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cDNA cloning and spatiotemporal expression during avian embryogenesis of hnRNP A1, a regulatory factor in alternative splicing

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Abstract

Alternative splicing and selective transport of RNA transcripts from cell nuclei are important regulatory mechanisms of gene expression during embryonic development. Here we report the molecular characterization and developmental expression in several tissue and organ systems of chicken hnRNP A1, a nucleo-cytoplasmic 'shuttle' protein which in mammalian systems has been shown to function in the regulation of RNA alternative splicing by antagonizing constitutive splicing factors such as SF2/ASF. We show that hnRNP A1 is represented in the chicken by a single gene which is widely expressed at early embryonic stages, with particularly high levels of expression in the brain, skin, developing gut, and other ectodermal and endodermal derivatives. At later stages, expression of its mRNA and protein product become progressively confined to specific organ primordia and cell types, where both transient and persistent expression patterns are observed. HnRNP A1 protein is expressed at sites of active neurogenesis in the developing central and peripheral nervous systems, regions of known extensive alternative splicing.

Keywords: Kidney development; Liver development; Neurogenesis; Natural antisense RNA

1. Introduction

Recent evidence suggests that more than half of human and other vertebrate genes are alternatively spliced (Modrek and Lee, 2002; Maniatis and Tasic, 2002). New information on the factors and mechanisms by which it is accomplished (Chabot, 1996; Lopez, 1998; Graveley, 2001; Dredge et al., 2001; Nissim-Rafinia and Kerem, 2002), and a growing understanding of the determinants of nucleo-cytoplasmic transport of particular mRNAs (Piñol-Roma and Dreyfuss, 1992; Siomi and Dreyfuss, 1997; Weis, 1998), has set the stage for a systematic analysis of how RNA processing factors contribute to regional and cell type specificity in the embryo. Ribonucleoprotein hnRNP A1 is of particular interest in this regard, as it functions in both RNA splice site selection and nucleus-to-cytoplasm transport of mRNA. In its capacity as a splicing factor this protein modulates 5′ splice site selection in a group of gene products, some of which contain a well-characterized RNA sequence determinant (Burd and Dreyfuss, 1994). Among these are the pre-mRNAs of the HIV type 1 tat protein (Del Gatto-Konczak et al., 1999; Tange et al., 2001), FGF receptor 2 (Del Gatto-Konczak et al., 1999), the receptor CD44 (Matter et al., 2000), and hnRNP A1 itself (Chabot et al., 1997). In its role in nucleus-to-cytoplasm transport, hnRNP A1 acts as a 'shuttle' protein (Piñol-Roma and Dreyfuss, 1992), and is characterized by a novel amino acid motif termed M9, which contains both nuclear localization and nuclear export activities (Michael et al., 1995; Siomi and Dreyfuss, 1995). We have now cloned cDNA specifying the full length of the chicken hnRNP A1 protein, and have examined its RNA and protein expression in whole embryos and in a number of developing organ primordia between 5 and 12 days of development.

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2. Results and discussion

2.1. cDNA and protein sequences of chicken hnRNP A1

A 683 bp reverse transcription–polymerase chain reaction (RT–PCR) product was amplified from 7-day chicken embryo brain mRNA using degenerate primers based on microsequence analysis of 11 different tryptic fragments of a single protein spot with apparent molecular weight ~35 kDa which was present in both precartilage and cartilage nuclei of 5–7-day embryonic limbs (Zhang et al., 1996). This sequence was extended to 1689 bp by amplifying overlapping PCR products from a chicken limb cDNA library (Fig. 1A). The 1689 bp sequence contained a 1134 bp open reading frame that specified a 378-amino-acid residue protein with a molecular weight of 39.4 kDa (Fig. 1B). Approximately half the predicted amino acid residues of this protein were represented in the 11 tryptic peptides on which the original PCR primers were based, and the entire sequence comprised by the tryptic peptides was ~99% identical to segments within the conceptual translation product of the cDNA.

This chicken protein had 78% sequence identity to the major 320-amino-acid human hnRNP A1 protein (Biamonti et al., 1989), and contained two RNA binding motifs at corresponding sites, as well as an RGG box (Dreyfuss et al., 1993). It also contained a ~40 amino acid residue M9 motif near its carboxy- terminal end, which contains nuclear import and export signals, and is a hallmark of the hnRNP A1 and A2/B1 classes of RNA binding proteins (Izaurralde et al., 1997; Siomi and Dreyfuss, 1995). Residues 272–318 of the chicken hnRNP A1 sequence do not have a counterpart in the human major A1 species but are similar in sequence to the corresponding region of the B variant of the human hnRNPA1 protein (Fig. 1B). This less abundant isoform is generated by alternative splicing in both human (Buvoli et al., 1990) and mouse (Chabot et al., 1997). This suggests that the sequence shown in Fig. 2 represents the B variant of chicken hnRNP A1. However, neither the antibody nor the cRNA probes used in the expression studies below bind sequences specific to the alternatively spliced region; they would therefore detect both potential hnRNP A1 isoforms. Chicken hnRNP A1 also contains a 25-residue N-terminal extension that has no counterpart in either of the human hnRNP A1 sequences. However this sequence is dissimilar to the N-terminal peptide of hnRNP B1 (Fig. 1B), which is the only human protein in this class with an analogous extension.

Southern hybridization of chicken genomic DNA cut with several different enzymes, using the central 683 bp RT–PCR product as probe, in all cases led to a simple pattern of 2–3 fragments, suggesting that the hnRNP A1 gene is present in a single copy in the chicken genome (Fig. 2A). To confirm this, we also performed Southern hybridization under nonstringent conditions (see Section 3), but were unable to detect additional bands (not shown). Therefore, unlike the human genome, which appears to contain a single active hnRNP A1 and a number of hnRNP A1 pseudogenes (Saccone et al., 1992), the chicken genome contains only a single copy of hnRNP A1 DNA. Northern blot hybridization of total RNA from 5-day embryos shows one major RNA species at ~3.7 kb (Fig. 2B). This species corresponded to the mature mRNA, as confirmed by Northern blot analysis of poly $(A)^+$ RNA isolated from whole embryos and several embryonic tissues (not shown).

2.2. Immunoblot and quantitative RT–PCR analysis of chicken hnRNP A1 in embryonic tissues at 5 and 7 days

Immunoblot analysis of 7-day embryos using affinity purified anti-hnRNP A1 antibody indicated a single reactive 39 kDa band in several tissues at this stage, including brain, limb and tail buds, heart (Fig. 2C), kidney and liver (not shown). The extraembryonic membranes contained hnRNP A1 at a lower percentage of total protein than the developing embryonic organs (Fig. 2C).

Relative abundance of hnRNP A1 poly$(A)^+$ RNA in selected organs of 9-day embryos was generally 10–20% than that in 5-day embryos, with the exception of intestine, where it remained high (Table 1). Because of the reported antagonistic relationship of proteins of the hnRNP A/B family to constitutive splicing factors such as SF2/ASF in regulation of alternative splicing (Mayeda and Krainer, 1992; Mayeda et al., 1993, 1994; Del Gatto-Konczak et al.,

Table 1
Quantitation of hnRNP A1 gene expression in whole embryos and embryonic organs

Sample	Primers	Mean no. of cycles[a]	T_m	A1/GAPDH
5-day embryo	hnRNP A1	23.1	88.4	0.022
	GAPDH	17.5	88.1	
Kidney	hnRNP A1	23.5	87.9	0.002
	GAPDH	15.9	87.7	
Skeletal muscle	hnRNP A1	22.8	88.0	0.004
	GAPDH	15.9	87.9	
Heart	hnRNP A1	23.2	88.2	0.004
	GAPDH	16.3	88.0	
Skin	hnRNP A1	22.7	88.0	0.003
	GAPDH	15.2	87.8	
Brain	hnRNP A1	21.8	88.1	0.004
	GAPDH	14.6	87.9	
Intestine	hnRNP A1	22.0	87.9	0.020
	GAPDH	17.1	88.1	
Liver	hnRNP A1	22.2	88.0	0.001
	GAPDH	12.7	87.8	

Samples consisted of total RNA derived from 5-day whole embryos or organs isolated from 9-day embryos. Equal amounts of RNA were used for each reverse transcription reaction and corresponding gene-specific sequences were quantitated by real time PCR using the LightCycler in the SYBR Green-I dye binding mode (see Section 3). Relative amplicon concentrations were calculated in relation to dilution curves from three replicate measurements for each primer set, with all SEMs for hnRNP A1 ≤ 7% and for GAPDH ≤ 13%.

[a] Initiation of exponential accumulation of amplification product.

A

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   1 gcgtctccac ccctcagcgg gcggcggtga gtgcgccagg ccagcgccgg cgtgggaccg
  61 agcgggcgtg aaggcgcgag ctgaacgctg gcacggtttc ctagatctaa aagaaaggcc
 121 gagttagagt acccttccaa aATGGCTGCT ATTAAGGAAG AGAGAGAGGT GGAAGATTAC
 181 AAGAGAAAAA GGAAGACGAT CAGCACAGGC CATGAGCCTA AGGAGCCAGA GCAGTTGAGA
 241 AAGCTGTTCA TTGGAGGTCT GAGCTTCGAG ACGACGGATG ATAGCTTGAG AGAGCACTTT
 301 GAAAAATGGG GCACACTCAC GGACTGTGTG GTGATGAGAG ACCCACAAAC AAAACGTTCC
 361 AGAGGCTTTG GCTTTGTTAC TTACTCTTGC GTGGAAGAGG TGGATGCGGC CATGAGCGCT
 421 CGACCACATA AGGTGGATGG ACGTGTGGTT GAACCAAAGA GAGCAGTTTC AAGGGAGGAT
 481 TCTGTAAAGC CTGGGGCGCA TCTCACAGTA AAGAAAATAT TTGTTGGTGG CATTAAAGAA
 541 GATACAGAAG AATATAATTT AAGGGGGTAC TTTGAAACAT ATGGCAAGAT CGAAACGATA
 601 GAAGTCATGG AAGACAGACA AAGTGGAAAG AAAAGAGGCT TCGCTTTTGT AACTTTTGAT
 661 GATCACGATA CAGTTGATAA AATTGTTGTT CAGAAATACC ATACTATAAA TGGTCATAAC
 721 TGCGAAGATA AAAAAGCACT CTCAAAACAA GAGATGCAGA CTGCCAGCTC TCAGAGAGGT
 781 CGTGGGGGTG GTTCAGGCAA CTTCATGGGT CGTGGAAATT TTGGAGGTGG TGGAGGAAAC
 841 TTTGGCCGAG GAGGAAACTT TGGTGGAAGA GGAGGCTATG GGGGTGGTGG TGGCGGTGGT
 901 GGGAGCAGAG GAAGCTTTGG GGGTGGTGAT GGATACAACG GATTTGGTGA TGGTGGCAAC
 961 TATGGAGGTG GTCCTGGCTA TGGCAGCAGA GGGGGTTATG GTGGTGGTGG AGGACCAGGA
1021 TATGGAAACC CAGGTGGTGG ATATGGAGGT GGAGGAGGAG GATATGGTGG CTACAATGAA
1081 GGAGGCAATT TTGGAGGTGG TAATTATGGA GGCAGTGGAA ACTACAATGA CTTTGGTAAC
1141 TACAGTGGAC AGCAGCAGTC CAATTACGGT CCCATGAAAG GTGGTGGCAG TTTTGGTGGT
1201 AGAAGTTCAG GCAGTCCCTA TGGTGGTGGT TATGGATCTG GAAGTGGAAG TGGGGGCTAT
1261 GGTGGTAGAA GATTCTaaaa atgctaccag aaaaagggct acagttctta gcaggagaga
1321 gagcgaggag ttgtcaggaa agctgcaggt tactttgaga cagtcgtccc aaatgcatta
1381 gaggaactgt aaaatctgcc acagaaggaa cgatgatcca tagtcagaaa agttactgca
1441 gcttaaacag gaaacccttc ttgttcagga ctgtcatagc cacagtttgc aaaaagagca
1501 gctattggtt aatgcaatgt agtgtcgtta gatgtacatc ctgaggtctt tatctgttgt
1561 agctttgtct ttcttttttc tttttatttt cccattacat caggtatatt gccctgtaaa
1621 ttgtggtagt ggtaccagga ataaacaaat taaggaattt ttggcttttc aaaaaaaaaa
1681 aaaaaaaaa
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B

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CHKA1   MAAIKEEREVEDYKRKRKTISTGHEPKEPEQLRKLFIGGLSFETTDDSLREQFEKWGTLTDCVVMRDPQTKRSRG  75
HUA1    ------------------MSKSESPKEPEQLRKLFIGGLSFETTDESLRSHFEQWGTLTDCVVMRDPNTKRSRG  56
HUA1B   ------------------MSKSESPKEPEQLRKLFIGGLSFETTDESLRSHFEQWGTLTDCVVMRDPNTKRSRG  56
HUA2    ------------------------MEREKEQFRKLFIGGLSFETTEESLRNYYEQWGKLTDCVVMRDPASKRSRG  51
HUB1    ------------MEKTLETVPLERKKREKEQFRKLFIGGLSFETTEESLRNYYEQWGKLTDCVVMRDPASKRSRG  63

CHKA1   FGFVTYSCVEEVDAAMSARPHKVDGRVVEPKRAVSREDSVKPGAHLTVKKIFVGGIKEDTEEYNLRGYFETYGKI 150
HUA1    FGFVTYATVEEVDAAMNARPHKVDGRVVEPKRAVSREDSQRPGAHLTVKKIFVGGIKEDTEEHHLRDYFEQYGKI 131
HUA1B   FGFVTYATVEEVDAAMNARPHKVDGRVVEPKRAVSREDSQRPGAHLTVKKIFVGGIKEDTEEHHLRDYFEQYGKI 131
HUA2    FGFVTFSSMAEVDAAMAARPHSIDGRVVEPKRAVAREESGKPGAHVTVKKLFVGGIKEDTEEHHLRDYFEEYGKI 126
HUB1    FGFVTFSSMAEVDAAMAARPHSIDGRVVEPKRAVAREESGKPGAHVTVKKLFVGGIKEDTEEHHLRDYFEEYGKI 138

CHKA1   ETIEVMEDRQSGKKRGFAFVTFDDHDTVDKIVVQKYHTINGHNCEDKKALSKQEMQTASS-QRGRGGGSGNFMG- 223
HUA1    EVIEIMTDRGSGKKRGFAFVTFDDHDSVDKIVIQKYHTVNGHNCEVRKALSKQEMASASSSQRGRSG-SGNFGGG 205
HUA1B   EVIEIMTDRGSGKKRGFAFVTFDDHDSVDKIVIQKYHTVNGHNCEVRKALSKQEMASASSSQRGRSG-SGNFGGG 205
HUA2    DTIEIITDRQSGKKRGFGFVTFDDHDPVDKIVLQKYHTINGHNAEVRKALSRQEMQEVQSSRSGRGG---NFGF- 197
HUB1    DTIEIITDRQSGKKRGFGFVTFDDHDPVDKIVLQKYHTINGHNAEVRKALSRQEMQEVQSSRSGRGG---NFGF- 209

CHKA1   RGNFGGGGGNFGRGGNFGGRGGYGGGGGGGGSRGSFGGGDGYNGFGDGGNYG--GG-PGYGSRGGYGGGGGPGYG 295
HUA1    RGGGFGGNDNFGRGGNFSGRGGFGGSRGGGG-YG--GSGDGYNGFGNDG-------------------------- 251
HUA1B   RGGGFGGNDNFGRGGNFSGRGGFGGSRGGGG-YG--GSGDGYNGFGNDGGYG--GGGPGYSGGSRGYGSGGQGYG 275
HUA2    -GDSRGGGGNFGPGPGSNFRGGSDGYGSGRG------FGDGYNGYGGGPGGGNFGGSPGYGGGRGGYGGGGPGYG 265
HUB1    -GDSRGGGGNFGPGPGSNFRGGSDGYGSGRG------FGDGYNGYGGGPGGGNFGGSPGYGGGRGGYGGGGPGYG 277

CHKA1   NPGGGYGGGGGGYGGYNEGGNFG-----GGNYGGSGNYNDFGNYSGQQQSNYGPMKGGGSFGG-RSSGSPYGGG- 363
HUA1    ----------------------------SNFGGGGSYNDFGNYN-NQSSNFGPMKGG-NFGG-RSSG-PYGGGG 293
HUA1B   NQGSGYGGSGS-YDSYNNGGGGGFGGGSGSNFGGGGSYNDFGNYN-NQSSNFGPMKGG-NFGG-RSSG-PYGGGG 346
HUA2    NQGGGYGGGYDNYG--------------GGNYG-SGNYNDFGNYN-QQPSNYGPMKSG-NFGGSRNMGGPYGGG- 322
HUB1    NQGGGYGGGYDNYG--------------GGNYG-SGNYNDFGNYN-QQPSNYGPMKSG-NFGGSRNMGGPYGGG- 334

CHKA1   -----------YGSGSGSGGYG-GRRF  378
HUA1    QYFAKPRNQGGYGGSSSSSSYGSGRRF  320
HUA1B   QYFAKPRNQGGYGGSSSSSSYGSGRRF  372
HUA2    --------NYGPGGSGGSGGYGGRSRY  341
HUB1    --------NYGPGGSGGSGGYGGRSRY  353
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Fig. 1. (A) Full-length cDNA sequence of chicken hnRNP A1. Capital letters indicate open reading frame. Underlined sequence represents the 683 bp fragment used as a probe in these studies. (B) Translation product of residues 142–1275 of the cDNA shown in (A). Protein sequence is aligned with the translation products of human hnRNPA1 (Biamonti et al., 1989; accession no. NP002127), hnRNP A1B hnRNPA1 (Biamonti et al., 1989; accession no. NP112420), hnRNP A2 (Biamonti et al., 1994; accession no. NP2128), and hnRNP B1 (Biamonti et al., 1994; accession no. NP112533) using the CLUSTALW program (Thompson et al., 1994). RNA binding motifs, underlined; RGG box, dotted underline; M9 sequence, thick underline. Highlighted residues represent peptide used for polyclonal antibody production.

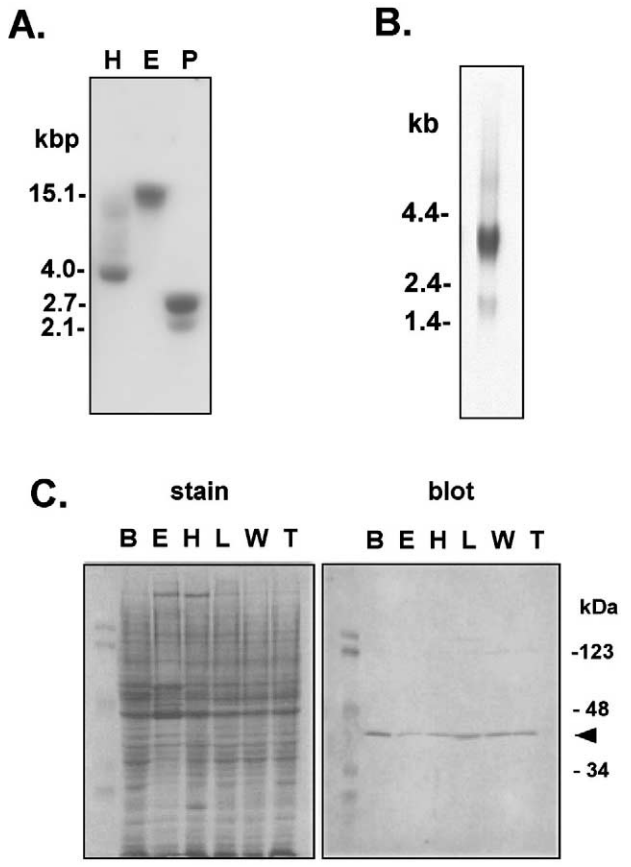

Fig. 2. (A) Southern blot analysis of hnRNP A1. Genomic DNA was digested with various restriction endonucleases: H, *Hin*dIII; E, *Eco* RI; P, *Pst* I. Fragments were detected using an [α-^{32}P]dCTP-labeled random primed probe generated from the cloned double-stranded 683 bp cDNA fragment. (B) Northern blot of total RNA from 5-day embryo. (C) Immunodetection of embryonic tissues using an affinity purified anti-hnRNP A1 antibody. Arrowhead indicates the 39 kDa band corresponding to chicken hnRNP A1. Coomassie gel (left) and Western blot (right) of 7-day embryonic tissue: B, brain; E, extraembryonic membrane; H, heart; L, leg; W, wing; T, tailbud.

1999), and the cellular complexity of the organs analyzed for bulk protein and mRNA comparisons in Fig. 2C and Table 1, a more detailed analysis of the expression of hnRNP A1 at both the RNA and protein levels during the period of active organogenesis was warranted.

2.3. Spatiotemporal expression of hnRNP A1 RNA during early development

The modified in situ hybridization procedure employed, which used RNA probes directly labeled with alkaline phosphatase, permitted whole-mount visualization of transcripts at more advanced embryonic stages than possible with conventional methods. Expression of hnRNP A1 mRNA in several tissues and organs of a 6-day embryo is seen in Fig. 3. High levels of expression are seen in the brain, the dorsal axis, the limb buds, the heart, and the extraembryonic membranes (Fig. 3A, left embryo). In addition, natural antisense RNA (Storz, 2002) was detected in some of the same regions in which mRNA was present but at a lower level of expression (Fig. 3A, right embryo). The authenticity of the antisense expression, which has also been reported for another splicing factor (Sureau et al., 1997), was confirmed by RNase protection (Fig. 3B). Its presence necessitated the use of an extraneous probe as a negative control for in situ hybridizations (see below).

In sectioned 4.5–6-day embryos in situ hybridization detected mRNA in heart muscle (Fig. 3C), the endothelial cells and subendothelial connective tissues of the aorta (Fig. 3E,F), the mesonephric kidney (Fig. 5A), the liver (Fig. 6A, B), the ventricular lining of the brain (Fig. 3C), the developing neural tube (Fig. 3A,C), as well as in the developing cranial nerves (Fig. 3H) and dorsal root ganglia (Fig. 3C). Messenger RNA expression was also detected in limb buds (Fig. 3A, left), vertebral bodies (Fig. 3C), and the epithelium of the gut, with particularly high expression levels detected in the crop and gizzard (Fig. 3C,G). Expression of hnRNP A1 message was also at a high level in the mandibular swelling of the developing pharyngeal arches (Fig. 3C,I) in contrast to the low level of expression of natural antisense RNA in this primordium (Fig. 3D,J). Both sense (Fig. 3C,K) and natural antisense hnRNP A1 RNA (Fig. 3D,L) were expressed in the developing heart ventricle. The latter was mainly confined to cell nuclei (Fig. 3L), confirming that staining was not the result of contaminating antisense probe sequences. Control sections probed with an extraneous digoxigenin-labeled cRNA showed no staining (Fig. 3M).

2.4. Localization of hnRNP A1 protein in 5–6-day embryos

Immunohistochemistry of 5–6-day sectioned embryos detected hnRNP A1 protein in a only a subset of tissues expressing the RNA (compare Fig. 4A with Fig. 3C). Localization of protein was primarily nuclear. As expected from the whole-mount in situ detection of sense transcript (Fig. 3A, left), hnRNP A1 protein was present in the extraembryonic membranes (Fig. 4B). The inset in Fig. 4B shows lack of staining of the extraembryonic membranes when a primary antibody reactive with a different class of RNA binding proteins, Hu (Wakamatsu and Weston, 1997), was used. Consistent with the in situ detection of sense transcript in sectioned tissue (Fig. 3D,J,L) hnRNP A1 protein was also detected in the epithelial lining of the intestine (Fig. 4D), and in both the smooth muscle of the crop and the gizzard as well as in the epithelial lining of these organs (Fig. 4A, higher magnification, not shown). In the developing lung bud, hnRNP A1 was detected throughout the parenchyma (Fig. 4C). Protein was also detected in the atrial and ventricular myocardial tissues (Fig. 4E,F), the developing mesonephric tubules (Fig. 5B), as well as in the developing gonads (not shown).

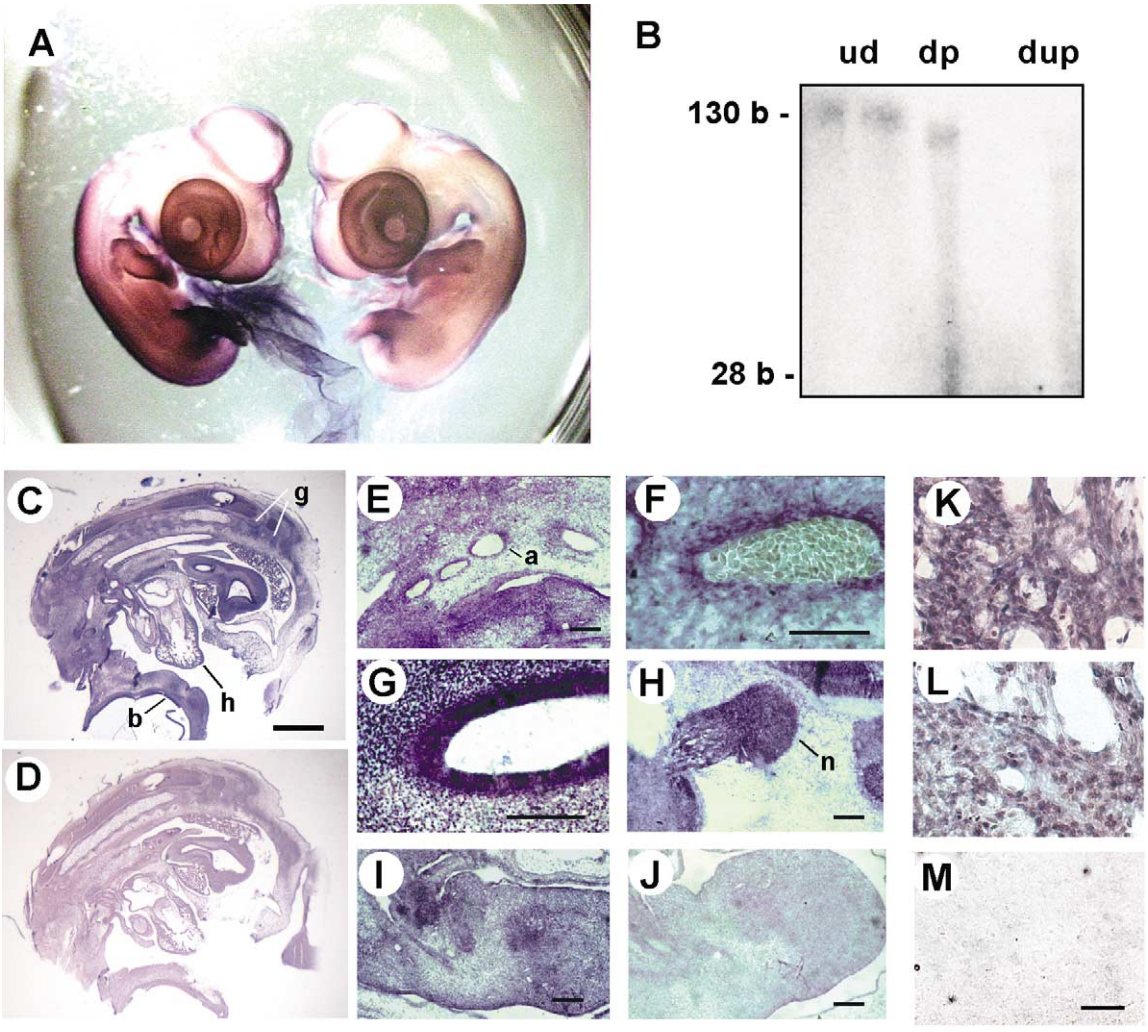

Fig. 3. (A) In situ hybridization of 6-day whole embryos. Left: detection of sense hnRNP A1 using alkaline phosphatase-labeled antisense cRNA probe. High levels of expression are seen in the dorsal axis, limb buds, developing brain, and extraembryonic membranes. Right: detection of natural antisense hnRNP A1 RNA using alkaline phosphatase-labeled sense cRNA probe. Antisense transcripts are present at numerous sites throughout the embryo, but at generally lower levels than sense transcripts. (C,D) Low magnification views of in situ hybridization of 5.5–6-day sectioned embryos. Sense and antisense transcripts were detected by digoxigenin-UTP labeled cRNA probes. (B) Detection of natural antisense hnRNP A1 transcript by RNase protection. Sense transcript (~130 b) labeled with [α-^{32}P]UTP was incubated under hybridization conditions with equal amounts of total RNA from 5-day embryos or yeast total RNA. Each reaction mixture contained 20 000 cpm of labeled probe. The chicken embryo RNA sample and one yeast RNA sample were digested with RNase A/T1, and another yeast RNA sample served as an undigested control (see Section 3). Following hybridization and digestion all samples were precipitated and resuspended in 20 μl of gel sample buffer. Lanes: ud, ~100 cpm of replicate samples containing undigested sense transcript hybridized with yeast total RNA; dp, 2 × 10 μl samples containing digested (protected) sense transcript hybridized with chicken embryo total RNA; dup, 20 μl of sample containing digested (unprotected) sense transcript hybridized to yeast total RNA. The band of ~130 b in the ud lane (arrow) comprised the full-length probe; the protected fragment had a size of ~110 b. Sizes of RNA molecules estimated relative to migration of bromophenol blue and xylene cyanole tracking dyes, the positions of which are indicated on the left. (C) Sagittal section of whole embryo stained for sense hnRNP A1 (b, brain ventricle; h, heart; g, dorsal root ganglia). Bar = 1 mm. (D) Sagittal section of whole embryo stained for antisense hnRNP A1. (E–M) Higher magnification views of various organs stained for sense hnRNP A1. (E) Aorta (a). Bar = 100 μm. (F) Aorta. Bar = 50 μm. (G) Gizzard. Bar = 100 μm. (H) Cranial nerve (n). Bar = 100 μm. (I) Mandibular process. Bar = 100 μm. (J) Stained for antisense hnRNP A1: mandibular process. Bar = 100 μm. (K) Sense transcript in the heart ventricle; hnRNP A1 RNA is localized to both nuclear and cytoplasmic regions. (L) Natural antisense transcript in the heart ventricle; antisense hnRNP A1 is localized primarily in nuclei. Bar = 25 μm. (M) Control section in which cRNA probe was a digoxigenin-UTP labeled *Xenopus* elongation factor RNA (see Section 3). Bar = 25 μm.

2.5. hnRNP A1 expression during kidney development

High levels of hnRNP A1 mRNA were detected in the developing 5-day mesonephros, or provisional kidney. Expression was seen throughout the tissue, including glomeruli and tubules (Fig. 5A). In contrast, hnRNP A1 protein was detected in some, but not all, nuclei of the glomeruli and tubules of the 6-day mesonephros (Fig. 5B). No nuclei were positive for the neural RNA binding protein Hu (Fig. 5B, left inset). While individual tubules at this stage tended to be either uniformly stained or unstained, counterstaining with periodic acid-Schiff (PAS) reagent indicated that both distal (PAS-negative) and proximal (PAS-positive) tubules were among those positive for

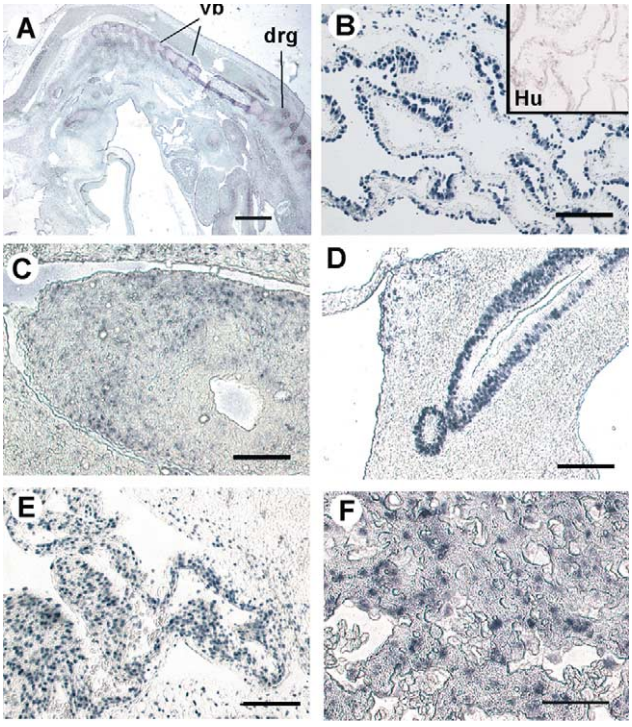

Fig. 4. Indirect immunohistochemistry of 6-day embryos. (A) Sagittal section. hnRNP A1 protein is detected in the developing vertebral bodies (vb), dorsal root ganglia (drg), the neural tube. Bar = 500 μm. (B) Extraembryonic membranes showing prominent nuclear staining. Inset shows lack of staining when Hu primary antibody was used. Bar = 100 μm. (C) Lung bud. Protein expression is seen throughout the parenchyma. Bar = 100 μm. (D) Intestinal loop. hnRNP A1 is localized to the gut epithelium. Some expression is also seen in the smooth muscle and mesothelium. Bar = 100 μm. (E) Atrium of heart. Bar = 100 μm. (F) Ventricle of heart. Bar = 50 μm.

hnRNP A1 (not shown). At 9 days of development the mesonephros still contained hnRNP A1 mRNA, but its abundance was only 10% that of the average 5-day embryonic level (Table 1). By 12 days, mRNA expression was no longer detected in the mesonephros (Fig. 5C,D) (but note the expression in condensing precartilage mesenchyme of vertebral bodies in the same section). Despite this, hnRNP A1 protein is still localized in the tubules at this stage (Fig. 5E). In addition, tubules of the developing metanephros are pervasively stained for hnRNP A1 protein at 12 days (Fig. 5F).

2.6. hnRNPA1 expression during liver development

Abundant hnRNP A1 mRNA was detected in the developing liver parenchyma and endothelium of 4.5 days (Fig. 6A) and 5.5 days (Fig. 6B) embryos. In the 5.5-day liver the endothelium lining both the sinusoids and central veins expressed the sense transcript, while antisense expression in this tissue was extremely low (Fig. 6B, inset). hnRNP A1 protein was present throughout the liver primordium in 6-day embryos, but was particularly concentrated in the peripheral regions of the developing lobes (Fig. 6C,D). At 9 days of development the abundance of hnRNP A1 mRNA in liver had declined relative to the 5-day whole embryo value (Table 1), although the magnitude of the decline may have appeared falsely high because of anomalously high levels of GAPDH in embryonic liver (Table 1). However, if the hnRNP A1 signal in 9-day liver is normalized to values of GADPH similar to that of other 9-day organs it is still among the lowest expressing of the organs surveyed. By 11 days hnRNP A1 protein varied in expression levels, appearing more abundant in the parenchyma of less mature regions (Fig. 6E) than in more mature regions (Fig. 6F), though in the latter expression was still detected in the endothelial tissue (Fig. 6E,F).

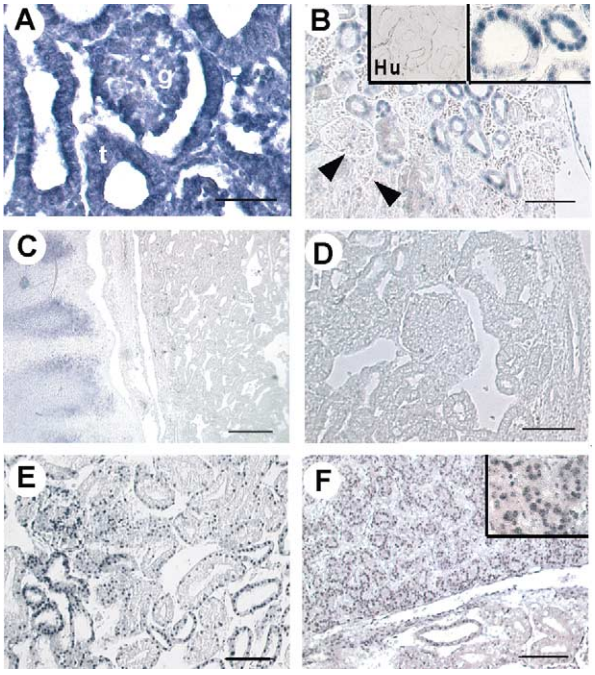

Fig. 5. hnRNP A1 mRNA and protein expression patterns in the 5–6-day and 12-day kidney. (A) 5.5-Day mesonephros: in situ hybridization; hnRNP A1 transcripts are detected at high levels in the cytoplasm of the developing tubules (t) and glomeruli (g). Bar = 50 μm. (B) Six-day mesonephros: immunohistochemistry; hnRNP A1 protein is present in tubules and glomeruli. Protein is detected in some, but not all nuclei. Insets: high magnification view of tubules showing lack of staining when Hu primary antibody was used (left) and nuclear staining when hnRNP A1 primary antibody was used. Arrows indicate glomeruli showing low levels of protein expression. Bar = 100 μm. (C) Low magnification view of in situ hybridization of coronal section containing 12-day mesonephros and developing vertebral bodies. hnRNP A1 transcripts are expressed in the precartilage condensations of the vertebral bodies but expression is no longer detected in the mesonephric kidney. Bar = 400 μm. (D) High magnification view of 12-day mesonephros in c, showing tubules and glomeruli. Bar = 100 μm. (E) Twelve-day mesonephros; immunohistochemical detection of hnRNP A1 protein in some but not all nuclei of tubules. Bar = 100 μm. (F) Twelve-day metanephric primordium: hnRNP A1 protein is present in the nuclei of all tubules (upper region of panel). Inset: high magnification of portion of this region. Lower region of panel: mesonephros. Bar = 100 μm.

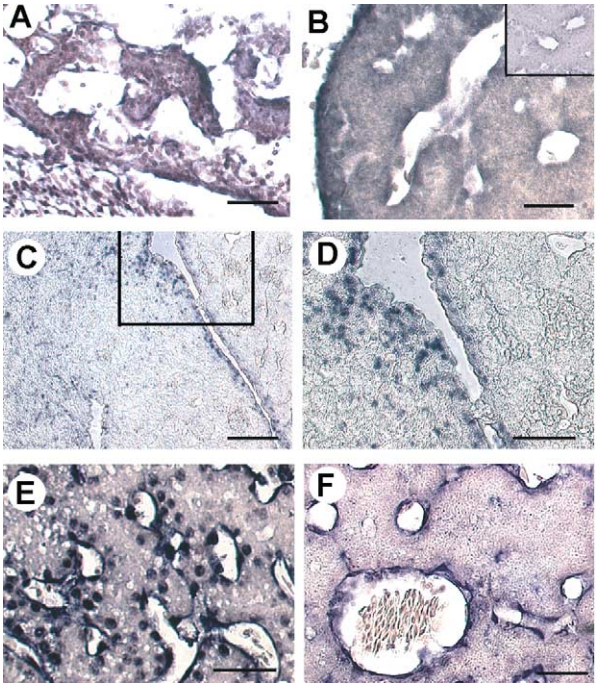

Fig. 6. hnRNP A1 mRNA and protein expression patterns in the 4–11-day developing liver. (A) 4-day liver: in situ hybridization detects high levels of hnRNP A1 transcript throughout the developing parenchyma. Bar = 100 μm. (B) 5.5-Day liver: in situ hybridization; hnRNP A1 mRNA continues to be expressed in the parenchyma, but even higher levels of message is detected in the capsular region and epithelium lining the sinusoids. Inset, in situ hybridization with sense strand probe, showing natural antisense expression in some nuclei. Bar = 25 μm. (C) Five-day liver: immunolocalization; hnRNP A1 protein is seen in nuclei of the parenchyma, particularly in the subcapsular region. Bar = 100 μm. (D) High magnification view of a region of tissue shown in c. Bar = 50 μm. (E) Eleven-day liver, immature region: immunolocalization; hnRNP A1 protein is seen in nuclei of hepatocytes and sinusoidal epithelial cells. Hepatocyte cytoplasm is also moderately positive for protein. Bar = 25 μm. (F) Eleven-day liver, mature region; hnRNP A1 protein expression is restricted to the endothelium lining the sinusoids and the central vein. Bar = 25 μm.

2.7. hnRNP A1 expression in the developing nervous system and axial skeleton

Both hnRNP A1 mRNA (Fig. 3B) and protein (Fig. 7A–C) were localized throughout the developing neuroepithelium of the 5–6-day embryo. High levels of protein were detected in the developing gray matter of the neural tube (Fig. 7A), the developing brain–particularly in the ventricular zone (Fig. 7G), and the dorsal root ganglia (DRG) of the peripheral nervous system (Fig. 7D,F). Expression of hnRNP A1 in the DRG overlapped spatially with expression of Hu (Fig. 7J), but the hnRNPA1-containing cell nuclei (Fig. 7F) were more numerous and generally larger than those containing Hu, suggesting that the distribution is not entirely overlapping. Furthermore, the cartilage of the vertebral bodies (Fig. 7E) between the DRGs were positive for hnRNP A1, but not Hu (Fig. 7F,J).

Expression in the lumbar dorsal roots exhibited an anterior to posterior gradient, with higher levels of expression in the more anterior regions (Fig. 7D). In some embryos two such anterior to posterior gradients were evident: one in the cervical to thoracic region and another in the lumbar region (not shown). In oblique sections through the lumbar portion of the vertebral column of 9-day embryos, hnRNP A1 protein was detected in the developing neural tube (Fig. 7H,I) with protein localized to nuclei in the gray matter of the interneuron region. Highest levels of expression were seen in the dorsal portion of the ventricular layer surrounding the central canal (Fig. 7F). A wave of labeled, presumably migrating, neuroblasts can be observed deep to the labeled ventricular layer of the mesometencephalic fold of the 6-day brain (Fig. 7G). In the more developmentally advanced thoracic region of a 9-day embryo, hnRNP A1 protein continued to be expressed in the gray matter of the neural tube and the DRG, but was no longer detected in the periventricular region, nor, significantly in cartilages of the developing ribs, except for their perichondria (Fig. 7K).

3. Experimental procedures

3.1. Protein and DNA sequence determination

Embryonated White Leghorn chicken eggs were obtained from Avian Services Inc., Frenchtown, NJ. Eggs were incubated in a humidified incubator at 39 °C for 4.5–12 days. Protein spots derived from embryonic limb bud nuclei that comigrated on nonequilibrium two-dimensional electrophoretic gels with the cyclic AMP-dependently phosphorylated protein (designated p35 by Zhang et al., 1996, and PCP 35.5 by Leonard and Newman, 1987) were excised from the dried gels and collected for tryptic fragment microsequencing (Tempst et al., 1990) and sequenced at the Microchemistry Laboratory of the Sloan Kettering Institute. Identity of the sequenced fragments was confirmed by mass spectrometry (Elicone et al., 1994). The partial protein sequence obtained was used to design degenerate PCR primers which amplified a 683 bp DNA fragment from a reverse transcript prepared from 7-day chicken embryo brain mRNA. The PCR product was cloned (TA Cloning kit, Invitrogen) and sequenced using the Sanger method (Sanger et al., 1977). The full-length protein sequence was determined by extending the cDNA sequence to the 3′ and 5′ ends using a chicken embryo limb bud cDNA library (Mackem and Mahon, 1991; a gift of Dr. Susan Mackem, NIH) and primers determined from the 683 bp sequence.

3.2. Southern and Northern hybridization

Total genomic DNA was isolated digested with *Hin*dIII, *Eco* RI, and *Pst* I and transferred to nitrocellulose for Southern hybridization as described by Sambrook et al. (1989). For Northern blot hybridization, total RNA (5 μg)

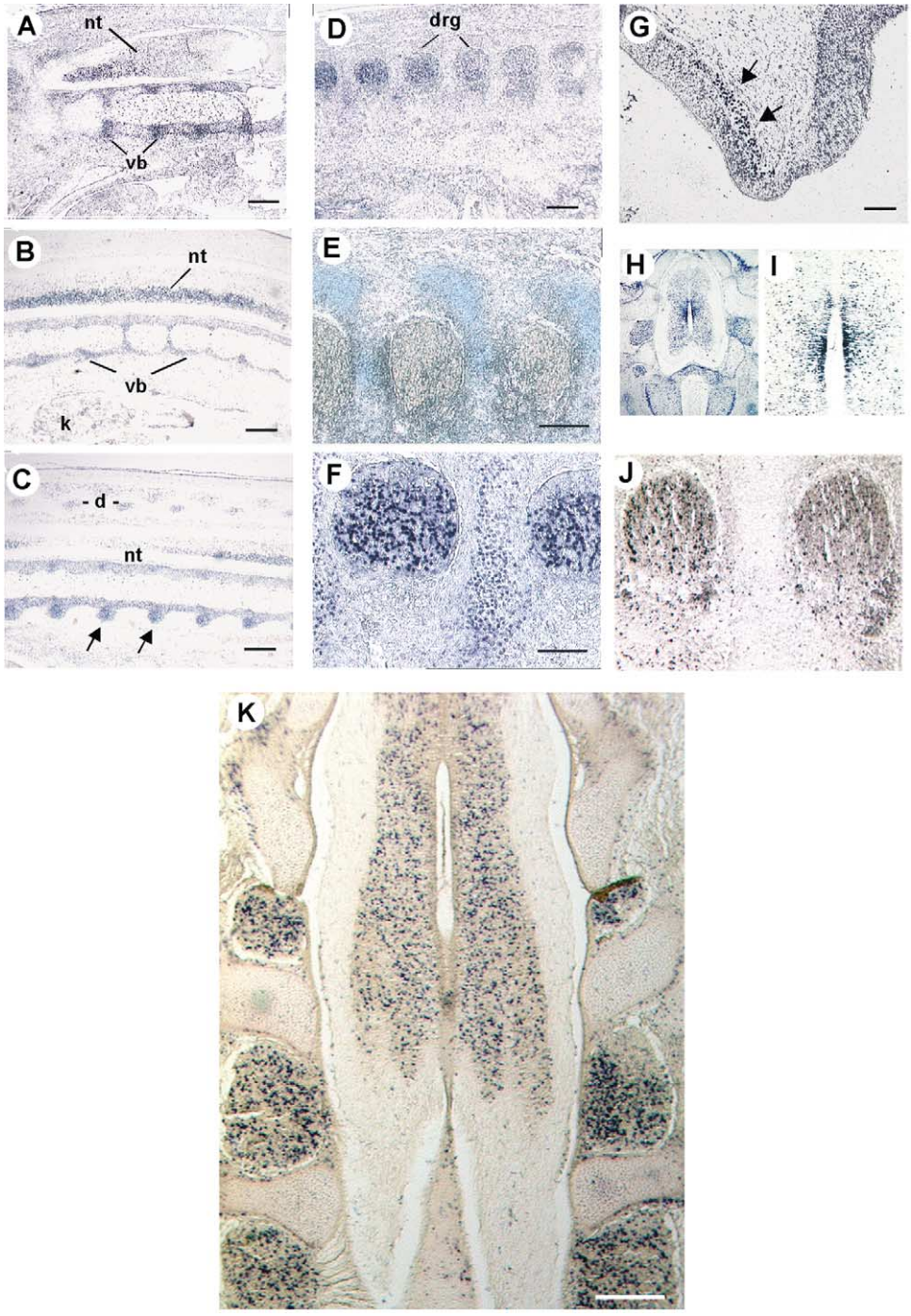

Fig. 7. hnRNP A1 protein expression patterns in 6- and 9-day developing neural tissue. (A–G) Sagittal sections through a 6-day embryo. (dorsal, top; anterior, left). (A–D) Low magnification views. (A) Neural tube (nt) and vertebral bodies (vb). (B) Neural tube (nt), vertebral bodies (vb) and mesonephric kidney (k). (C) Dermatomes (d), neural tube (nt), and vertebral bodies (arrows). (D) Developing dorsal root ganglia (drg). Lung bud is seen in the lower left corner. Bars = 100 μm. (E,F) Higher magnification of developing axial region. (E) Vertebral bodies are Alcian blue-positive. DRG are located between the vertebral bodies. (F) Nuclei of both vertebral body cartilage and DRG are positively immunostained for hnRNP A1 protein. Bars = 100 μm. (G) Meso-metencephalic fold of the 6-day brain. hnRNP A1 protein expression is detected throughout the developing neuroepithelium. Migrating cells are indicated by arrows. Bar = 100 μm. (H) Low magnification view of an oblique section through the lumbar region of a 9-day embryo showing the neural tube. Bar = 250 μm. (I) Higher magnification view of portion of section in (H), showing expression of hnRNP A1 in the interneuron region, with particularly high levels of expression in the dorsal portion of the ventricular zone surrounding the central canal. Bar = 100 μm. (J) Staining of DRG in 6-day embryo using primary antibody directed against Hu protein. Typical stained nuclei are smaller than those stained by hnRNP A1 antibody (F) and vertebral body cartilage is not stained. (K) Coronal section of thoracic region of 9-day embryo. Localization of hnRNP A1 protein is evident in gray matter of developing spinal cord, and in DRG. Bar = 250 μm.

was isolated from 5-day embryos with RNAzol (Tel-Test Inc., Friendswood, TX), denatured with 6% formamide, electrophoresed on a 1.2% agarose gel and transferred to nitrocellulose, as described (Sambrook et al., 1989). The probe for Southern blot hybridization was prepared by the random-primer method (Gibco-BRL) with [α-^{32}P]dCTP, using the 683 bp hnRNP A1 cDNA fragment as template. The probe for Northern blot hybridization was an antisense cRNA prepared using T3 polymerase and the T3 MegaScript kit (Ambion) from plasmid pRNP683, containing the 683 bp chicken hnRNP A1 insert between the *Bam* HI and *Hin*dIII sites of pBluescript KS (Stratagene). Single-stranded RNA probe synthesis (using 75 mM ribonucleotides except for UTP where [α-^{32}P]UTP was substituted), was carried out at 37 °C for 2 h using *Bam* HI linearized plasmid, followed by a 30-min incubation at 37 °C with 0.1 unit per μl RNase-free DNase 1 to remove the DNA template. Hybridizations were carried out overnight at 42 °C with cDNA or cRNA probes. After hybridization, filters were washed in 4× SSC, 0.2% SDS at 45 °C for 15 min, 2× SSC, 0.1% SDS at 50 °C for 30 min, and 1× SSC, 0.1% SDS at 55 and 65 °C for 30 min each. For low stringency Southern hybridization the final two washes were performed at 50 °C.

3.3. Quantitative analysis of hnRNP A1 gene expression

Expression of the hnRNP A1 gene was measured using RT followed by real-time quantitative analysis of the PCR with the LightCycler System (Roche Laboratories). The SYBR Green-I dye binding method was used, followed by melting curve analysis to confirm fragment-specific melting temperature (T_m). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as an internal standard. Total RNA was isolated from 5-day embryos and from liver, mesonephric kidney, breast and leg skeletal muscle, heart, skin, intestine, and brain isolated from 9-day embryos. Equivalent amounts (4 μg) of each RNA was used to synthesize cDNA using AMV reverse transcriptase (Stratagene) and oligo(dT) and random hexamer primers. For real-time PCR the following primers used for reverse transcription and amplification of chicken hnRNP A1: forward primer, 5′-GGTGGTTCAGGCAACTTCAT-3′; reverse primer, 5′-ATCCGTTGTATCCATCACCA-3′, amplifying a 157 bp fragment from within the 683 bp region of the hnRNP A1 cDNA. These primers were also used to generate cRNA probes for the Northern blot and in situ hybridization assays (see below). The sequence of the amplified product was confirmed by direct DNA sequencing. For chicken GAPDH the primers were as follows (Munsterberg and Lassar, 1995): forward primer, 5′-AGTCATCCCTGAGCTGAATG-3′; reverse primer, 5′-AGGATCAAGTCCACAACACG-3′, amplifying a 330 bp fragment.

3.4. Ribonuclease protection assay

Radiolabeled sense strand RNA probe was synthesized from plasmid pRNP683 linearized with *Hin*dIII, using T7 polymerase and the T7 MegaScript kit, as described above. Following DNase 1 treatment the probe was gel purified on a 5% acrylamide−8 M urea gel (Bio-Rad), 130 V for 2 h, and exposed to X-ray film. The most abundant band (probably cognate to 5′ sequences of the insert) was excised and eluted from the gel. This was a fragment of ~130 b as determined by migration relative to xylene cyanole and bromophenol blue. Aliquots of the purified probe (20 000 cpm) were precipitated in the presence of 10 μg of total RNA prepared from whole 5-day chicken embryos or 10 μg total yeast RNA, and hybridized overnight at 42 °C. Following hybridization, single-stranded RNA was digested by the addition of a 1:50 dilution of RNase A/RNase T1 (Ambion) to final concentrations of 0.5 and 200 unit/ml, respectively, at 37 °C for 30 min, with one yeast control left untreated. Following inactivation and precipitation, the samples were separated by electrophoresis on a 5% acylamide−8 M urea gel (Bio-Rad) at 130 V for 2 h, dried, and exposed to Kodak BioMax MS film at −80 °C using intensifying screens.

3.5. In situ hybridization

Embryos (4.5−12 days) for whole-mounting or sectioning were fixed overnight in 4% formaldehyde and stored in 100% ethanol at −20 °C (whole embryos), or processed in a Tissue-Tek VIP embedding system (Miles Scientific, Tarrytown, NY) and sectioned at 4 or 10 μm. Digoxigenin-UTP-labeled single-stranded sense and antisense cRNA probes were synthesized as described (Wedeen and Figueroa, 1998) from linearized plasmid pRNP683. In some in situ hybridizations a control digoxigenin-labeled sense strand probe was synthesized from plasmid pTRI-Xef containing a cDNA specifying a *Xenopus* elongation factor (Ambion). For whole-mount in situ hybridization unlabeled sense and antisense probes were synthesized as above and labeled using the AlkPhos Direct kit (Amersham, Piscataway, NJ). Elimination of indirect immunostaining steps made it possible to do whole-mount staining of 6-day embryos. Transcripts were covalently linked to alkaline phosphatase following the manufacturer's instructions. Hybridizations and washes were performed using the hybridization and post-hybridization solutions described in Wedeen and Figueroa (1998) rather than those suggested by the manufacturer. Color development of embryos was then performed directly by incubation in BCIP/NBT liquid substrate system (Sigma). For sections, an additional overnight incubation of 4 °C was performed in blocking solution containing 1:200 dilution of AP-conjugated anti-digoxigenin antibody (Roche Laboratories) followed by incubation in BCIP/NBT. Whole embryos were stored in 80% glycerol/PBS after washing in PBS and clearing in

80% ethanol. Sections were serially dehydrated to 100% ethanol, dipped in Histosol or xylene and mounted using Permount (Fisher).

3.6. Antibody production and purification

The decapeptide NH_2-TTDDSLREQF-COOH, representing amino acid residues 44–53 of the chicken hnRNP A1 sequence was synthesized by the SynPep corporation (Dublin, CA), conjugated to keyhole limpet hemocyanin, and used for production of a rabbit polyclonal antibody. Sera from bleeds reactive with a 39 kDa protein on immunoblots were affinity purified by passage over a column that had been conjugated with the original decapeptide.

3.7. Indirect immunolocalization and other staining procedures

Tissue sections were cleared in Histosol and rehydrated in distilled water. Immunostaining was performed as described (Wedeen and Figueroa, 1998). Following incubation with affinity purified anti-hnRNP A1 antibody (1:100) slides were incubated with AP-conjugated goat anti-rabbit IgG (1:200) (Jackson Immunoresearch, West Grove, PA). Protein was detected using the BCIP/NBT liquid substrate method. Some 6-day embryo sections were incubated with anti-human neuronal RNA binding protein HUC/HUD mouse monoclonal antibody 16A11 (Wakamatsu and Weston, 1997; obtained from Molecular Probes, Eugene, OR) and detected using a horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch) and the DAB substrate method. Sections were stained using standard histological protocols were 4 μm; those stained with Alcian blue were 10 μm.

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