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Mechanisms of Development 95 (2000) 231–237

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Gene expression pattern

Conservation of sequence and expression of Xenopus and zebrafish dHAND during cardiac, branchial arch and lateral mesoderm development

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Received 1 February 2000; received in revised form 17 March 2000; accepted 7 April 2000

Abstract

dHAND and eHAND are related basic helix-loop-helix transcription factors that are expressed in the cardiac mesoderm and in numerous neural crest-derived cell types in chick and mouse. To better understand the evolutionary development of overlapping expression and function of the *HAND* genes during embryogenesis, we cloned the zebrafish and *Xenopus* orthologues. Comparison of dHAND sequences in zebrafish, *Xenopus*, chick, mouse and human demonstrated conservation throughout the protein. Expression of dHAND in zebrafish was seen in the earliest precursors of all lateral mesoderm at early gastrulation stages. At neurula and later stages, dHAND expression was observed in lateral precardiac mesoderm, branchial arch neural crest derivatives and posterior lateral mesoderm. At looping heart stages, cardiac dHAND expression remained generalized with no apparent regionalization. Interestingly, no eHAND orthologue was found in zebrafish. In *Xenopus*, dHAND and eHAND were co-expressed in the cardiac mesoderm without the segmental restriction seen in mice. *Xenopus* dHAND and eHAND were also expressed bilaterally in the lateral mesoderm without any left-right asymmetry. Within the branchial arches, XdHAND was expressed in a broader domain than XeHAND, similar to their mouse counterparts. Together, these data demonstrate conservation of HAND structure and expression across species.

Keywords: dHAND; eHAND; *Xenopus*; Zebrafish; Heart; Branchial arches; Neural crest

1. Results

The bHLH transcription factors dHAND and eHAND (*d*eciduum/*e*xtra-embryonic membrane, *h*eart, *a*utonomic nervous system, *n*eural crest-derived cell types), also called HAND2 and HAND1 (Cserjesi et al., 1995; Cross et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995), respectively, share high homology within their bHLH regions and are encoded by genes with similar intron-exon organization. The dHAND proteins from mouse and chick share greater than 95% homology, while eHAND is much less conserved across species (Srivastava et al., 1995). In chick, dHAND and eHAND are co-expressed in the heart and loss-of-function studies in chick suggest some genetic redundancy (Srivastava et al., 1995). In mice, cardiac expression of dHAND and eHAND is complementary in the right (pulmonic) and left (systemic) ventricles, respectively. Consistent with an essential role of dHAND in the right ventricle, *dHAND*-null mouse embryos display hypoplasia of the right ventricle (Srivastava et al., 1997). dHAND and eHAND are also expressed in post-migratory neural crest cells that populate the pharyngeal arches and aortic arch arteries. Here, they play a role in development of the pharyngeal arches and in remodeling of the aortic arch arteries (Srivastava et al., 1997; Thomas et al., 1998), derivatives of the primitive gill arches of fish and amphibians.

Although zebrafish have only one atrium and one ventricle that pumps blood in series through the gills and systemic

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0925-4773/00/$ - see front matter
PII: S0925-4773(00)00334-8

circulation, we searched for zebrafish orthologues of dHAND and eHAND. A 30–36 h post fertilization (hpf) zebrafish cDNA library was screened at low stringency using the bHLH region of chick dHAND. Numerous clones were obtained, however sequencing revealed only a single cDNA species. The sequence of these clones most closely matched known dHAND sequences in chick (Srivastava et al., 1995), mouse (Srivastava et al., 1995) and human (Russell et al., 1998). Because only a dHAND orthologue, zdHAND, was found, we searched for evidence of a relative that might share sequence similarity with eHAND, but found none. Zebrafish genomic DNA was digested and subjected to electrophoresis and Southern analysis was performed using zdHAND as a probe under low stringency conditions. In spite of analysis with multiple restriction enzymes, no evidence was found of cross-hybridization of the zdHAND probe with other relatives (data not shown), suggesting that only one *HAND* gene might be present in the zebrafish genome.

In contrast to zebrafish, *Xenopus* has two atrial and one ventricular chamber resulting in a partially divided circulation. To identify potential orthologues of the *HAND* genes in *Xenopus*, a stage 28 embryonic *Xenopus* cDNA library was screened under conditions of low stringency using the bHLH region of chick dHAND. Two distinct populations of clones were identified. Sequence analysis revealed four overlapping clones that shared high sequence identity with mouse and chick dHAND. Three other clones were sequenced and encoded the *Xenopus* orthologue of eHAND as previously reported (Sparrow et al., 1998). No other related family members were found in this screen. As a result, we believe the novel clones represent the *Xenopus* orthologue of dHAND and will refer to it as XdHAND.

The amino acid sequence of zdHAND and XdHAND was

Fig. 1. Amino-acid sequence alignment of dHAND. Amino acid sequence comparison of zebrafish, *Xenopus*, chick, mouse and human dHAND is shown (A). Identical amino acids are shaded in boxes. Similar residues are in unshaded boxes. The basic and helix-loop-helix (HLH) domains are indicated in brackets. Polyalanine tract is marked with asterisk (*). Comparison of XdHAND and XeHAND sequence is also shown (B). Zebrafish dHAND (accession no. AAF67130) and *Xenopus* dHAND (accession no. AAF67131) nucleotide sequences have been submitted.

almost entirely conserved within the bHLH region (Fig. 1A) compared to human, mouse and chick dHAND. Interestingly, cross-species comparisons revealed that even outside the bHLH region, the proteins were highly conserved. Overall, zdHAND and XdHAND were 87 and 94% identical, respectively, to mouse and human dHAND. XdHAND shared only 56% homology with XeHAND (Fig. 1B). It is notable that zdHAND contains a proline residue rather than a conserved glycine residue in the basic region. Although XdHAND and XeHAND also contain a glycine at this position, mouse and chick eHAND have a proline residue, similar to zdHAND, in the basic region. Because the basic region of bHLH proteins is the DNA-binding domain and typically interacts with the canonical E box DNA binding site (CANNTG) (Murre et al., 1989), a proline residue may alter the DNA-binding specificity of bHLH proteins. Proteins containing a proline residue in the basic region often bind a slightly different N box (CACNAG) and sometimes serve as transcriptional repressors (Oshako et al., 1994). In addition, N-terminal of the bHLH is a region of conserved polyalanines. This polyalanine tract, although initially reported as a polyhistidine tract in mouse and chick (Srivastava et al., 1995), was found to be conserved in all other species upon repeated sequencing. Polyalanine tracts are found in several transcriptional repressors, including engrailed and Kruppel (Licht et al., 1990; Han and Manley, 1993a,b), and are essential for their repressor activities. Finally, the carboxyl terminus of the protein is almost entirely conserved between XdHAND and XeHAND (Fig. 1B) and across multiple species, suggesting a functional role for this region.

Whole-mount in situ hybridization was performed on zebrafish embryos ranging from the 75% epiboly stage (8 hpf) to 48 hpf to compare the expression of zdHAND to that of other species. zdHAND expression was first seen at 8hpf in a circumferential pattern in the lateral mesoderm (Fig. 2A). At 12 hpf, zdHAND continued to be expressed in the anterior and posterior lateral plate mesoderm which is partly detached from the yolk at this stage (Fig. 2B). By 18–20 hpf, the bilateral cords of cardiac primordia fuse at the midline forming an aortic ring which is defined by the cardiac marker, Nkx2.5. A dorsal view of the embryo at this stage showed zdHAND expression in the cardiac ring and in a layer of lateral mesoderm surrounding the cardiac region (Fig. 2C,G), as well as in the posterior lateral mesoderm (not shown). At 24 hpf, zdHAND expression was seen throughout the nascent cardiac tube and in bilaterally symmetric clusters of ventrolateral cells that condense to form branchial arch mesoderm which gives rise to jaw and pharyngeal bones (Fig. 2D). zdHAND expression continued bilaterally in the posterior lateral mesoderm (Fig. 2E,H). At 36–48 hpf, zdHAND expression was seen in defined branchial arch mesoderm populations in a pattern complementary to that of Nkx2.3 which is expressed in the endoderm of the pharyngeal pouches (Fig. 2F,I) (Lee et al., 1996). Histologic analysis of the cardiac region at the looped stage showed strong zdHAND expression throughout the heart tube (Fig. 2J), consistent with its earlier cardiac expression. Overall, the domains of zdHAND expression were remarkably similar to those observed in both chick and mouse and may be indicative of a conserved role and regulation of this gene between species.

To continue to investigate the expression of dHAND and eHAND across species, whole-mount in situ hybridization was performed on *Xenopus* embryos from stage 15 (early neurulation) to stage 47 (tadpole). At stage 15, there was faint expression of XdHAND in the antero-ventral mesoderm (data not shown). By stage 20, after neural tube closure, expression of XdHAND was apparent in the ventro-lateral mesoderm of the mid-embryo with punctate expression in anterior clusters of cells that were subepidermal and may represent migrating neural crest cells (Fig. 3A). By stage 24, or early tailbud stage, the expression pattern of XdHAND had expanded to include lateral plate mesoderm in the mid-embryo and the heart anlage in the ventral midline (Fig. 3B). The punctate groups of subepidermal cells persisted at this stage. Expression in the lateral plate mesoderm at stage 24 was symmetric along the left-right axis in all embryos tested (Fig. 3B). By the late tail bud stages (stage 28–30), the region of XdHAND expression in the lateral mesoderm had expanded into a symmetrical arc of ventral and lateral expression which fused in the midline where the straight heart tube had formed. The branchial arches had high levels of XdHAND expression (Fig. 3C). Linear bands of punctate expression leading to the branchial arches and anterior cardiac region were again observed, similar but not identical to the migratory pattern described previously for neural crest cells (Vallin et al., 1998). By stage 33, the heart had begun to loop and XdHAND expression was present in the heart, lateral mesoderm and branchial arches (Fig. 3D). At stage 37, expression in the looped heart was evident as was branchial arch expression (Fig. 3E). XdHAND was most abundant in the heart and outflow tract at stage 47 (Fig. 3F).

Xenopus eHAND (Sparrow et al., 1998) was previously described to diverge in its expression pattern with left-right asymmetry in the lateral mesoderm, and absence of branchial arch expression. Although the sequence of XeHAND obtained in this study was identical to that reported, our analysis of gene expression differed in some respects. Expression of XeHAND was similar to XdHAND at stage 15, with mRNA apparent in the anterior and ventral mesoderm near the heart primordia (Fig. 4A,B). At stage 22 there was a central band of XeHAND expression in the lateral plate mesoderm that was confined to the mid-embryo (Fig. 4C). It extended more dorsally but less anteriorly than XdHAND expression. Like XdHAND, the left and right lateral plate mesoderm had expression of XeHAND in all embryos studied, and no LR asymmetries of expression were observed. XeHAND expression was not

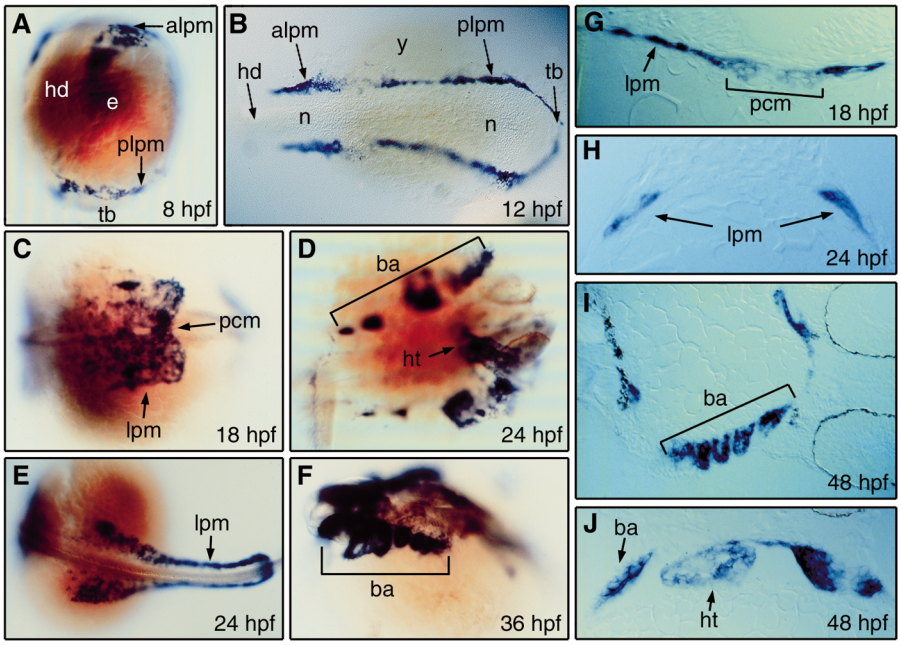

Fig. 2. dHAND expression in staged zebrafish embryos. Whole-mount in situ hybridization (A–F) revealed expression of dHAND in the lateral plate mesoderm (lpm), pre-cardiac mesoderm (pcm), branchial arches (ba) and in the heart (ht). As early as 8 h post fertilization (hpf), expression was evident circumferentially in the anterior lateral plate mesoderm (alpm) and posterior lpm (plpm) in an oblique head-on view (A); positions of head (hd), eye (e) and tailbud (tb) are shown. At 12 hpf expression was again seen in the alpm and plpm (B); embryo is oriented with the head to the left and the tailbud to the right. Notochord (n) and yolk (y) are also noted. Expression in the pcm became evident at 18 hpf (C) and persisted into the heart tube at 24 hpf (D) seen ventrally, when branchial arch expression also became apparent. The embryo at 24 hpf demonstrated bilaterally symmetric expression of dHAND in the lateral plate mesoderm (E) in a dorsal view of the caudal region. Branchial arch expression persisted at 36 hpf (F) in the rostral region as seen dorsally. Transverse sections of 18 (G) and 24 (H) hpf embryos in the rostral and caudal regions, respectively, confirmed expression in the pcm and lpm. (I) demonstrates branchial arch expression of zdHAND at the histological level. (J) Shows expression of zdHAND throughout the heart tube.

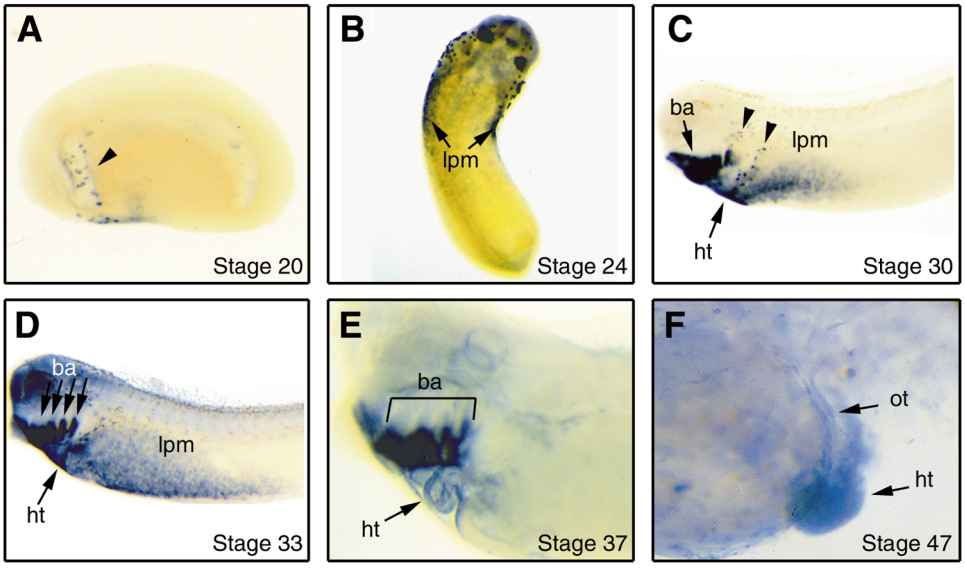

Fig. 3. dHAND expression in staged *Xenopus* embryos. Whole-mount in situ hybridization in *Xenopus* embryos revealed dHAND expression in punctate subepidermal cells (arrowheads) as early as stage 20 (A). The punctate cells later appeared to be migrating to the branchial arches and heart (C). dHAND expression was observed in the branchial arches throughout their development (C–E). dHAND was expressed bilaterally in the lateral plate mesoderm (lpm) at stage 24 through stage 33 (B–D). High magnification of the anterior embryo better demonstrated expression in the looped heart tube and branchial arches at stage 37 (E). Even higher magnification of the cardiac region (F) demonstrated expression in the heart tube (ht) and outflow tract (ot) at stage 47 without any chamber restriction.

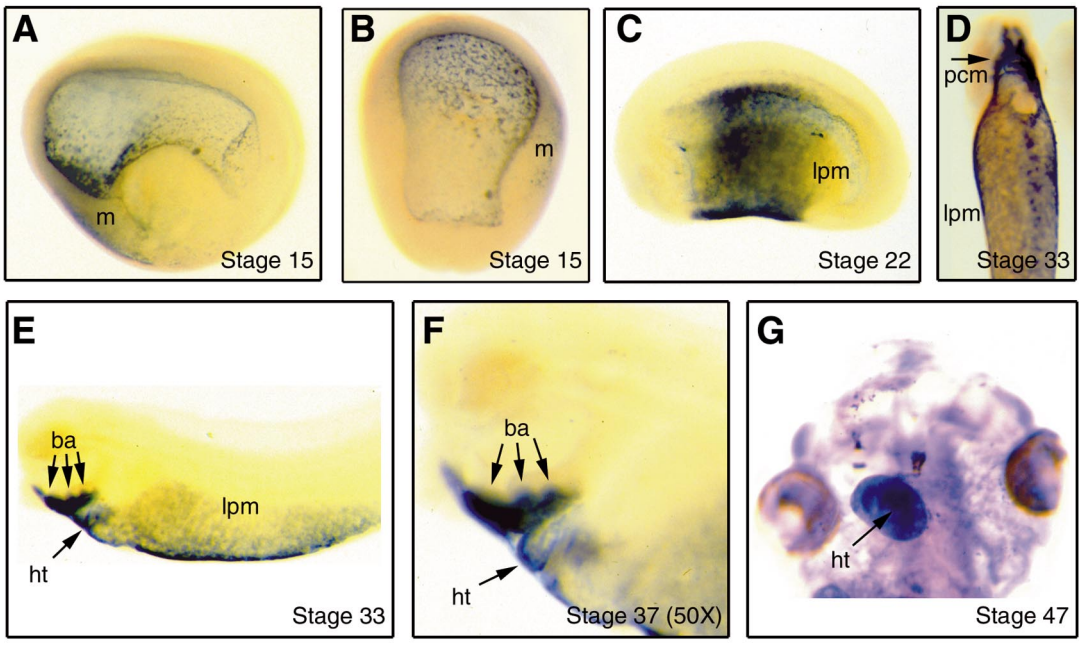

Fig. 4. eHAND expression in staged *Xenopus* embryos. eHAND expression in stage 15 *Xenopus* embryos was evident in the antero-lateral mesoderm (m) in lateral (A) and frontal (B) views. Bilaterally symmetric expression in the lateral plate mesoderm (lpm) was observed at stage 22 (C) and stage 33 in a ventral view (D). Expression in the precardiac mesoderm (pcm) became apparent at stage 30–33 (D). In a lateral view at stage 33 (E), branchial arch (ba) expression was observed in addition to the heart (ht) and lateral plate mesoderm expression. Magnification of the anterior region (F) better illustrated branchial arch and heart expression. High levels of heart expression were seen at stage 47 (G).

observed in the branchial arch precursors at stage 22. Expression of XeHAND in the heart anlage and lateral plate mesoderm during stages 28–33 was similar to the expression pattern of XdHAND (Fig. 4D) with no asymmetries. In contrast to XdHAND, however, there was no punctate expression noted in the subepidermal region anteriorly. Branchial arch expression of XeHAND was present at stage 33 but was more medial than XdHAND (Fig. 4E). These subdomains of XdHAND and XeHAND in the branchial arches were reminiscent of the pattern observed in chick (Srivastava et al., 1995) and mouse (Thomas et al., 1998). At heart looping stages (stage 37) the myocardium and cardiac outflow tract expressed XeHAND uniformly (Fig. 4F). In the tadpole (stage 47), XeHAND expression was like that of XdHAND, with expression in the heart and cardiac outflow tract (Fig. 4G).

Analysis of XdHAND expression in adult *Xenopus* revealed specific expression in several adult tissues (Fig. 5). Unlike adult mouse, XdHAND was detectable in the adult heart. Interestingly, transcripts were also observed in the liver and spleen, but not in skeletal muscle or ovary.

The cardiovascular systems of organisms have evolved with increasing complexity in order to adapt to specific environments. The remarkable conservation of dHAND's amino acid sequence in species ranging from fish to humans is indicative of a critical role for this transcription factor throughout evolution. Recent identification of a dHAND deletion in a zebrafish mutant exhibiting cardiac defects supports a conserved role for dHAND (Yelon et al., 2000). Analysis of the function of zebrafish and Xenopus HAND proteins may provide additional insights into the molecular mechanisms underlying the early steps of cardiogenesis.

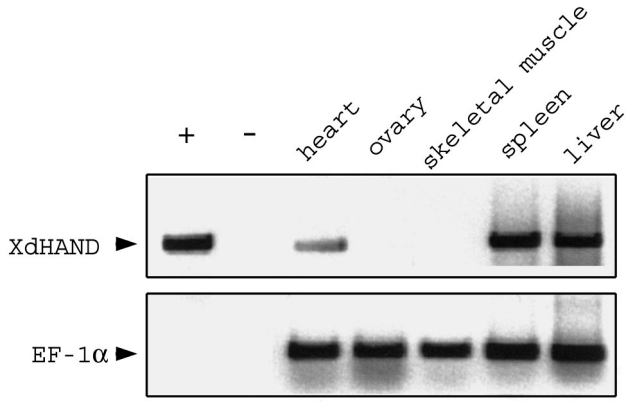

Fig. 5. Expression of dHAND in adult *Xenopus* tissues. Reverse transcriptase polymerase chain reaction was performed on total RNA extracted from adult *Xenopus* organs as indicated. XdHAND was detected in the adult heart, spleen and liver, but not in the ovary or skeletal muscle. Non-reverse transcribed RNA was used as a negative control (−) and XdHAND cDNA as positive control (+). RNA quality and quantity was assessed by amplification of the ubiquitous transcript, EF-1α.

2. Materials and methods

2.1. Zebrafish and Xenopus cDNA library screen

The bHLH region of chick dHAND was radiolabeled and used to screen 1.2×10^6 recombinants from a 30–36 h whole embryo zebrafish lgt11 cDNA library (gift of K. Zinn) or 2.0×10^5 recombinants from stage 28 *Xenopus* cDNA library at low stringency according to previously described methods (Benton and Davis, 1977). Positive clones were plaque purified, subcloned into plasmid vectors and sequenced.

2.2. Whole-mount RNA in situ hybridization

Xenopus embryos were obtained as described previously (Lohr et al., 1997) and RNA probe synthesis and whole-mount in situ hybridization were performed using a standard protocol (Harland, 1991; Sive and Bradley, 1996). Antisense XdHAND riboprobe was synthesized using a BamH1 linearized plasmid and T7 RNA polymerase. Antisense XeHAND riboprobe was synthesized using a Xho1 linearized plasmid and T3 RNA polymerase. *Xenopus* embryos were staged according to Nieuwkoop and Faber (1967) and embryos from stage 15 (early neurulation) to stage 47 (tadpole) were used for in situ hybridization.

In situ hybridization of various staged zebrafish embryos was performed as previously described (Lee et al., 1996). Antisense RNA probes were synthesized by in vitro transcription of full-length zebrafish dHAND cDNA as above.

2.3. Histologic analysis

Embryos were dehydrated and embedded in paraffin after whole-mount in situ hybridization. Transverse sections were made at 5–7 micron intervals. Sections were not counterstained in order to preserve visibility of labeled RNA transcripts.

2.4. Reverse transcriptase polymerase chain reaction

RNA from adult male Albino frogs was obtained by isolating the heart, liver, spleen, ovary, and skeletal muscle. Reverse transcribed RNA was used to generate cDNA for polymerase chain reaction (PCR) amplification of XdHAND. Primer pairs were as follows: 5′-ATG AGT CTG GTT GGG GGG TTT C-3′ 5′-GTC CTG CCT TTG GTT TTC TTA TCG-3′.

PCR conditions were: 94°C for 5 min, 25–35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. A final extension was done at 72°C for 10 min. Non-reverse-transcribed RNA samples were used as negative controls. RNA quantity and quality was assessed by amplification of EF-1α. A range of amplification cycles was performed to provide semi-quantitative results.

Acknowledgements

The authors wish to thank S. Johnson for manuscript preparation and graphics; and D. Stanier and D. Yelon for sharing unpublished data. D.S. was supported by grants from the NIH/NHLBI (1-P50-HL61033-01, 1-R01-HL62591-01, 5-R01 HL57181-04), March of Dimes Birth Defects Foundation, and Smile Train Inc.

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