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Feature Article

Neurofilament M mRNA is Expressed in Conduction System Myocytes of the Developing and Adult Rabbit Heart*

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M. Vitadello, S. Vettore, E. Lamar, K. R. Chien and L. Gorza. Neurofilament M mRNA is expressed in conduction system myocytes of the developing and adult rabbit heart. *Journal of Molecular and Cellular Cardiology* (1996) 28, 1833–1844. We previously demonstrated that conduction-system myocytes of the rabbit heart express cytoskeletal proteins immunologically related to neurofilaments. In order to determine more precisely the nature of these proteins, we screened an expression cDNA library, prepared from the sino-atrial node region of the rabbit heart, using a monoclonal antibody which reacts with the M subunit of neurofilaments. Sequence analysis of the isolated cDNA clones shows high homology with rat and human neurofilament M mRNAs. Northern blot analysis demonstrates hybridization with a transcript expressed in brain, with the size expected for neurofilament M mRNA. An mRNA species of the same size is also detectable in the Northern blot of the sino-atrial node region RNA. *In situ* hybridization documents that in the adult rabbit the transcript accumulates in neurons and is localized in myocytes of the sino-atrial and atrio-ventricular nodes and of the atrio-ventricular bundle and bundle branches, but not in working atrial and ventricular myocytes. Developmental analysis was undertaken in order to determine the distribution of the neurofilament M mRNA in the rabbit embryonic heart. *In situ* hybridization shows that neurofilament M mRNA is detectable in a few ventricular myocytes in proximity to the atrio-ventricular groove after 9.5 days of embryonic development and it is accompanied by the presence of the protein. At subsequent stages of development neurofilament M mRNA is detectable in a number of cardiac myocytes, which are mainly localized at the atrio-ventricular junction and in the ventricular subendocardium and presumably correspond to myocytes of the heart conduction system.

Key Words: cDNA library; Heart conduction system; Neurofilament M mRNA; *In situ* hybridization; Heart development.

Introduction

The precise sequential contraction of atria and ventricles in the heart is determined by a system of specialized myocytes. Pacemaker cells localized at the sino-atrial (SA) node activate at first; conducting myocytes of the atrio-ventricular (AV) node, AV bundle and peripheral Purkinje system regulate the activation of ordinary cardiomyocytes by slowing or accelerating the diffusion of stimuli (Carmeliet and Veerecke, 1979).

Although specialized myocytes of the conduction system cannot be distinguished morphologically from working myocytes in many mammalian species, they are characterized by unique features that are adopted as cellular markers, among which are some embryonic cardiac proteins and unique isoforms of muscle proteins, as well as markers

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expressed by neural cells (see Gorza *et al.*, 1994, for a review). Our laboratory demonstrated that the conduction system myocytes of the rabbit heart can be identified by antibodies reacting with the light (L) and middle (M) molecular weight subunits of neurofilaments (NF) (Gorza *et al.*, 1988; Vitadello *et al.*, 1990). NFs belong to class IV intermediate filaments, together with α-internexin (Fliegner *et al.*, 1990). Until recently, NFs have been thought to be expressed only in post-mitotic neurons where they would play a structural role in determining the axonal diameter (Hoffman *et al.*, 1987). Non-neuronal expression of NF-M has been described in rat myelin-forming Schwann cells by means of specific antibodies and through the isolation of NF-M cDNA from a Schwann cell cDNA library (Kelly *et al.*, 1992).

Our previous studies showed that anti-NF-M antibodies react in Western blot analysis of SA node and AV bundle regions of the rabbit heart with a polypeptide which displays electrophoretic mobility in SDS gel identical to neuronal NF-M. Immunocytochemical studies revealed co-localization of NF-immunoreactivity with desmin, the muscle-specific class III protein of intermediate filaments in conduction system myocytes (Gorza *et al.*, 1988). In these myocytes, anti-NF-M antibodies also decorate 10-nm filaments at the ultrastructural level (Vitadello *et al.*, 1990). In order to identify unambiguously the nature of the immunoreactive protein expressed in heart conduction myocytes, we prepared an expression cDNA library from the SA node region of the adult rabbit heart and screened it with the anti-NF-M monoclonal antibody iC8 (Gorza *et al.*, 1988). Sequence analysis of the isolated clones indicates that they encode for the rabbit homolog of NF-M mRNA. *In situ* hybridization studies show that NF-M mRNA is detectable in myocytes of the heart conduction system, namely in the SA and AV nodes and AV bundle and bundle branches. The expression of NF-M mRNA and protein in the heart has been then investigated at early stages of embryonic development.

Materials and Methods

Tissue source

Rabbit embryos were obtained after 9, 9.5, 10, 11 and 12 days of gestation; time of mating was considered as time 0. For immunocytochemistry, embryos were fixed in freshly prepared 2% paraformaldehyde in phosphate buffer saline (PBS) pH 6.5 with 5% sucrose for 2 min at room temperature, then in 2% paraformaldehyde in 0.1 M sodium borate pH 9.5 with 5% sucrose for 12 min at room temperature and washed in PBS pH 6.5 with 18% sucrose overnight at 4°C (Gorza and Vitadello, 1989). For *in situ* hybridization, embryos were fixed with 4% paraformaldehyde in PBS. Some embryos from the same litter were embedded in OCT (Miles Lab.) and frozen in liquid nitrogen, other embryos were dehydrated and embedded with paraffin (Gorza *et al.*, 1993).

Samples from central (brain, cerebellum and medulla) and peripheral nervous system (superior cervical ganglion), liver, hindlimb muscles, and heart, i.e. the atrial and the ventricular myocardium, the SA node region, the AV node and AV bundle regions, were obtained from adult rabbits. All specimens were immediately frozen without fixation.

Antibodies used

Monoclonal antibody iC8 reacted with rabbit neurofilament M subunit in brain and with a related polypeptide in the heart (Vitadello *et al.*, 1986; Gorza *et al.*, 1988). Secondary antibodies were rabbit anti-mouse Ig conjugated with peroxidase (Dakopatts) and goat anti-mouse Ig conjugated with rhodamine (Cappel).

Library preparation and screening

PolyA$^+$ RNA was obtained from the SA region isolated from 120 adult rabbit hearts, as described (Ausubel *et al.*, 1987). Methylated cDNA was reverse transcribed from 5 μg of polyA$^+$ RNA using an oligodT primer containing a Xho I site in presence of 5-methyl dCTP (Zap-cDNA synthesis kit, Stratagene). Double strand cDNAs were blunted, ligated with EcoRI adapters and digested with XhoI and EcoRI. cDNAs longer than 500 bp were isolated by a Sephacryl S-400 spin column, directionally ligated with EcoRI-XhoI prepared arms of UniZapII XR and packaged with Gigapack packaging extracts (Stratagene). 1×10^6 pfu of the primary library were amplified once.

Screening was performed after induction of the synthesis of the recombinant protein using IPTG-soaked nitrocellulose filters (Schleicher and Schuell), as described by Mierendorf *et al.* (1987). Filters were first saturated with 3% bovine serum albumin in TBS (Tris-HCl 10 mM pH 8.0, NaCl 150 mM, Tween 20 0.05%) for 1 h at room temperature and subsequently incubated with adequate

(a)

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                                  coil 1b
rabbit    VKVELDKKVQSLQDEVAFLRTNHEEEVADLLAQIQASHITVERKDYLKTDISSAL
rat       VKVELDKKVQSLQDEVAFLRSNHEEEVADLLAQIQASHITVERKDYLKTDISTAL
man       VKVELDKKVQSLQDEVAFLRSNHEEEVADLLAQIQASHITVERKDYLKTDISTAL

rabbit    KEIRSQLECHSDQNMAQAEEWFKCRYAKLTEAAEQNKEAIRSAKEEIAEYRRQLQ
rat       KEIRSQLECHSDQNMAHAEEWFKCRYAKLTEAAEQNKEAIRSAKEEIAEYRRQLQ
man       KEIRSQLESHSDQNMAHAEEWFKCRYAKLTEAAEQNKEAIRSAKEEIAEYRRQLQ

rabbit    SKSIELESVAWHKESLERHVSDIEERHNHDLSSYQDTIQQLENELRGTKWEMARH
rat       SKSIELESVRGTKESLERQLSDIEERHNHDLSSYQDTIQQLENELRGTKWEMARH
man       SKSIELESVRGTKESLERQLSDIEERHNHDLSSYQDTIQQLENELRGTKWEMARH
                                  coil 2
rabbit    LREYQDLLNVKMALDIEIAAYRKLLEGEETRFSTFSGSITGPLYTHRQPSVTISS
rat       LREYQDLLNVKMALDIEIAAYRKLLEGEETRFSTFSGSITGPLYTHRQPSVTISS
man       LREYQDLLNVKMALDIEIAAYRKLLEGEETRFSTFAGSITGPLYTHRPP-ITISS

rabbit    KIQKTKVEAPKLKVQHKFVEEIIEETKVEDEKSEMEDALTAIAEELAVSVKEEEK
rat       KIQKT-VEAPKLKVQHKFVEEIIEETKVEDEKSEMEDALTVIAEELAASAKEE-K
man       KIQKTKVEAPKLKVQHKFVEEIIEETKVEDEKSEMEEALTAITEELAASMKEEKK

rabbit    EEEAEGKEEEQEAEEE-VAAKKSPVKATTPEIKEEE-GEKEEE--GQEEEEEEED
rat       EE-AEEKEEEPEV--------KSPVK--SPEAKEEEEGEKEEEEEGQEEEEEEED
man       E--AEEKEEEPEAEEEEVAAKKSPVKATAPEVKEEE-GEKEEEE-GQEEEEEE-D

rabbit    EGVKSDQAEEGGSEKEGSS-KNEGEQEE-GETEAEGEVEEAEAKEEKKTEEKSEE
rat       EGVKSDQAEEGGSEKEGSSEKNEGEQEEEGETEAEGEGEEAEAKEEKKTEGKVEE
man       EGAKSDQAEEGGSEKEGSSEKEEGEQEE-GETEAEAEGEEAEAKEEKKVEEKSEE

rabbit    VAAKEE-PVTEA--KVGKPEKAKSPVPKSPVEE----------------------
rat       MAIKEEIKV-E------KPEKAKSPVPKSPVEE----------------------
man       VATKEEL-V--ADAKVEKPEKAKSPVPKSPVEEKGKSPVPKSPVEEKGKSPVPKS
                            **********^^^^^^^^^^^^^^^^^^^^^^^^^^

rabbit    ----------------------------------------VKP-KAEATAGKGEQ
rat       ----------------------------------------VKP-KPEAKAGKDEQ
man       PVEEKGKSPVPKSPVEEKGKSPVSKSPVEEKAKSPVPKSPVEEAKSKAEVGKGEQ
          **********^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

rabbit    QKEEEEKVEEEKKKAAKESPKEEKVEKKEEKPKDVP-KKKAESPVKEEA-A-EEA
rat       -KEEE-KVEE-KKEVAKESPKEEKVEKKEEKPKDVPDKKKAESPVKEEK-AVEEM
man       -KEEE-KEV---K--EA--PKEEKVEKKEEKPKDVPEKKKAESPVKEEAVA-EVV

rabbit    ATITKPTKV-GLEKETKEGEKPLQQEKEKEKAGEEGGSEEEGSDQGSKRAKKEDI
rat       ITITKSVKVSGLEKDTKE-EKPQQQEKVKEKAEEEGGSEEEVGDKSPOESKKEDI
man       -TITKSVKVH-LEKETKEEGKPLQQEKEKEKAGGEGGSEEEGSDKGAKGSRKEDI

rabbit    AVNGEGEGKEEEEPETKEKGSGREEEKGVVTNGLDLSPADEKKGGDRSEEKVVVT
rat       AINGEVEGKEEEEQETQEKGSGQEEEKGVVTNGLDVSPAEEKKGEDRSDDKVVVT
man       AVNGEVEGKEEVEQETKEKGSGREEEKGVVTNGLDLSPADEKKGGDKSEEKVVVT

rabbit    KKVEKITTEGGDGATKYITKSVTA-QKVEEHEETFEEKLVSTKKVEKVTSHAIVK
rat       KKVEKITSEGGDGATKYITKSVTVTQKVEEHEETFEEKLVSTKKVEKVTSHAIVK
man       KTVEKITSEGGDGATKYITKSVTVTQKVEEHEETFEEKLVSTKKVEKVTSHAIVK

rabbit    EVTQSD
rat       EVTQGD
man       EVTQSD
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(b)

tggtcaaggt ggagctggac aagaaggtcc agtcgctgca ggatgaggtg gccttcctgc ggacgaacca cgaggaggag 80
gtagcggacc tgctggccca gatccaggcg tcgcacatca cggtggagcg caaagactac ctgaagacgg acatctcgtc 160
ggcgctgaag gagatccgct cccagctcga gtgccactcc gaccagaaca tgcatcaggc cgaagagtgg tttaagtgcc 240
gctacgccaa gctcaccgaa gccgccgagc agaacaagga ggccatccgc tccgccaagg aagagatcgc cgagtaccgg 320
cgccagctgc agtccaagag catcgagctc gagtcggtcg cgtggcacaa ggagtccctg gagcggcacg tcagcgacat 400
cgaggagcgc cacaaccacg acctcagcag ctaccaggac accattcagc agctggaaaa tgagcttcgg ggaacgaagt 480
gggaaatggc ccgccacttg cgagagtacc aggatctcct caatgtcaag atggctctgg atatcgagat cgcagcctac 560
agaaaactcc tggagggtga agagaccaga ttcagcacat tttcaggaag catcactggg ccactgtata cacaccgaca 640
gccctcagtc accatatcca gtaagattca gaagacaaag gtggaagctc ccaagctcaa agtccaacac aaatttgttg 720
aggagatcat agaggaaacc aaagtggagg atgagaagtc agaaatggaa gatgcactga cagccattgc agaggaactg 800
gccgtgtctg tgaaggaaga ggagaaggaa gaagaggcag aaggaaagga agaggagcaa gaagctgaag aagaagttgc 880
agctgccaag aagtctccag tgaaggctac cacacccgag attaaagagg aagaagggga aaaggaagaa gaaggccagg 960
aggaggaaga agaggaagaa gatgaaggtg ttaagtcaga ccaagctgaa gagggaggat cagagaagga aggctctagc 1040
aagaacgagg gtgagcagga agaaggagaa accgaggctg aaggtgaagt agaagaagca gaagccaagg aggaaaagaa 1120
aaccgaggag aagagtgaag aagtggctgc taaagaggag ccagtgacag aagccaaggt gggaaagcca gagaaagcca 1200
agtcccctgt gccaaaatca ccagtggaag aggtgaagcc aaaagctgaa gccacagcag ggaaagggga gcagaaagag 1280
gaagaagaga aggttgagga agaaaagaaa aaggcagcca aggaatctcc aaaggaagag aaggtggaga agaaggagga 1360
gaaaccaaaa gatgtgccaa agaagaaagc tgaatccccg gtaaaagagg aggccgcaga agaggctgcc accatcacca 1440
aacccacaaa ggtgggcttg gagaaagaga ccaaagaagg ggagaagccg ctgcagcagg agaaggaaaa ggagaaagca 1520
ggagaggagg gagggagtga ggaggaaggg agcgaccagg ggtcaaagag ggccaagaag gaagacatag cagtcaatgg 1600
ggagggcgaa gggaaagagg aggaagagcc ggagaccaag gaaaagggca gtgggcgaga agaggagaaa ggcgtcgtca 1680
ccaatgggtt agacctgagc ccagcagacg agaagaaggg gggtgacaga agcgaggaga aagtggtggt gaccaaaaag 1760
gtagaaaaaa tcaccactga ggggggcgat ggtgctacca aatacatcac taaatctgta accgctcaaa aggtcgaaga 1840
gcatgaagag acctttgagg agaaactagt gtctactaaa aaggtagaaa aagtcacttc acacgccatt gtaaaggaag 1920
tcacccagag tgactaagat ctgagtccat tgcaaaaggt taagccatag acaatttcaa aatgcatgtg attgacagct 2000
tcaaaacaga acgggttctc cataggggct ccaagacatt gtattttgct ttgtgcaata tgaggggact gcatgcaagc 2080
gcagggtgct ccctcctcag tcttcggggg attcaaatgc atgatattgt acgtacctgg ggaatttgcc agtttcctaa 2160
gctgttggaa gggggtactc agggggggatg tcttgagatg tattatgcaa agtaccaact gagccaaaaa taataaatga 2240
aacacagaac tctcttagcc ttaagaaagc tatatatgaa taattacgtt tacctcactg gtgcatttaa aatggacttt 2320
tgttcatggg agaacctcgc tgacatgcac agtttgcaat cttatgttga ttgatgtaaa acgtcacagc agtatttgct 2400
caataaaggt catattggaa acatagtc 2428

Figure 1 (a) Deduced aminoacid sequence of the longest rabbit pNF cDNA (clone 9.2) and comparison with sequences of rat and human NF-M. The first aminoacid in rabbit sequence corresponds to the aminoacid residue 216 in rat sequence (Napolitano *et al.*, 1987). Aminoacid differences are underlined. Dotted lines are used to obtain the best alignment. * and ^ indicate the R domain, which is present six times in human NF-M. Note that the R domain is present once in the tail region of rabbit NF-M, similarly to rat, whereas the domain is repeated eight times in chicken NF-M (Zopf *et al.*, 1990); (b) nucleotide sequence of the 9.2 cDNA clone, dots indicate stop codon and potential polyadenylation sites are underlined.

dilution of iC8 ascite fluid (1:1000). Unbound antibody was removed after extensive rinses with TBS and filters were incubated with secondary anti-mouse Ig conjugated with peroxidase. Peroxidase activity was revealed using diaminobenzidine, as previously described (Gorza *et al.*, 1993). Phages from reactive plaques were isolated to purity after a further plating and screening. The cDNA insert was rescued from lambda phage together with the pBluescript phagemid using coinfection of SolR *E. coli* strain with ExAssist helper phage (Stratagene).

Sequence analysis of cDNA inserts was performed with the dideoxychain termination method (Sanger *et al.*, 1987) using modified T7 DNA polymerase (Pharmacia) on the isolated clones and on subclones obtained either after restriction endonuclease or Ex-oIII-Mung bean digestion (Pharmacia) and with the aid of sequence-specific oligonucleotides as primers.

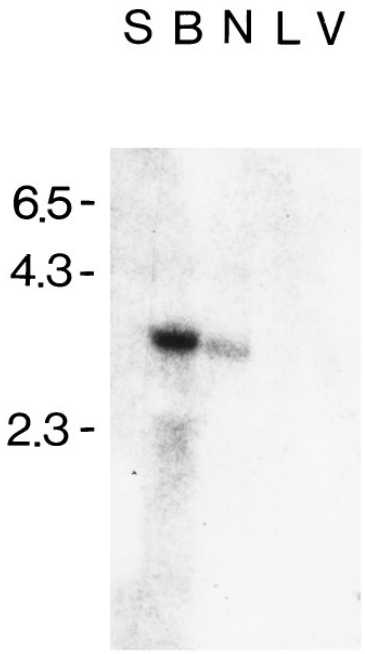


Figure 2 Northern blot analysis. Radiolabeled pNF 9.2 cDNA was hybridized with total RNA prepared from rabbit skeletal muscle (S), brain (B), SA node region (N), ventricular myocardium (V) and liver (L). The probe hybridized with a single mRNA species of about 3.0 kb abundant in brain and detectable in SA node region. The size was determined using λ Hind III-digested DNA and 28S and 18S RNA as markers.

Northern blot

Total RNA was isolated from rabbit brain, SA node region, ventricular myocardium, skeletal muscle and liver following the procedure described by Chomczynski and Sacchi (1987). Equal amounts (about 10 μg) of each sample, as determined by ethidium bromide staining of formaldehyde agarose gels, were transferred to nylon filters (Hybond N^+, Amersham) by capillary elution with 20 × SSPE. Blotted RNA was fixed following manufacturer's instructions and hybridized overnight at 42°C in presence of 50% formamide, 5 × SSPE, 5 × Denhardt's, 0.5% SDS and 5×10^5 cpm/ml of pNF-9.2 insert, labeled by random priming with ^{32}P-dCTP. High stringency washes were performed at 65°C with 0.1 × SSPE. Filter were exposed to *X*-ray film from 6 h to 2 days.

In situ hybridization

Radiolabeled antisense and sense cRNA probes were transcribed from clone pNF-6 after linearization with PstI and KpnI (Promega), respectively, using T3 or T7 RNA polymerase (Promega) and ^{35}S UTP, as previously described (Gorza *et al.*, 1993). The antisense probe was about 1800 nt long; the sense probe was 2200 nt long; both probes were digested to 50–100 nt by mild alkaline hydrolysis. Cryosections were fixed with formaldehyde and digested with 20 μg/ml of proteinase K, except for sections of early embryos which were not treated with proteinase K. Sections were hybridized overnight at 52°C with 10 μl of probe at a concentration of 1×10^5 cpm/μl, and washed at 65°C with 50% formamide, 2 × SSC and 0.1 M DTT. Sections were dehydrated, dipped in autoradiographic emulsion (Kodak NTB-2) diluted 1:1 with water, and exposed for 5–7 days at 4°C. Slides were developed with D 19 (Kodak) for 3.5 min, fixed and examined with a Zeiss Axioplan microscope equipped with dark field optics. Serial sections of the embryos were processed for routine hematoxylin-eosin staining.

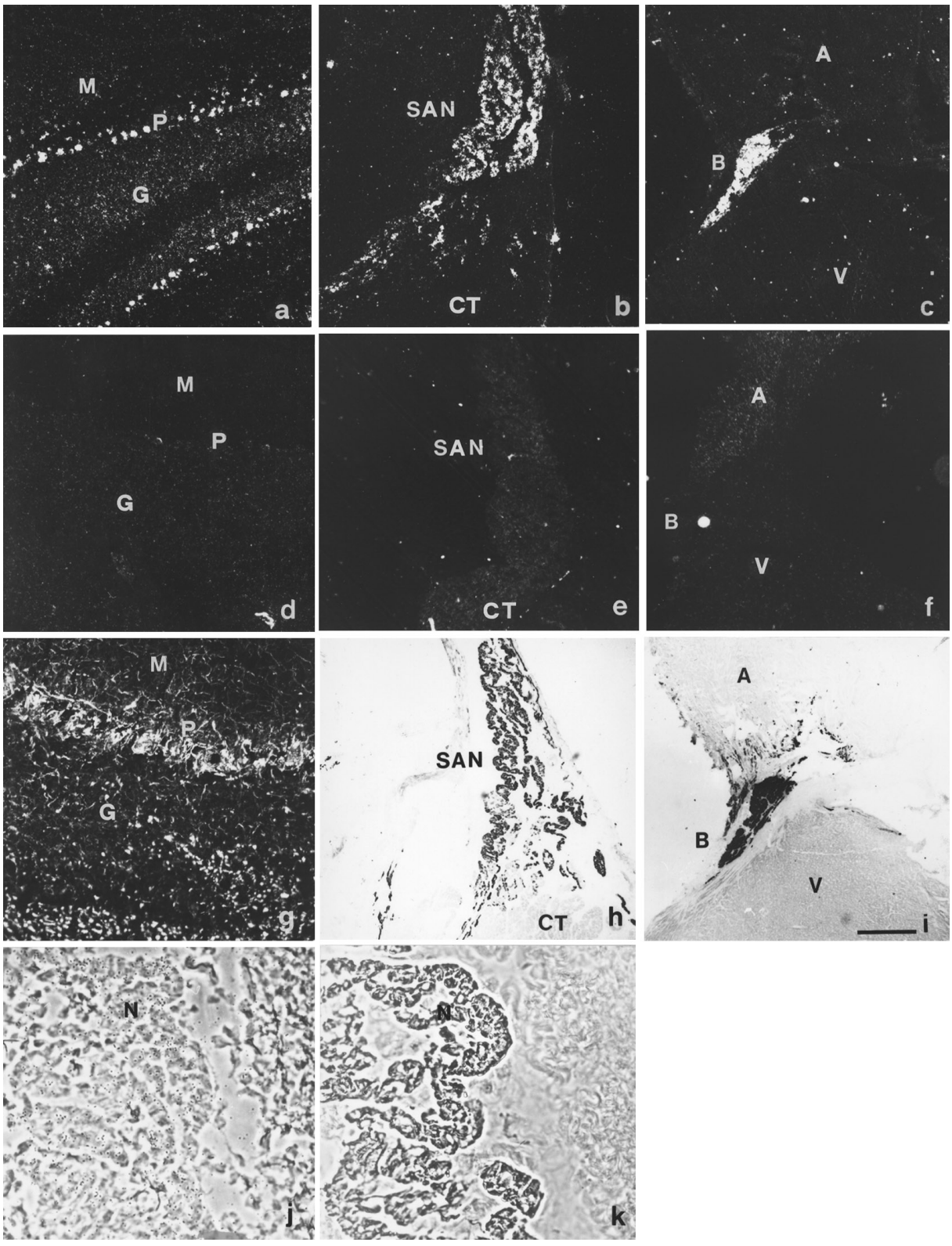
Immunohistochemistry

Cryosections (10 μm thick) from adult and embryonic specimens were used. Sections were incubated with iC8 antibody diluted with 0.5% bovine serum albumin in PBS. Incubation was carried out in a humidified chamber at 37°C for 30 min. After 30 min rinsing in PBS, sections were incubated with appropriate dilutions of secondary antibodies coupled with FITC or peroxidase.

Results

Conduction system myocytes of the rabbit heart express NF-M mRNA

An expression cDNA library enriched in SA node myocyte transcripts was screened with the anti-NF-M monoclonal antibody iC8, an antibody that has been demonstrated to be reactive with conduction system myocytes of the rabbit heart (Vitadello *et al.*, 1990). Three clones, pNF-9.2, pNF-6 and pNF-9.1 (2.4, 2.2 and 1.9 kb in length, respectively) were isolated from 2×10^5 recombinants examined. cDNA sequence analysis shows that the three clones overlap from the 3′ region and reveal a close similarity to NF-M cDNA sequence of man, rat, mouse and chicken (Myers *et al.*, 1987; Napolitano *et al.*, 1987; Levy *et al.*, 1987; Zopf *et al.*, 1990) both in the coding and in the 3′ untranslated flanking region (not shown). Alignment of deduced aminoacid sequence (Fig. 1)


M
P
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SAN
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b
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B
V
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M
P
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d
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CT
e
A
B
V
f
M
P
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g
SAN
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V
i
N
j
N
k

shows that the longest pNF cDNA spans from the rod coding region of the NF-M molecule, namely to aminoacid residue 216 of rat NF-M sequence (Napolitano *et al*., 1987), to the end of the tail region. The deduced aminoacid sequence shows 97% identity with rat and human NF-M in the rod region, while the tail domain shows 80% identity.

Total RNAs from adult rabbits were used in Northern blot analysis with pNF 9.2 cDNA. A single mRNA species of about 3 kb, a dimension similar to that calculated for NF-M mRNA in man and rat (Myers *et al*., 1987; Napolitano *et al*., 1987) is detectable in brain RNA (Fig. 2). Hybridization to a mRNA species of the same size is also observed with total RNA prepared from the SA node region of the rabbit heart, whereas no signal is detectable with RNAs prepared from the free wall of the left ventricular myocardium, from skeletal muscle and from liver (Fig. 2).

NF-M mRNA expression and protein localization in adult rabbit tissues

In situ hybridization experiments were undertaken to identify which cell type expresses the transcript. In the cerebellum, hybridization with the antisense probe shows strong signals in the somata of Purkinje cells, in a number of small cells of the inner third of the molecular layer, probably corresponding to basket cells, and in the granule cell layer [Fig. 3(a)]. This distribution of the transcript corresponds to that described for NF-M protein [Fig. 3(g) and Vitadello and Denis-Donini, 1990]. Positive labeling is also observed in spinal cord motor neurons and in superior cervical ganglion neurons whereas no signal is detected in non-neuronal structures of the brain or in other tissues, such as liver, skeletal muscle and kidney (not shown).

In the heart, the probe hybridizes with myocytes of the SA node [Fig. 3(b) and (j)], the AV node (not shown) and AV bundle [Fig. 3(c)], whereas no hybridization signal is detectable with working atrial and ventricular myocytes [Fig. 3(b) and (c)], confirming at the nucleic acid level the localization of the immunoreactivity observed at these same regions with anti-NF-M antibodies [Fig. 3(h)–(i) and (k)].

No hybridization signal is detected when the sense probe is used [Fig. 3(d)–(f)].

NF-M mRNA and protein expression in the developing heart

In order to determine whether NF-M mRNA is detectable in embryonic cardiac myocytes we extended our *in situ* hybridization studies to hearts of rabbit embryos obtained from embryonic day (E) 9 to 11 of development. To this purpose, cryosections had to be used instead of paraffin sections, because of the low intensity of the hybridization signal for the NF-M transcript observed after embedding of the embryos in paraffin.

No hybridization signal with NF-M antisense cRNA is apparently detectable in the heart of E9 embryos (not shown). At E9.5 of rabbit development, the heart tube displays the S-shaped conformation (Sissman, 1970): at this stage, hybridization signals for NF-M mRNA are present in the neural tube and are detectable in the heart only in rare myocytes [Fig. 4(b), (d) and (e)]. Serial section examination reveals that the myocytes positive for NF-M mRNA are localized in the ventricular myocardium in proximity to the right side of the AV junction [Fig. 4(e)]. Immunofluorescence analysis for cardiac NF-M expression, performed in embryos from the same litter, displays a detectable

Figure 3 (*opposite*) Parallel *in situ* hybridization analysis with antisense and sense pNF-6 cRNA probes (a–f and j) and immunolabeling with anti NF-M iC8 antibody (g–i and k) of adult rabbit tissues. Panels (a), (d) and (g) show cryostat sections of rabbit cerebellum; panels (b)–(c), (e)–(f) and (h)–(k) illustrate different regions of the rabbit heart: the SA node region (b, e, h, j–k), as it appears after sectioning the crista terminalis (CT) at its origin along a perpendicular plane to its major axis, and the AV bundle region (c, f and i). Darkfield micrograph of rabbit cerebellum shows hybridization signals with antisense NF-M cRNA in the somata of Purkinje (P) and in the granule cells (G) and in the inner third of molecular (M) layers (a), whereas no hybridization signal is apparently detectable with the sense probe (d). Darkfield micrographs of antisense NF-M cRNA show hybridization with myocytes of the SA node (SAN) and AV bundle (B), but not with working myocytes of either the crista terminalis, atrial (A) and ventricular (V) myocardium (b–c). No hybridization signal is apparently detectable in serial sections with the sense probe (e and f). The correspondence between NF-M mRNA and protein localization is confirmed by immunocytochemical analysis. (g) illustrates NF-M immunofluorescence in rabbit cerebellum; (h) and (i) correspond to serial sections of the SA node region (b, e) and AV bundle region (c, f) processed for immunoperoxidase: NF-M positive myocytes of the SA node and AV bundle appear dark, whereas working atrial and ventricular myocytes are unstained. Brightfield, phase contrast analysis of SA node region in consecutive sections confirms that silver grains corresponding to NF-M mRNA (j) and dark staining due to NF-M immunoreactivity (k) are localized in nodal myocytes (N). Bar: 270 μm (a, d); 400 μm (b,c,e,f,h,i); 190 μm (g); 35 μm (j–k).

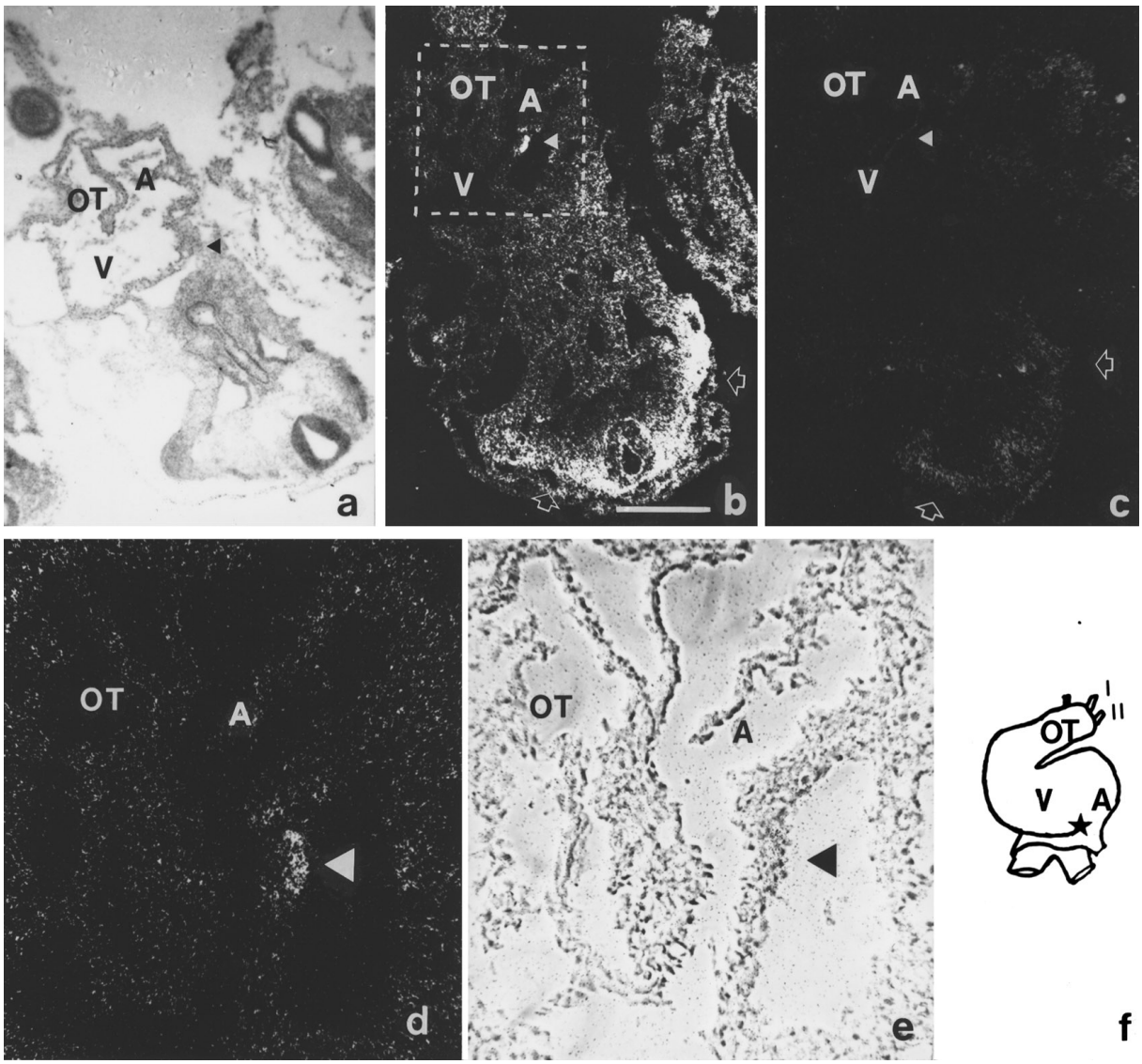

Figure 4 NF-M mRNA expression in E9.5 rabbit heart. Serial cryostat sections cut maintaining the heart in a frontal plane were stained with hematoxylin and eosin (a) and hybridized with antisense and sense pNF-6 cRNAs (b–e). Dark field micrographs show hybridization for NF-M mRNA at the level of the neural tube and in the heart (b, arrowhead). Arrows indicate regions showing strong hybridization signals which may correspond to migrating neural crest precursors (Sechrist *et al.*, 1993). Higher magnification of the cardiac region boxed in (b) is shown in dark (d) and bright fields (e). Only rare myocytes (arrowhead) localized in the ventricular myocardium (V) in proximity to the AV groove hybridized with the probe. A: atrium; OT: outflow tract. No hybridization signal is apparently detected with the sense probe (c). Panel (f) shows a diagram of E9.5 rabbit heart. Roman numbers indicate definitive aortic arches; star indicates the localization of myocytes expressing NF-M mRNA, deduced after serial section examination. Bar: a–c 300 μm; d–e: 75 μm.

signal only in rare ventricular myocytes: these myocytes are near to the AV junction and appear to be localized in the outer layer of the two-cell thick myocardial wall (Fig. 5).

At E10 of rabbit embryonic development, the heart looping is completed and hybridization signals with NF-M antisense probe are detected in the heart only in a small population of ventricular myocytes which are localized in proximity to and around the AV junction [Fig. 6(b) and (c)]. At E11, strong signal for NF-M mRNA is detectable in a larger number of myocytes which are mainly localized all around the AV junction, in the subendocardial layer of the ventricle and bulbus that corresponds to the growing interventricular septum, whereas weak signal is present in the SA region. Conversely, the outer layer of the ventricular and bulbar walls and a large portion of the atrial myocardium do not

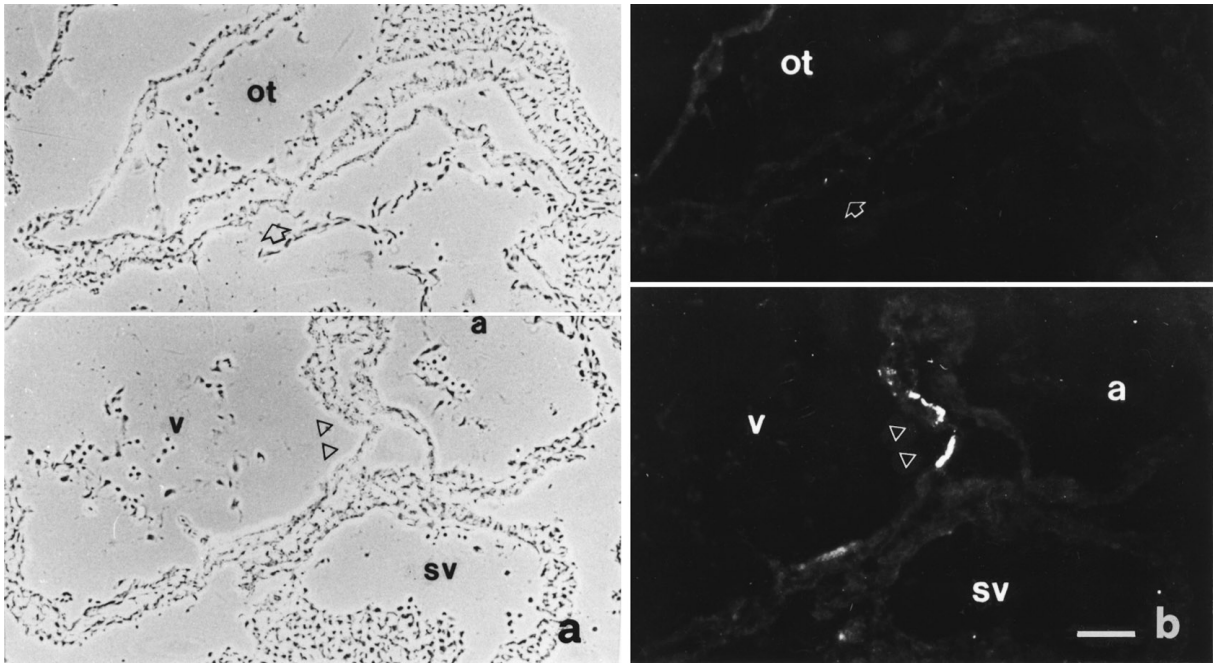

Figure 5 NF-M expression in E9.5 rabbit heart. Phase contrast (a) and NF-M immunofluorescence (b) of a sagittal cryostat sections from a E9.5 rabbit heart. Note NF-M positive myocytes at the ventricular side of the AV groove (arrowheads). Arrow indicates very weakly labeled myocytes localized at the opposite side of the AV junction. a: atrium; ot: outflow tract; sv: sinus venosus; v: ventricle. Bar: 50 μm.

show apparently any hybridization [Fig. 6(e)–(f)]. Also the myocytes of the outflow tract do not display accumulation of NF-M transcript, except for rare myocytes which appear to be localized very near to the embryonic pharyngeal arches [Fig. 6(e) and (f), arrows].

Discussion

This study shows that the intermediate filament network of the myocytes of the rabbit heart conduction system is composed of true neurofilament M protein, in addition to desmin. In fact, from a rabbit heart cDNA library enriched in SA node mRNAs we isolated three overlapping incomplete cDNA clones, which encode for the rabbit homologous of NF-M mRNA. Although the longest pNF cDNA lacks the 5′ untranslated region and a small portion of the coding region, its deduced aminoacid sequence shows high homology with NF-M of man and rat (Myers *et al*., 1987; Napolitano *et al*., 1987). Nucleic acid sequence analysis shows that the highest degree of identity (97%) is concentrated in the region coding for the rod domain, which is highly conserved in many species (Geisler *et al*., 1984) presumably because the formation of filaments requires multiple interactions in this region (Steinert and Roop, 1988). In adult animals, Northern blot analysis identifies a single mRNA species that is expressed in both the brain and the SA node region, but not in the ventricular myocardium and other tissues such as skeletal muscle and liver. The size of rabbit NF-M mRNA (about 3.0 kb) is consistent with those reported for rat and human NF-M mRNAs (Myers *et al*., 1987; Napolitano *et al*., 1987). *In situ* hybridization experiments confirmed that in the adult brain the expression of NF-M mRNA was restricted to neurons and showed that NF-M transcript is detectable in conduction system myocytes, i.e. in myocytes of the SA and AV nodes and of the AV bundle and bundle branches, but not in working atrial and ventricular myocytes of the adult heart. These findings validate our previous observations concerning NF-M immunoreactivity of conduction system myocytes of the rabbit heart (Gorza *et al*., 1988; Vitadello *et al*., 1990) and indicate that the immunolabeling was not due to spurious cross-reactions.

Other major findings about NF-M mRNA expression in the heart concern the distribution in myocytes during embryonic development. Our investigation shows that NF-M mRNA accumulation (i) is detectable in cardiac myocyte since the S-shape stage of heart development (ii) appears to be restricted to a subset of cardiac myocytes and (iii) is accompanied by the presence of the protein.

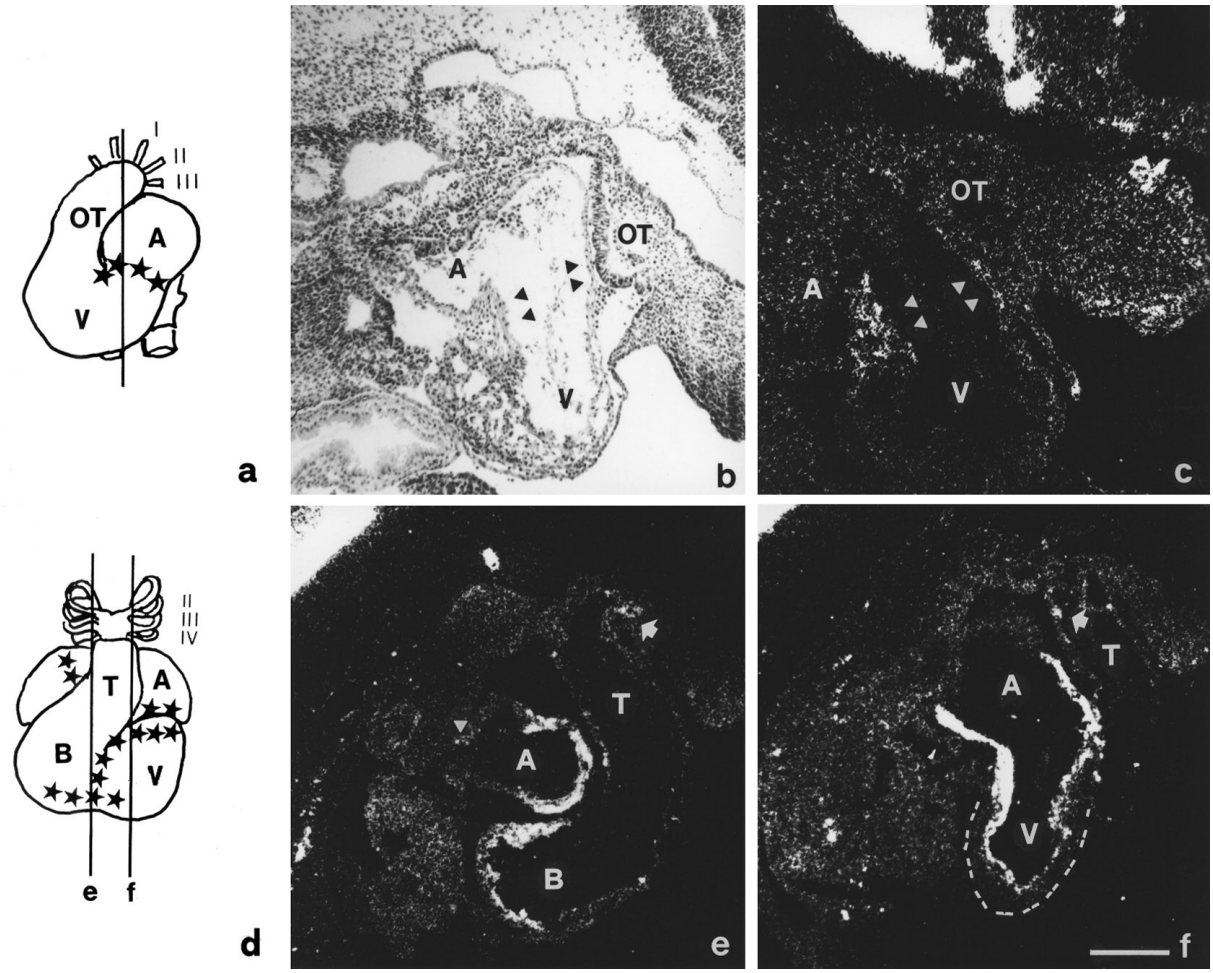

Figure 6 NF-M mRNA expression in E10 and E11 rabbit hearts. Diagrams illustrating ventral views of rabbit heart at E10 and E11 are shown in panels (a) and (d), respectively. Vertical bars in diagrams indicate the levels at which sagittal sections illustrated in (b–c), (e) and (f) were cut. Roman numbers indicate definitive aortic arches; stars indicate the localization of myocytes expressing NF-M mRNA, deduced from serial section examination. (b): E10 rabbit heart stained for hematoxylin-eosin. (c): darkfield view of a serial section hybridized with antisense pNF-6 cRNA; in addition to the neural tube, which remains on the upper part of the micrograph, NF-M mRNA is detected in the heart in the myocytes at the level of the AV junction (arrowheads). A: atrium; V: ventricle; OT: outflow tract. (e) and (f): darkfield views of E11 rabbit embryo heart hybridized with antisense pNF-6 cRNA; NF-M mRNA is detected in the heart in a large subset of subendocardial myocytes, localized at the AV junction, in the bulbus (B) and in the ventricle (V). In (e) arrowhead indicates a weak hybridization signal at the level of the SA junction. In (f) a dotted line was drawn to indicate the outer ventricular wall which does not hybridize with the probe. Note that the truncus (T) does not display hybridization signals, except for few myocytes (arrows in e and f) near to the pharyngeal arch. Bar: 150 μm (b,c); 300 μm (e, f).

Several genes, such as slow skeletal troponin I (Gorza *et al.*, 1993; Zhu *et al.*, 1995) and smooth α-actin in the rat and connexin isoform Cx 42 in the chicken (reviewed in Gorza *et al.*, 1994), are expressed in every embryonic cardiomyocyte during heart development and are down-regulated in the adult heart in working atrial and ventricular myocytes, but not in the myocytes of the conduction system. Here we report that NF-M transcript is detectable in a subpopulation of cardiac myocytes since early stages of embryonic development. Such evidence represents the first example of a gene which in the adult is expressed in all the compartments of the heart conduction system, i.e. the SA and AV nodes and the AV bundle and bundle branches, and in the embryo is restricted to a subpopulation of cardiac myocytes. Moreover, the embryonic myocytes displaying NF-M mRNA are not randomly distributed; they are localized in proximity of the right-ventral side of the AV groove already at E9.5 of development. At E10 and E11 stages of development, myocytes expressing NF-M

transcript surround the AV junction and are localized in the subendocardial layer of the ventricular and bulbar myocardium, namely in correspondence of the growing interventricular septum, but not in the truncal myocardium. NF-M mRNA expression is accompanied by the presence of the protein, which can be detected since E9.5 stage of development by immunohistochemistry and whose distribution follows the regional distribution of the transcript (this study and Gorza and Vitadello, 1989). Previous immunohistochemical observations showed that the population of myocytes reactive for NF-M in E15 rabbit embryonic hearts could be unequivocally identified as myocytes of the heart conduction system on the basis of their anatomical distribution (Gorza and Vitadello, 1989). We thus speculate that the myocytes expressing NF-M mRNA and protein before E15 of development correspond to heart conduction system myocytes. A study performed on embryonic human hearts, at a developmental stage approximately corresponding to E10 in the rabbit embryo (Sissman, 1970), showed that myocytes of the heart conduction system, identified by the monoclonal antibody G*l*N2, were localized near to both the right side of the AV groove and the bulboventricular groove (Wessels *et al*., 1992). NF-M positive ventricular myocytes are indeed detected at the same sites where G*l*N2 immunoreactivity was reported; however, NF-M is expressed by a larger population of myocytes which surrounds the AV junction, and is detectable in myocytes of the SA node region (this study and Gorza and Vitadello, 1989), whereas the G*l*N2 epitope appears to be transitorily expressed only by myocytes of the AV node and AV bundle of the heart conduction system (Wessels *et al*., 1992).

A major question concerns the significance of NF-M expression in myocytes of the conduction system of the rabbit heart. No evidence for the presence of NF-M and L proteins and their corresponding mRNAs has been obtained in rats and humans (Aoyama *et al*., 1993; Gorza L and Vitadello M, unpublished observations), although transient expression of a NF-M-like protein has been described in myocytes of the developing chicken heart (Bennett and DiLullo, 1987). This fact may indicate that the expression of NF-M protein is not essential for the function of specialized heart myocytes. Until recently, NFs have been considered to be expressed only in post-mitotic neurons where they play a structural role (Hoffmann *et al*., 1987). However, NF protein subunits were identified in other non-neuronal cells: rat myelin-forming Schwann cells, which express NF-M protein and transcript (Kelly *et al*., 1992) and the β cells of the islets of Langerhans in the embryonic rat, which express NF-L (Escurat *et al*., 1991).

The co-expression of neural and muscle genes in heart conduction system myocytes raises questions about the myogenic or neurogenic origin of these myocytes. Recently, Gourdie and co-workers (1995), using retroviral marking, demonstrated that in the chick heart the peripheral component of the conduction system (i.e., the ventricular intramural Purkinje myocyte network) shares a common myogenic precursor with ventricular working myocytes. Unfortunately, viral tagging of myogenic precursors in E3 embryonic chicken heart did not succeed in tracking the origin of the central component of the heart conduction system (i.e., the SA and AV nodes and AV bundle), leaving open the question about the origin of this important component of the heart conduction system. We interpreted the NF expression in heart conduction system as suggestive for the neuroectodermal origin of this myocyte population (Gorza *et al*., 1988). In the present study we show that NF-M expression, even at the mRNA level, remains circumscribed to a subset of cardiac myocytes since early stages of heart development. While this evidence does not add further arguments to unveil the origin of these myocytes, the apparently restricted up-regulation of the NF-M gene underlines the precocious diversification of this myocyte population during heart development.

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