expression (Mark et al., 1989; Caterson et al., 1990; Sorrell et al., 1990; Fernandez-Teran et al., 1993), thus TC2 reactivity with any given proteoglycan does not necessarily dictate that the epitope will be present on the same proteoglycan core protein at a different stage of development or in all tissues, nor does it imply that the TC2 epitope is exclusive to any particular proteoglycan. However, in the early stages of cardiac development examined in this study, TC2 reactivity was noted to correlate well with staining for the large chondroitin sulfate proteoglycan, versican, using an antibody specific to a peptide sequence encoded by the CS-α domain (Zanin et al., 1998). Interestingly, TC2 reactivity was present in the heart tube of wild-type mice at 8.5–9.0 days postcoitum, but was absent from homozygous *hdf* mutants that have a disrupted versican gene (Mjaatvedt et al., 1998), suggesting that at this stage of development versican is the only TC2-reactive proteoglycan found in the early mouse heart. The morphological consequence of this disruption is that both the conotruncus and the endocardial cushions are absent, emphasizing the importance of versican in the normal development of these heart segments. While close correlation between the staining patterns of TC2 and anti-versican in the chick existed in the early tubular heart and reactivities of both antibodies overlapped in most instances as development proceeded, some differences were also apparent. Such differences could arise by regional alternative processing of versican GAG attachment domains (Zako et al., 1997), masking of the TC2 epitope by matrix components other than hyaluronic acid, or expression of different proteoglycans bearing the TC2 epitope.

The present study demonstrates a striking heterogeneity in the localization of chondroitin sulfate GAG epitopes in the developing heart. Many of the functions of proteoglycans are due to their associated GAG chains (Rouslahti, 1988; Kjellen and Lindahl, 1991), which may be specifically modified in response to changing functional needs during tissue differentiation (Mark et al., 1989; Caterson et al., 1990; Sorrell et al., 1990; Fernandez-Teran et al., 1993). Expression of specific sulfated GAG sequences is a

dynamic process during cardiac development and the differential localization of chondroitin sulfate GAG epitopes recognized by Mabs TC2, d1C4, and CS-56 reflect a microheterogeneity of proteoglycan expression, turnover, and intermolecular associations in the preseptated heart. These patterns may be linked to modulation of proteoglycan function in different regions of the heart, including establishment and modification of matrix structure or interaction with other molecules, such as the binding of specific growth factors. Studies are under way to characterize the TC2 epitope, identify alternative TC2-positive proteoglycans in the preseptation endocardial cushions, and evaluate the functional significance of TC2 epitope masking.

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