Myosin Types and Fiber Types in Cardiac Muscle. III. Nodal Conduction Tissue

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Abstract. The sinoatrial (SA) and atrioventricular (AV) nodes are specialized centers of the heart conduction system and are composed of muscle cells with distinctive morphological and electrophysiological properties. We report here results of immunofluorescence and immunoperoxidase studies on the bovine heart showing that a large number of SA and AV nodal cells share a distinct type of myosin heavy chain (MHC) which is not found in other myocardial cells and can thus be used as a cell-type–specific marker. The antibody used in this study was raised against fetal skeletal myosin and reacted with fetal skeletal but not with adult skeletal MHCs. Both atrial and ventricular fibers, as well as fibers of the ventricular conduction tissue were unlabeled by this antibody. Specific reactivity was exclusively seen in most cells in the central portions of the SA and AV nodes and rare cells in perinodal areas. However, a number of nodal cells, particularly those located in the peripheral nodal regions, were unreactive with this antibody. The myosin composition of nodal tissues was also explored using two antibodies reacting specifically with α-MHC, the predominant atrial isoform, and β-MHC, the predominant ventricular isoform. Most nodal cells were reactive for α-MHC and a number of them also for β-MHC. Variation in reactivity with the two antibodies was also observed in perinodal areas: at these sites a population of large fibers reacted exclusively for β-MHC. These findings point to the existence of muscle cell heterogeneity with respect to myosin composition both in nodal and perinodal tissues.

THE sinoatrial (SA)[1] and atrioventricular (AV) nodes are morphologically differentiated regions of the atrial myocardium of higher vertebrates. As first described by Tawara (46) and Keith and Flack (21), the nodes consist of thin, palely stained muscle fibers, arranged in slender anastomosing bundles and surrounded by abundant connective tissue. The SA node, located at the junction of the superior vena cava with the right atrium, is the primary cardiac pacemaker; the AV node, located at the junction of interatrial and interventricular septa, is a region of slow conduction, which regulates the ventricular activation through a gate mechanism on arriving impulses, and of latent pacemaker activity. The AV node is continuous with the His bundle that transmits the impulse to the ventricular myocardium. Nodal muscle cells are smaller than ordinary myocardial cells and have sparse myofibrils, poorly developed sarcotubular system and few nexus regions (see reference 43). They display low amplitude and slowly rising action potentials due to slow inward currents; in the SA node they also show spontaneous diastolic depolarization responsible for pacemaker activity (see reference 4). Nodal tissues are not homo-

geneous with respect to cell composition. Electrophysiological studies have disclosed wide heterogeneity both in passive and active membrane properties of nodal fibers (16, 31) and both light and electron microscopy observations have revealed the presence of several different fiber types within the nodal conduction tissue (for reviews see references 1, 48, and 49). The heterogeneous cellular composition of the nodes has been confirmed by combined electrophysiological and morphological studies on the same preparations (2, 20, 27) but precise markers for identifying the various fiber types are lacking. No information is available concerning the biochemical composition of nodal cells: biochemical studies are complicated by the small size of the nodes and by lack of well-defined anatomical boundaries with the surrounding ordinary myocardium.

Polymorphism of cardiac myosin has proved to be a powerful tool for investigating the cellular composition of the myocardium. Immunocytochemical studies have shown that different myosin heavy chain (MHC) isoforms are variably distributed within the atrial and ventricular myocardium of the avian and mammalian heart. The chicken heart appears to contain at least three distinct MHC isoforms, one present in ventricular muscle cells, another in atrial muscle cells, and still another in Purkinje fibers (12, 38). Two distinct MHC isoforms, corresponding to the α- and β-MHC originally

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1. *Abbreviations used in this paper:* anti–bA, anti–bovine atrial myosin antibody; anti–bF, anti–bovine fetal skeletal myosin antibody; anti–bV, anti–bovine ventricular myosin antibody; AV node, atrioventricular node; MHC, myosin heavy chain; PAP, peroxidase–antiperoxidase; SA node, sinoatrial node.
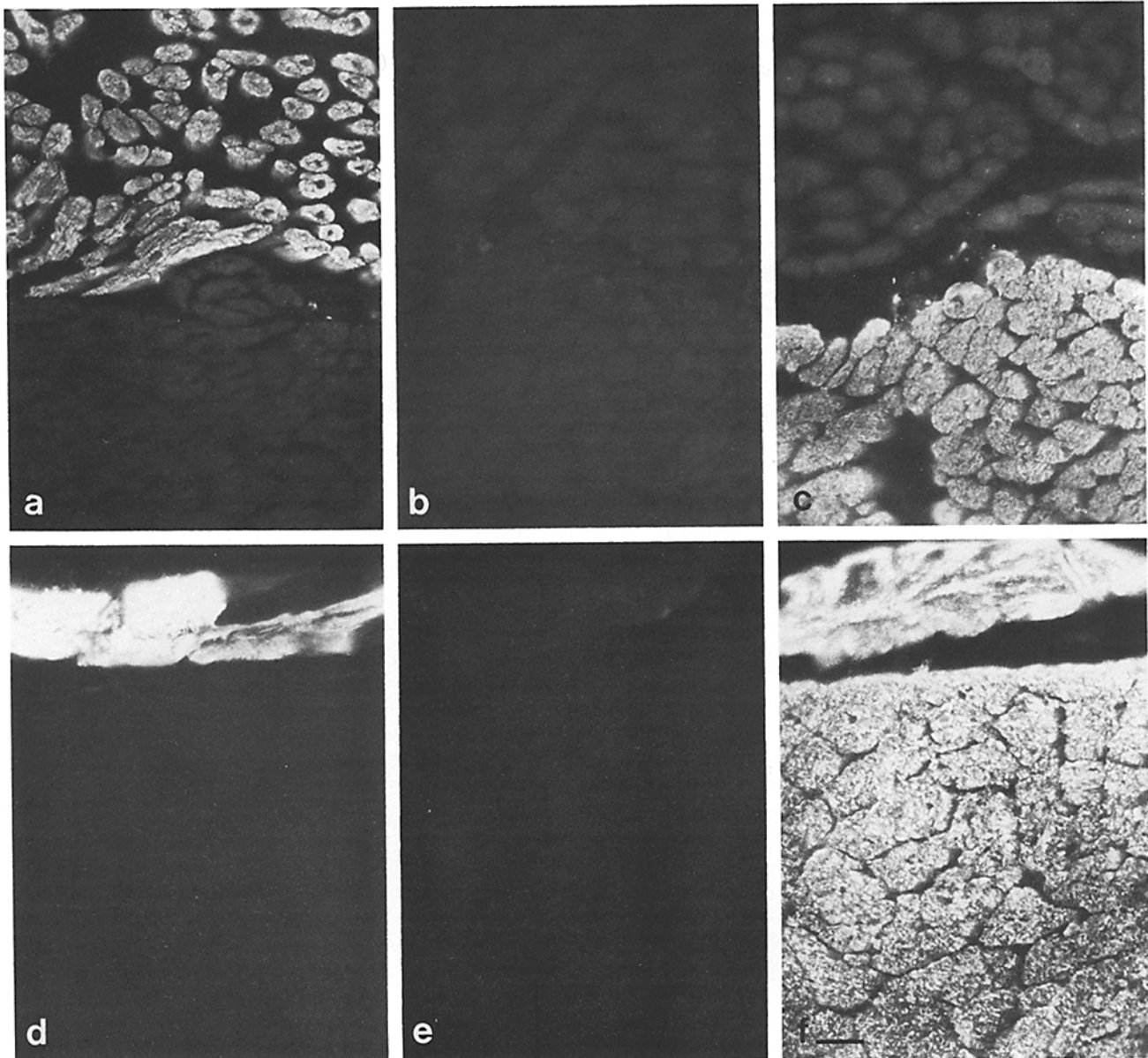
Figure 1. Immunofluorescence, ordinary atrial and ventricular myocardium, and Purkinje fibers. (*a–c*) Sections of a composite block of bovine left atrial myocardium (auricular appendage, upper field) and left ventricular myocardium (lower field) processed for indirect immunofluorescence with anti-bA (*a*), anti-bF (*b*), and anti-bV (*c*). (*d–f*) Sections through the subendocardial region of the left ventricular myocardium including a bundle of Purkinje fibers (*top*) stained with anti-bA (*d*), anti-bF (*e*), and anti-bV (*f*). Atrial fibers stain with anti-bA, ventricular fibers with anti-bV, and Purkinje fibers either with anti-bV or with both antibodies. No fiber is labeled by anti-bF. Bar, 30 μm.

described by Hoh (17, 18), are visualized in the ventricular myocardium of different mammalian species (13, 14, 36); antigenically similar α- and β-MHCs can be identified in the atrial myocardium of the bovine and human heart (13, 15).

Using a panel of antimyosin antibodies, specifically directed against different MHC isoforms, we have now investigated the myosin composition of the SA and AV nodal regions in the adult bovine heart. The main finding of this study is that nodal fibers express a unique type of MHC immunologically related to fetal skeletal myosin.

Materials and Methods

Antimyosin Antibodies

Three different polyclonal antibodies specific for the MHC subunit were used in this study. Anti–bovine atrial myosin (anti-bA) and anti–bovine ventricular

myosin (anti-bV) antibodies were described in previous studies on the bovine heart (15, 36): they reacted selectively with atrial and ventricular MHCs, respectively. Anti–bovine fetal skeletal myosin (anti-bF) antibodies were also described previously (37): they reacted with MHCs from rat and bovine fetal skeletal muscle and not with MHCs from adult skeletal muscle.

Immunohistochemistry

Immunohistochemical procedures were performed on fresh-frozen cryostat sections of different specimens obtained from 10 adult bovine hearts. Samples of SA and AV nodes were excised with the perinodal regions in order to preserve any relationship with the surrounding myocardium. Samples from the crista terminalis, the left and right auricles, the orifices of the pulmonary veins, and different subendocardial and subepicardial regions of right and left ventricles were also examined.

Antibody binding was revealed either by indirect immunofluorescence or by the peroxidase–antiperoxidase (PAP) technique, the latter procedure being especially useful for low magnification study of large sections. For indirect immunofluorescence sections were incubated with appropriate dilutions of the antimyosin antibodies for 30 min at 37°C. After rinsing twice in phosphate-
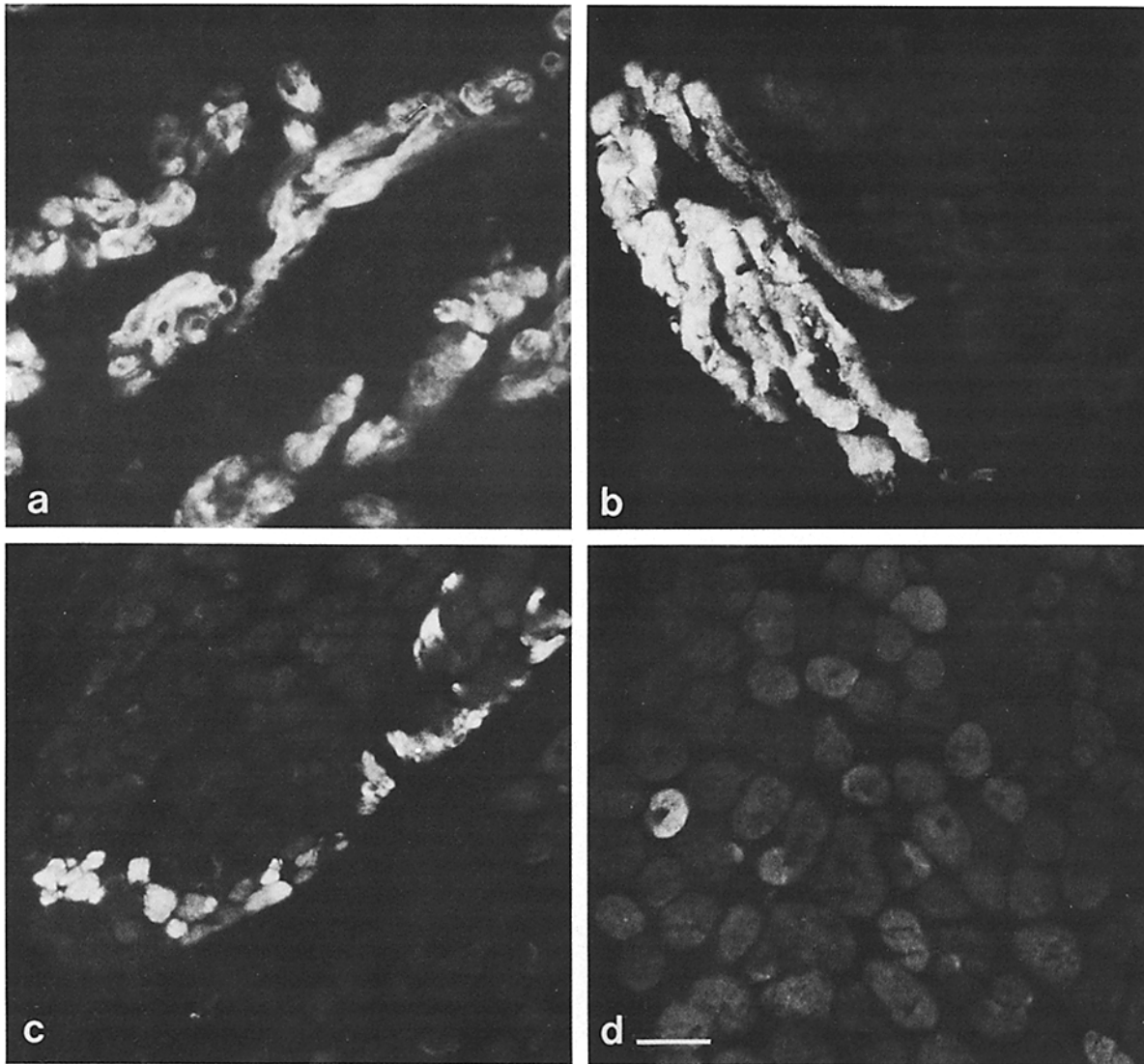
Figure 2. Immunofluorescence, nodal, and perinodal tissues. Sections of SA node (*a*), AV node (*b*), a region contiguous to the SA node at the origin of the crista terminalis (*c*), and a region of the left atrium at the orifice of a pulmonary vein (*d*), stained with anti-bF. Bar, 30 μm.

buffered saline, they were incubated with appropriate dilutions of fluorescein-labeled anti-rabbit immunoglobulins (Miles Laboratories Inc., Elkhart, IN) for 30 min at 37°C. The sections were washed twice with buffered saline, fixed for 10 min in paraformaldehyde 1.5% in buffered saline, mounted with Elvanol, and examined with a Leitz Dialux microscope equipped with epifluorescence optics. The PAP technique was performed following the procedure described by Sternberger (44). In brief, sections were incubated with appropriate dilutions of the antimyosin antibodies overnight at room temperature, then treated with appropriate dilutions of goat anti-rabbit immunoglobulins (Miles Laboratories Inc.) for 30 min at room temperature and finally with rabbit PAP complex (Dakopatts, Glostrup, Denmark) for 30 min at room temperature. Bound antibody was revealed using 1.2 mg/ml of *p*-phenylenediamine-pyrocatechol (Hanker Yates Reagent; Polysciences, Inc., Warrington, PA) as substrate in 0.05 Tris-HCl, pH 7.6, 0.01% H_2O_2 for 20 min. Controls for immunofluorescence and PAP technique included sections treated with preimmune rabbit immunoglobulins and sequential omission of each step for the PAP procedure. No endogenous peroxidase activity was observed in the presence of the substrate alone.

Results

Immunofluorescence reactivity of ordinary bovine atrial and ventricular myocardium with anti-bA, anti-bV, and anti-bF

is summarized in Fig. 1. As previously described (15, 36), anti-bA stained all atrial fibers and a minor population of ventricular fibers (Fig. 1 *a*), whereas anti-bV stained all ventricular fibers and a minor population of atrial fibers (Fig. 1 *c*). In contrast, anti-bF did not react with atrial or ventricular ordinary myofibers (Fig. 1 *b*). Components of the ventricular conduction system, i.e., subendocardial Purkinje fibers and fibers of the His bundle and bundle branches, were also unlabeled by anti-bF (Fig. 1, *d–f*). Specific reactivity with anti-bF was found exclusively in muscle fibers of the SA and AV nodes and in occasional fibers in the perinodal regions of the atrial myocardium (Fig. 2). Rare, weakly labeled fibers were observed around the orifices of the pulmonary veins (Fig. 2 *d*). No labeled fiber was found in the auricles, in the interatrial septum, and along the crista terminalis, except for the origin of the crista contiguous to the SA node (Fig. 2 *c*). The cellular composition of the nodal and perinodal regions was examined in greater detail with the three antibodies.
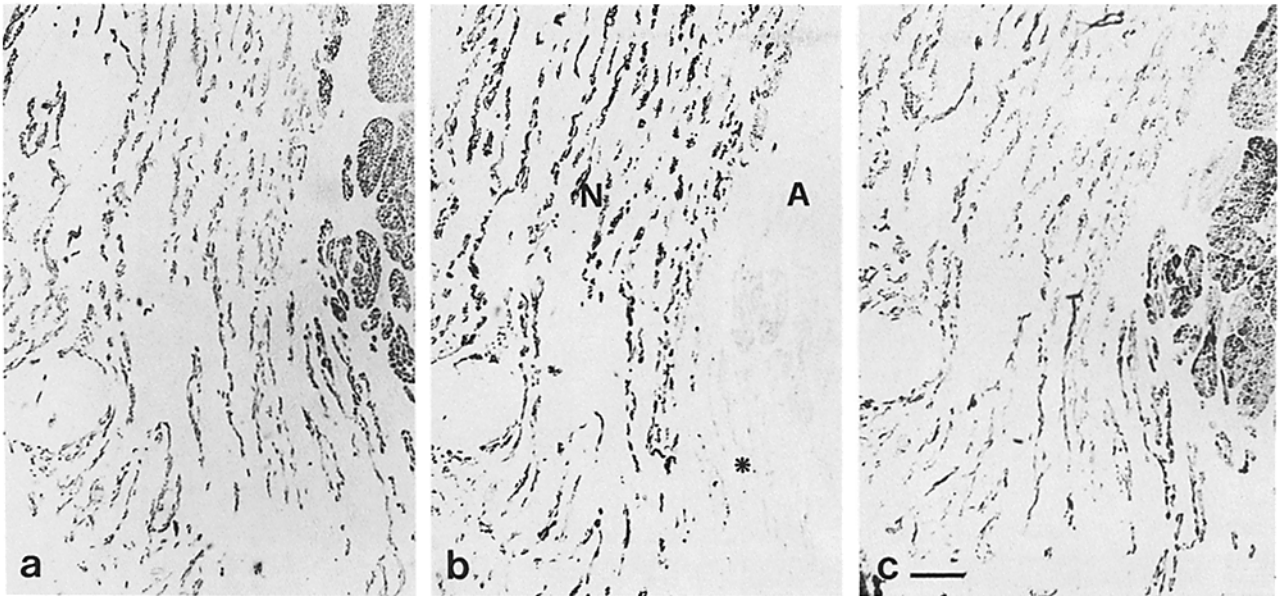
Figure 3. PAP staining, SA node. Serial sections through part of the SA node (*N*) and neighboring atrial myocardium (*A*) stained with anti-bA (*a*), anti-bF (*b*), and anti-bV (*c*). Asterisk marks a portion of nodal tissue composed of cells which are morphologically indistinguishable from other nodal cells but are unreactive with anti-bF. Bar, 300 μm.

SA Node

The contrasting response of SA nodal tissue and ordinary atrial myocardium to anti-bF is illustrated in Fig. 3. Both nodal and atrial fibers were stained by anti-bA whereas only nodal fibers reacted with anti-bF. However, there were regional variations in the reactivity of the latter antibody. Two fiber types could be distinguished in the SA node on the basis of their reactivity with anti-bF. Nodal fibers reactive with anti-bF were especially abundant in the central region of the SA node, oriented from the sulcus terminalis towards the endocardial surface; these fibers were also brightly stained by anti-bA and showed variable reactivity with anti-bV (Fig. 4, *a–c*). Muscle fibers in the peripheral portions of the node, which were morphologically indistinguishable from those present in the central areas, were usually unreactive with anti-bF: all these fibers were labeled by anti-bV and anti-bA (Fig. 4, *d–f*).

The atrial myocardium surrounding the SA node showed a very heterogeneous fiber composition. Small fibers labeled by anti-bF were occasionally observed at these sites (Fig. 2*c*). However, most perinodal fibers reacted with both anti-bA and anti-bV and were unlabeled by anti-bF, like most fibers in the crista terminalis. A population of characteristically large perinodal fibers were found to be reactive almost exclusively with anti-bV (Fig. 4, *g–i*): these fibers were especially numerous in the wall of the superior vena cava and at the origin of the crista terminalis.

AV Node

In the bovine heart, the AV node consists of a loose network of small-sized muscle fibers embedded in densely packed connective tissue (33). In the lower portion of the node there is an abrupt transition in muscle fiber morphology between the nodal tissue and the His bundle, composed of large Purkinje fibers, whereas in the upper portion of the node

there is a smooth transition between the nodal tissue and the surrounding atrial myocardium. The pattern of anti-myosin immunoreactivity of AV nodal cells was similar to that of SA nodal cells. In the central and lower portions of the node, most cells were labeled by anti-bF and anti-bA and were unlabeled by anti-bV. As shown in Fig. 5, the transition from the AV node to the His bundle was outlined by clearcut differences in antimyosin reactivity. With anti-bF, the small nodal fibers were positive and the large His bundles fibers were negative, whereas a reverse pattern of reactivity was observed with anti-bV. All nodal fibers were also reactive with anti-bA whereas only a minority of His bundle fibers stained with this antibody.

As in the SA node, the reactivity of anti-bF with nodal fibers showed regional variation: the portion of the AV node connected with the His bundle was found to be homogeneously labeled by anti-bF, whereas in the upper portion of the node and in the transitional areas towards the atrial myocardium, nodal fibers were found to vary in their reactivity with anti-bF (Fig. 6, *a–f*). These two regions differed in reactivity also with anti-bV antibodies: whereas the nodal fibers of the central and lower portion of the node were usually unlabeled, many fibers were labeled in the regions where transitional-type fibers were abundant.

Clusters of fibers labeled by anti-bF were also consistently observed at the periphery of the AV node, especially along the ridges of the Eustachian valve and the coronary sinus. However, the distinguishing feature of these regions was the presence of bundles of large fibers labeled by anti-bV and unlabeled by anti-bA and anti-bF (Fig. 6, *g–i*). As previously reported (15), these fibers were distributed mainly in the subendocardial layers of the right atrium, from the coronary sinus to the central fibrous body and in the more posterior and upper perinodal regions close to the interatrial septum. These fibers must be distinguished from the other fiber types reactive with both anti-bA and anti-bV, especially abundant
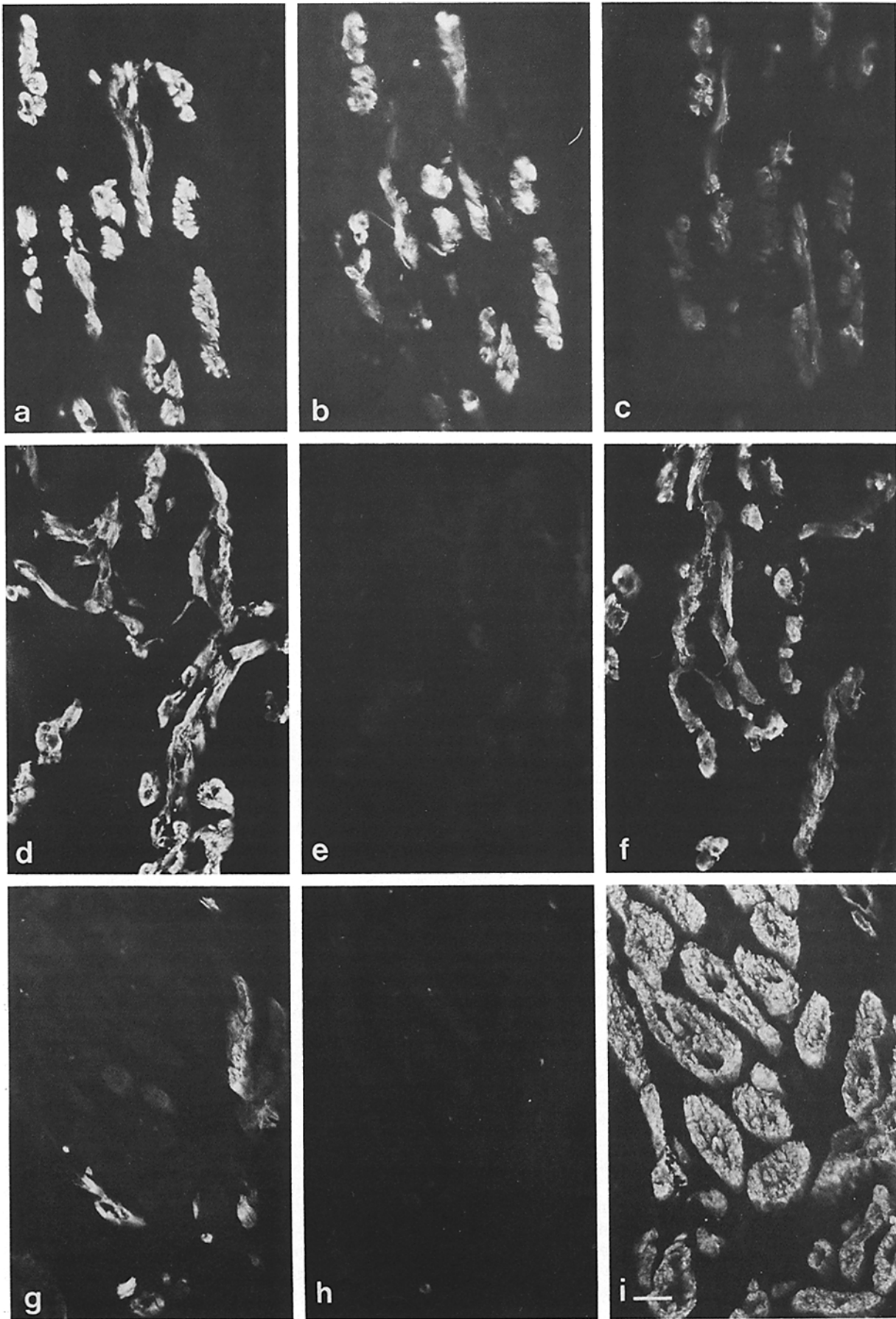
Figure 4. Immunofluorescence, SA node. Sections from the central portion of the node (*a–c*), peripheral portion of the node (*d–f*), and perinodal area (*g–i*), stained with anti-bA (*a, d,* and *g*), anti-bF (*b, e,* and *h*), and anti-bV (*c, f,* and *i*). Bar, 30 μm.
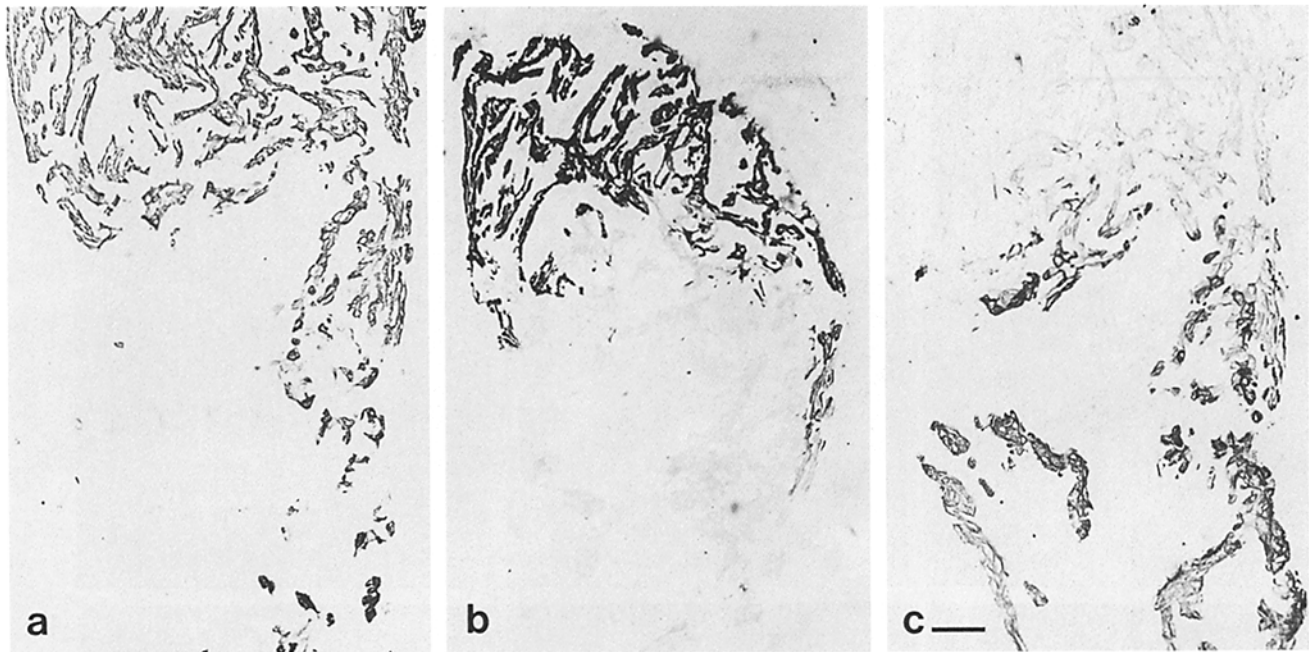
Figure 5. PAP staining, AV node. Serial sections through the junctional area between the AV node (*upper field*) and the His bundle (*lower field*) stained with anti-bA (*a*), anti-bF (*b*), and anti-bV (*c*). Note the reversed staining pattern of anti-bF and anti-bV. Bar, 300 μm.

along the crista terminalis and in the interatrial septum (15), and from the ordinary atrial fibers reactive only with anti-bA.

Discussion

Two distinct MHC isoforms, called α- and β-MHCs, are present in the ventricular myocardium of the mammalian heart (17, 18, 35). These MHCs are encoded by two closely associated genes (25), whose expression is developmentally regulated and can be modulated by hormonal and mechanical influences (11, 24). Immunocytochemical studies have revealed muscle cell heterogeneity in the normal ventricular myocardium with respect to the expression of α- and β-MHC (13, 14, 36) and the frequent coexistence of the two isoforms within the same myocyte (34).

The relative proportion of α- and β-MHC in the adult ventricular myocardium varies according to species: α-MHC is the predominant isoform in mouse and rat ventricles, whereas β-MHC is the predominant isoform in ventricles of larger mammals, including the beef (7, 23). The two antibodies used in the present study, anti-bA and anti-bV, appear to react with α- and β-MHC, respectively. In fact, anti-bV reacts strongly with bovine ventricular myosin and stains all ventricular myocytes, whereas anti-bA reacts only with a minor population of ventricular myocytes (15, 36). The presence of a minor α-type MHC component has also been demonstrated in the human ventricles (13, 29). Specificity of anti-bA for α-MHC has been confirmed by enzyme immunoassay studies (29) showing that anti-bA reacts with rat V1 myosin, the ventricular isoform corresponding to the αα homodimer, but not with rat V3 myosin, the ββ homodimer. Furthermore we have found that the anti-bA stains strongly and homogeneously the ventricular myocardium of thyrotoxic rabbits (36), known to contain almost exclusively α-MHC (5, 22, 26), whereas it does not react with ventricles of hypothyroid

rabbits, in which β-MHC is the predominant component (10, 42).

Two MHC isoforms, antigenically similar to ventricular α- and β-MHC, have been identified in the atrial myocardium (15). Myosin isolated from bovine auricular appendages reacts strongly with anti-bA whereas it does not react with anti-bV, thus it appears to consist essentially of α-type MHC; accordingly, by immunofluorescence most atrial myocytes stain with anti-bA and are negative with anti-bV (15). A similar response was seen in the human heart (3, 13). Using specific cDNA probes and S1 nuclease mapping analysis, it has been shown that the predominant MHC mRNA expressed in rat and rabbit atrial muscle is indistinguishable from ventricular α-MHC and appears to be the product of the same α-MHC gene (24, 41). Accordingly, immunochemical and peptide mapping studies have confirmed that, in the rabbit, atrial MHC and ventricular α-MHC are identical proteins (6, 9). In the bovine heart a β-type MHC, recognized by anti-bV, is expressed by a minor population of atrial myocytes, which are especially concentrated in the right atrium and in the interatrial septum (15). Low levels of β-MHC mRNA have also been detected in the rat atrial myocardium (24). As previously reported (15) and confirmed in the present study, a very minor population of bovine atrial myocytes is stained by anti-bV but not by anti-bA, and thus appears to contain exclusively β-type MHC. These cells, which are generally larger in size than ordinary atrial muscle cells, are specifically distributed in the transitional areas between the SA and AV node and the atrial myocardium, whereas they are more rarely seen along the crista terminalis where most cells appear to contain both α- and β-MHC. Based on the distribution of β-MHC in the normal atrial myocardium, it has been suggested that β-positive atrial cells may correspond to cells specialized for faster conduction along the internodal tracts (15).

We report here immunohistochemical evidence for an ad-
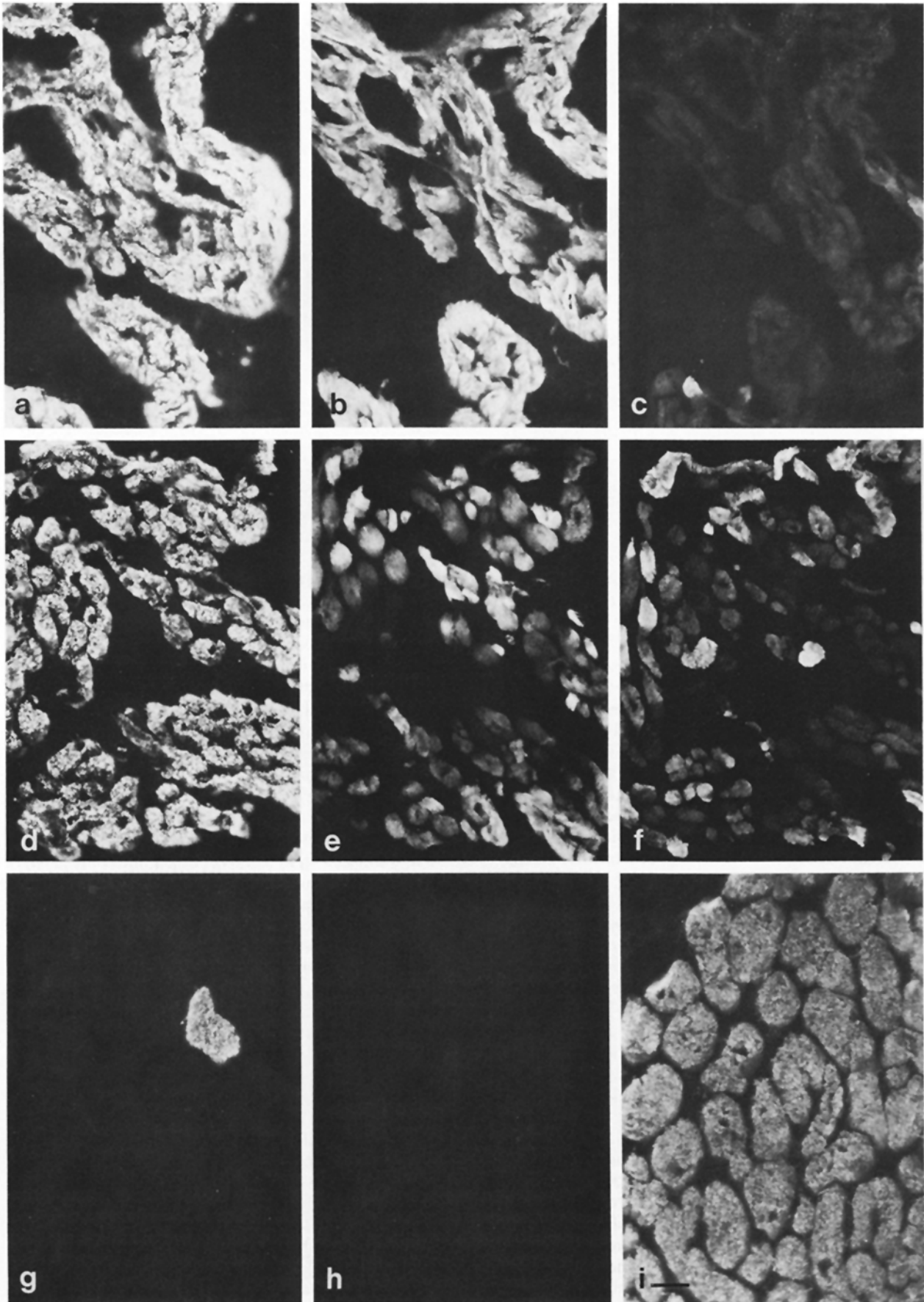
Figure 6. Immunofluorescence, AV node. Sections from the central portion of the node (*a–c*), the transitional region towards the atrial myocardium (*d–f*), and a perinodal region towards the coronary sinus (*g–i*), stained with anti-bA (*a, d,* and *g*), anti-bF (*b, e,* and *h*), and anti-bV (*c, f,* and *i*). Bar, 30 μm.

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ditional type of cardiac MHC, which is antigenically related to fetal skeletal MHC and is specifically distributed in the nodal conduction tissue. A large proportion of muscle cells in the SA and AV nodes of the bovine heart were stained by anti-bF. Rare cells labeled by anti-bF were also found in the transitional regions between nodal tissue and atrial myocardium and around the orifices of the pulmonary veins. The latter finding may be related to the fact that the area of the left atrium surrounding the outlet of the pulmonary veins has a common origin with the sinus venosus region (49) and may correspond to the site of formation of an abortive left-sided SA node (50).

The nature of the MHC present in nodal cells and recognized by anti-bF is not known. In particular, it remains to be determined whether this "nodal" MHC is identical to one of the MHC isoforms which have been identified in developing skeletal muscle (52). It is known that skeletal and cardiac muscle may express similar myosin isoforms: a fetal skeletal myosin light chain is also present in atrial muscle and in fetal ventricles (8, 51, 53), and the ventricular β-MHC is apparently identical to the slow twitch skeletal MHC (24). The finding that most nodal cells also stained with anti-bA and a number of them with anti-bV points to the presence of other MHC types in nodal tissue and to the possible coexistence of different MHCs in the same cell.

The presence of a fetal-like myosin isoform in nodal conduction tissue is of particular interest since nodal cells and embryonic cardiac muscle cells display similar morphological features and electrophysiological properties (for reviews see references 4 and 43). One possibility is that the fetal-like myosin identified in the adult nodes corresponds to a primordial type of myosin present in the developing heart. There is at present no evidence for embryonic- or fetal-specific MHC isoforms in mammalian cardiac muscle (40), but early developmental stages have not been investigated. In preliminary immunohistochemical studies on bovine fetal hearts of various ages we have found that reactivity with anti-bF is always confined to small clusters of fibers in specific regions of the atrial myocardium, most atrial and ventricular fibers being completely unreactive. In the youngest specimens investigated, from embryos 4–6 wk old, reactive fibers were only seen in the sinus venosus region and in the closely associated areas of the posterior atrial wall (39). These findings indicate that from early stages the developing SA node contains a special myosin isoform that may be used as a specific marker to investigate the ontogeny of the atrial conduction system. Further studies on the very initial stages of heart morphogenesis are required to determine whether nodal MHC indeed represents a vestige of a primordial cardiac myosin.

An alternative interpretation is that nodal muscle cells are not embryonic remnants but specialized cell types resulting from the differentiation of a particular cell lineage early during embryonic development (28, 30). The fetal-like nodal myosin might have a specific functional role related to particular contractile properties. It has been suggested that the contractile activity of sinus node cells may be important in the synchronization of the pacemaker action through the mechanical effect of stretch on the activation of spontaneous depolarization (32). The significance of the myosin composition, thence of the contractile function, of conduction tissue cells should be reconsidered also in the light of antimyosin immunofluorescence studies in the chicken heart. Purkinje fibers in the chicken heart contain a distinct MHC isoform, antigenically related to slow tonic skeletal MHC (38), which is absent in the early embryonic heart and is first detected in the differentiating Purkinje fibers during later stages of embryonic development (12, 47). It should be stressed that in the bovine heart the large Purkinje fibers of the His bundle and its branches, including the final arborizations of the ventricular conduction tissue, react with anti-bV and a number of them also with anti-bA, but none of them with anti-bF; thus Purkinje fibers differ in myosin composition from nodal muscle cells. Taken together with results of peptide mapping studies showing that MHC from bovine His bundle is apparently identical to ventricular MHC (53), our findings indicate that the ventricular conduction tissue consists essentially of β-MHC with a minor α-MHC component, but does not contain "nodal" MHC.

The results of this study confirm the existence of cellular heterogeneity within the SA and AV nodes, which has previously been demonstrated by ultrastructural and electrophysiological studies (1, 2, 16, 19, 31, 49). We find that only a subset of nodal muscle cells, predominantly localized in the central areas of the nodes, is labeled by anti-bF; other subsets of nodal and perinodal cells are unlabeled. By analogy with studies on the rabbit heart, it is tempting to speculate that the SA nodal fibers labeled by anti-bF correspond to the population of nodal fibers electrophysiologically characterized as true pacemaker cells and situated in the central part of the node (2, 27). In the same way, reactive fibers in the AV node, abundant in the central and inferior portion of the node, abutting on the His bundle, may correspond to the "N cells" or "typical nodal cells" electrophysiologically identified in the rabbit and human heart (1, 20). The heterogeneous distribution of α- and β-MHC, as well as "nodal" MHC, at the periphery of the nodes is consistent with the marked electrophysiological polymorphism described in these transitional regions (1, 45). Correlated electrophysiological and antimyosin immunofluorescence studies of single nodal cells should contribute to clarify the significance of these various muscle cell populations.

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