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Identification of New Genes *Ndr2* and *Ndr3* Which Are Related to *Ndr1/RTP/Drg1* but Show Distinct Tissue Specificity and Response to N-myc

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***Ndr1* was isolated as a gene upregulated in *N-myc* mutant mouse embryos and is repressed by N-myc and c-myc. Consistent with Myc regulation, the same gene was also isolated as one sensitive to transformation (*Drg1*), and in addition as one induced under a few stress conditions (*RTP*). Two new genes, *Ndr2* and *Ndr3*, were identified which encode proteins highly related to Ndr1/RTP/Drg1 and constitute the *Ndr* gene family. *Ndr2* and *Ndr3* are under spatio-temporal regulations distinct from *Ndr1*, and are not activated in *N-myc* mutants. When whole embryo RNA was analyzed, *Ndr3* expression was already high at 9.5 days postcoitus (dpc), while expression of *Ndr2* and *Ndr1* became significant after 12.5 dpc and 13.5 dpc, respectively. At 14.5 dpc, expression of these genes partially overlaps, but many tissues are unique to one of them. For instance, *Ndr1* is strongly expressed in the liver and gut epithelium, *Ndr2* in the ventricular zone throughout the CNS, and *Ndr3* in the spinal cord and the thymus rudiment. Genes of the *Ndr* family probably have tissue-dependent allotments of the possibly related functions.**

***Key Words: Ndr1; RTP; Drg-1;* N-myc; embryo; cell differentiation.**

To gain insight into the action of N-myc involved in oncogenesis and embryonic development, we and other groups generated N-myc deficient mice by gene targeting (1–3). N-myc deficient mouse embryos grow to day 11 but then die primarily by cardiovascular failure, but earlier embryos provided good materials to identify genes regulated by N-myc. We carried out cDNA subtraction between wild-type and N-myc deficient mutant embryos and identified *Ndr1* gene, the expression of which is highly augmented in the mutant embryos (Shimono and Kondoh, database submission number U60593, July 1996 (4)). In normal embryonic tissues, expression of *Ndr1* gene usually takes place in the group of tissues where N-myc is expressed, but *Ndr1* expression initiates only when N-myc expression falls from its peak and the cells begin to differentiate. These observations suggested us that *Ndr1* is normally repressed by N-myc, becomes derepressed during the course of cell differentiation and plays a certain role in the differentiated cell state. Analysis of *Ndr1* gene regulation revealed in fact that N-myc and Max repress *Ndr1* promoter activity in 10T1/2 cells (4). It was also observed that this promoter is equally repressed by c-myc and Max, suggesting that if N-myc or c-myc activity is augmented during malignant transformation of the cells, then *Ndr1* expression would be repressed.

In fact, *Drg1*, the human homologue of *Ndr1*, was identified by differential display analysis as one of those significantly upregulated during colon carcinoma cell differentiation (5). Differential display and analogous analyses have repeatedly identified the same *Ndr1/Drg1* gene as the one whose expression increases in differentiation of neoplastic cells: retinoic-acid induced differentiation of myeloma cell lines (5, 6), in androgen-induced prostatic adenocarcinoma cells (7), and in forskolin-induced choriocarcinoma cells (8). Similarly, comparison of normal and tumor tissues of mammary and prostate epithelia identified *Ndr1/Drg1* (called *rit42* by the authors) as the one down-regulated in tumor cells (9). *Ndr1/Drg1* is also shown to be induced by p53 (9).

Thus, repression by N-myc/c-myc and by cell transformation, as well as activation by cell differentiation and by p53 expression, all indicate that *Ndr1/Drg1* expression is inversely related to cell transformation. Moreover, it has been reported that forced expression of *Ndr1/Drg1 (rit42)* in tumor cells reduced cell growth (9), suggesting an anti-oncogenic effect of *Ndr1/Drg1*.

Besides inverse relationship with cell transformation, *Ndr1/Drg1* expression is modulated under various conditions which may impose a stress on the cells.

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The first report along this line was the human gene *RTP*, identical to *Drg1*, which is upregulated by homocysteine and tunicamycin in vascular endothelial cells (10). *Ndr1/RTP/Drg1* was also shown to be induced by nickel compounds (called *Cap43* (11)), and downregulated by androgens in T cell hybridoma (called *TDD5* (12)). Therefore, *Ndr1/RTP/Drg1* is regulated in at least two modes, during differentiation-transformation transition and by chemical stimulation.

An important gene function is often managed by a family of related genes. In this study, we investigated the possible occurrence of a family of *Ndr1*-related genes. In fact, two genes, *Ndr2* and *Ndr3*, were identified showing significant conservation of the overall characteristic of the amino acid sequences compared to Ndr1. In contrast to *Ndr1*, however, *Ndr2* and *Ndr3* were not under negative regulation by N-myc. Their expression during mouse development indicates that three members of the family are under distinct spatiotemporal regulations, implying that the *Ndr1* gene family bears different allotments of the possibly related functions.

MATERIALS AND METHODS

cDNA cloning. cDNA sequences related to *Ndr1* were looked for by BLAST search in the dbEST database on Genomenet WWW server (http://www.genome.ad.jp). This identified a mouse cDNA fragment of *Ndr1*-related gene which we termed *Ndr2*. A *Ndr2* cDNA fragment of 619 bp was amplified by PCR of cDNA pool of adult mouse kidney (gift of Dr. H. Moribe) using the primers 5′-CCAGGGACAGACTCACTCTG-3′ and 5′-GTCTCGGCGGTTGTTGTAGC-3′. This cDNA sequence, which is fairly distant from *Ndr1*, was used as probe to screen λgt22 cDNA library of 11.5 dpc mouse embryos (gift of Dr. H. Sasaki). Two distinct levels of intensities of hybridization signals were observed among positive plaques, each at a frequency of roughly 30 in 8×10^5 plaques. Those with strong hybridization signals had *Ndr2* cDNA inserts, and those with much weaker signals represented the third *Ndr1*-related cDNA sequence which we called *Ndr3*. No *Ndr1* clones were obtained in this screen.

Mice and embryos. Heterozygous N-myc mutant mice carrying the allele described by Sawai *et al.* (3, 13) in C57BL6 background were mated to obtain homozygous mutant embryos. Individual embryos derived from the heterozygous crosses were genotype-determined using yolk sac DNA (3).

Northern blot analysis. A whole embryo was frozen in liquid nitrogen, homogenized, then total RNA extracted using an RNeasy kit (QIAGEN). Isolated RNA was electrophoresed in 1% agarose/formaldehyde gel (4 μg/lane) and transferred onto nylon membrane using a VacuGene XL vacuum blotting system (Amersham Pharmacia). Probes of *Ndr1*, *Ndr2* and *Ndr3* cDNAs were labeled with [α-^{32}P]dATP using a Strip-EZ DNA probe synthesis kit (Ambion). Hybridization was performed at 65°C in QuickHyb hybridization solution (Stratagene) for 3 h, membrane was washed with 0.2× SSC/1% SDS at 65°C, and an autoradiogram taken using an X-ray film (Fuji Film) by overnight exposure. Transcript size was determined using an RNA ladder (0.24–9.5 kb, Gibco-BRL).

In situ hybridization. Embryos of ICR mice were fixed in 4% paraformaldehyde in phosphate-buffered saline at 4°C for overnight. The probes labeled with digoxigenin-11-UTP (Boehringer Mannheim) were synthesized using as templates 2.7 kb *Ndr1* cDNA (m113-4 fragment (4)), 2.0 kb *Ndr2* cDNA, and 2.6 kb *Ndr3* cDNA by transcription of linearized plasmids by T3 or T7 RNA polymerases (Stratagene). *In situ* hybridization of whole mount specimens and of histological sections was performed according to Wilkinson (14) and Uwanogho *et al.* (15), respectively, except that glycine treatment after Protease K digestion was omitted. After hybridization, specimens were subjected to high stringency washes in 50% formamide, 2× SSC and 10 mM dithiothreitol at 70°C.

RESULTS

Identification of Ndr1-Related Genes

In a search for *Ndr1*-related genes, we inquired into EST databases and found a few overlapping sequences which are different from *Ndr1*. We called this sequence *Ndr2*, generated a cDNA fragment by PCR of a mouse kidney cDNA pool using primers based on EST database sequences, and screened an oligo dT-primed λgt22 cDNA library of 11.5 day mouse embryos using as probe the portion of *Ndr2* cDNA which is diversified from *Ndr1* (corresponding to the region from codon 37 to codon 242 of the *Ndr2* sequence to be described below). Strongly hybridizing clones representing *Ndr2* sequences were obtained at the frequency of 30 in 8.0×10^5 clones, and weakly hybridizing clones were obtained at almost the same frequency. The latter clones were found to represent the third related sequence, which was called *Ndr3*.

The majority of the *Ndr2* and *Ndr3* clones had inserts of roughly 2.0 kb and 2.6 kb, respectively, which differ slightly at the 5′ end termini. The longest of the *Ndr2* and *Ndr3* cDNA sequences are deposited in the databases (DDBJ/EMBL/GenBank accession numbers, AB033921 and AB033922, respectively). These sizes are consistent with the Northern blot data described below. The cDNA sequences had open reading frames encoding proteins which resemble Ndr1. *Ndr2* and *Ndr3* are expected to encode 371 and 375 amino acid proteins, respectively (Fig. 1). Amino acid sequences of Ndr2 and Ndr3 proteins are similar to Ndr1, showing 54% and 64% identity to Ndr1, respectively. Therefore, these three proteins can be grouped as the Ndr family. Three repeats of the GXRSRSHTSE sequence found in Ndr1/RTP/Drg1 (4, 5, 10) were not present in Ndr2 or Ndr3.

Expression of Ndr Family Genes in Relation to N-myc Activity

Ndr1 was identified as a gene significantly upregulated in N-myc deficient mutant embryos (4). To see whether *Ndr2* and *Ndr3* are under an analogous regulation, we examined expression of these genes in wild type, heterozygous and homozygous N-myc mutant embryos at 11.5 dpc. Confirming the previous report on 10.5 dpc embryos (4), *Ndr1* was highly augmented in N-myc homozygotes (Fig. 2A). In contrast, low expression of *Ndr2* which was similar to *Ndr1* at this stage in wild type embryos was not affected at all by the N-myc

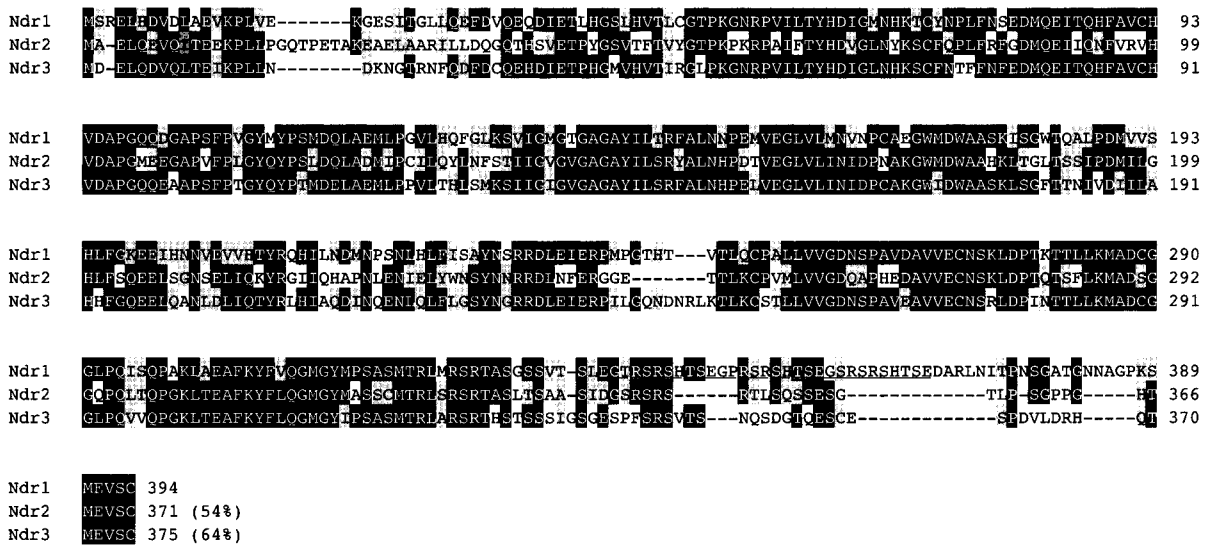


FIG. 1. Comparison of amino acid sequences among Ndr1, Ndr2 and Ndr3. Amino acid residues identical in at least two of the three proteins at the position are shown in black, and those related to the amino acid residues of one of the other proteins are shown in gray. Amino acid identity was 64% between Ndr1 and Ndr3, 57% between Ndr2 and Ndr3, and 54% between Ndr1 and Ndr2. Note three repeats of GXRSRSHTSE sequence (underlined) unique to Ndr1.

genotype. Expression of *Ndr3* was slightly lower in homozygous mutants in repeated experiments (Fig. 2A). Thus, repression by N-myc is unique to *Ndr1* among the related genes.

In wild type mouse embryos, *Ndr1* expression increases after 13.5 dpc. when N-myc expression in the embryo is largely downregulated (4), as confirmed in Fig. 2B and C. *Ndr2* expression was quite low at the stage of 9.5 dpc, but began to increase after 11.5 dpc, earlier than *Ndr1*. However, *Ndr3* expression was already significantly high at 9.5 dpc and showed only a slight increase in later developmental stages (Fig. 2B and C).

Expression in Mouse Embryos of 9.5 to 10.5 dpc

Expression of all three members of the *Ndr1* family at these stages was examined by whole mount *in situ* hybridization. In 9.5 dpc mouse embryos, somites showed some signals but otherwise *Ndr1* expression was generally low (Fig. 3A). *Ndr2* expression was also low, but a strong expression was observed in the atrium of the heart (Fig. 3B). Fairly strong expression of *Ndr3* was widespread in the embryo, and was prominent in the tail bud (Fig. 3C). In 10.5 dpc mouse embryo, all three members of the *Ndr1* family were expressed in somites (Figs. 3D–F). *Ndr1* was also significantly expressed in primordia of liver, stomach and intestine. *Ndr2* was expressed in the heart, but the major site of expression was shifted from the atrium to the ventricle at this stage. Expression of *Ndr3* covered the entire embryos but, interestingly, was excluded from the heart (Fig. 3F, white arrowhead). Relatively high expression of *Ndr3* in embryos of these stages is consistent with the northern blot data (Figs. 2B and C).

Expression in the Limb of 12.5 dpc Embryo

Whole mount *in situ* hybridization is informative for tissues close to the surface of the embryos at 12.5 dpc. The limb buds showed an interesting difference of expression among the genes. In the limb bud, *Ndr1* was barely expressed, *Ndr2* was expressed in the perichondrium of the phalanges, while *Ndr3* was expressed in the interdigit mesenchyme (Figs. 2E and F). Other remarkable sites were follicles of vibrissae and the nasal pits expressing *Ndr1* (data not shown).

Expression in Tissues of 14.5 dpc Embryo

To identify tissues expressing individual members of the *Ndr* gene family, we analyzed 14.5 day embryos by *in situ* hybridization of transverse sections. Expression at a significant level of all *Ndr1*, *-2* and *-3* was expected at this stage (Fig. 2C).

In the nervous system, *Ndr1* to *3* were differentially expressed with partial overlap. In the cortex of the cerebrum, strong *Ndr1* expression and moderate *Ndr3* expression were observed in the cortical plate (Figs. 4A and C). *Ndr2* was expressed strongly in the ventricular zone (Fig. 4B) where it continued from the brain to the spinal cord (Fig. 4E). *Ndr3* was expressed broadly from the ventricular zone to the marginal zone in the spinal cord (Fig. 3F). Dorsal root and other sensory ganglia expressed *Ndr2* fairly strongly (Fig. 4E) and *Ndr1* weakly (Fig. 4D).

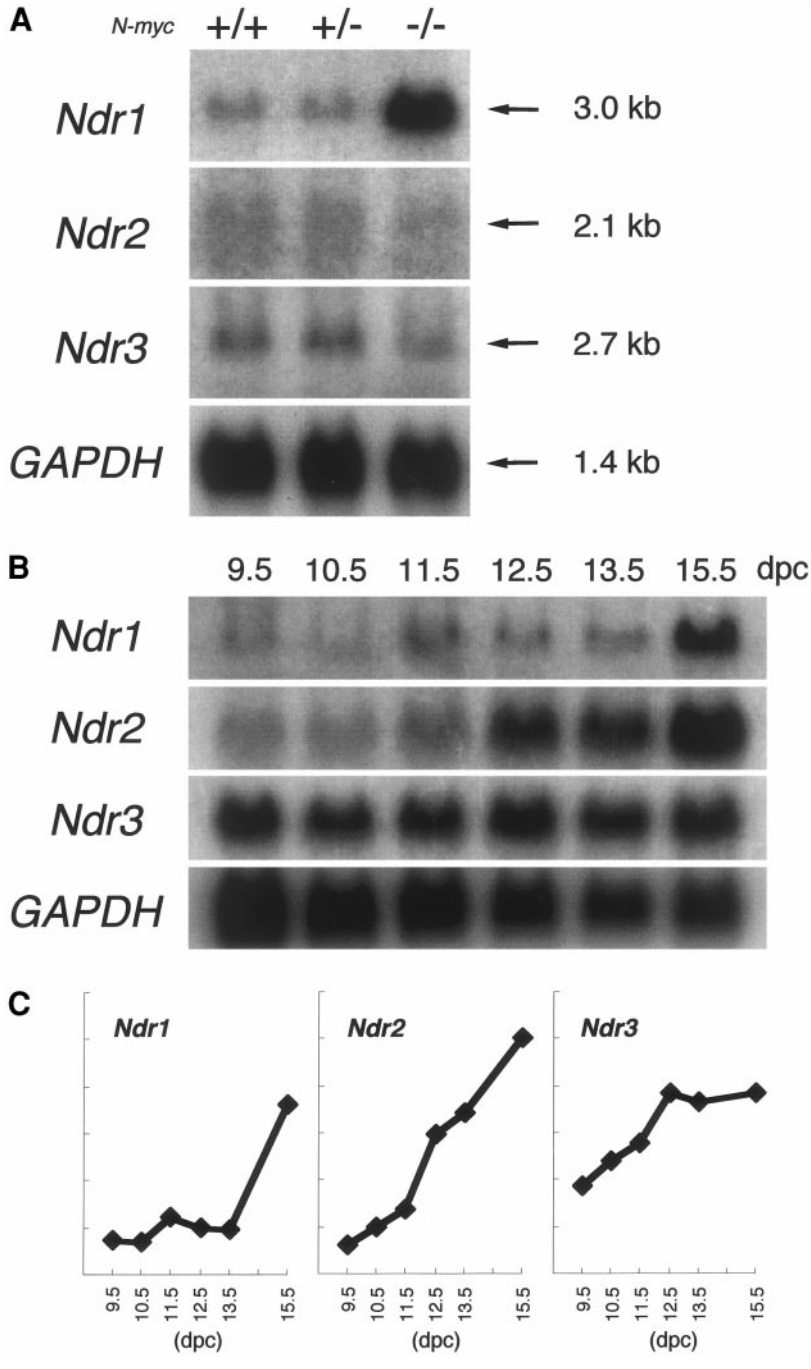


FIG. 2. Regulation of Ndr family genes in N-myc deficient mutant embryos and during organogenesis as analyzed by Northern blots. (A) Comparison of expression of *Ndr1, Ndr2* and *Ndr3* among wild type (+/+), heterozygous (+/−) and homozygous (−/−) N-myc mutant embryos at 11.5 dpc. *Ndr1* expression was highly augmented in the homozygous embryo, while *Ndr2* expression was not affected by the genotype, while *Ndr3* expression was slightly lowered in the homozygote. The sizes of the RNAs, as determined by comparison with an RNA ladder are indicated on the right. (B) Expression of *Ndr1, Ndr2* and *Ndr3* at various stages of development of wild type embryos. Data for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA were included to monitor the RNA load. (C) Temporal changes of *Ndr1, Ndr2* and *Ndr3* expression as indicated by relative expression levels normalized to *GAPDH* expression.

There are tissues expressing only one of the three *Ndr1* family members. The rudimentary thymus uniquely expressed *Ndr3* (Fig. 4I), myocardium and trabeculae of the heart ventricle expressed *Ndr2* (Fig. 4K), while liver cells expressed *Ndr1* (Fig. 4M).

Along the alimentary tract, the epithelia of stomach, midgut and hindgut expressed *Ndr1* at a high level

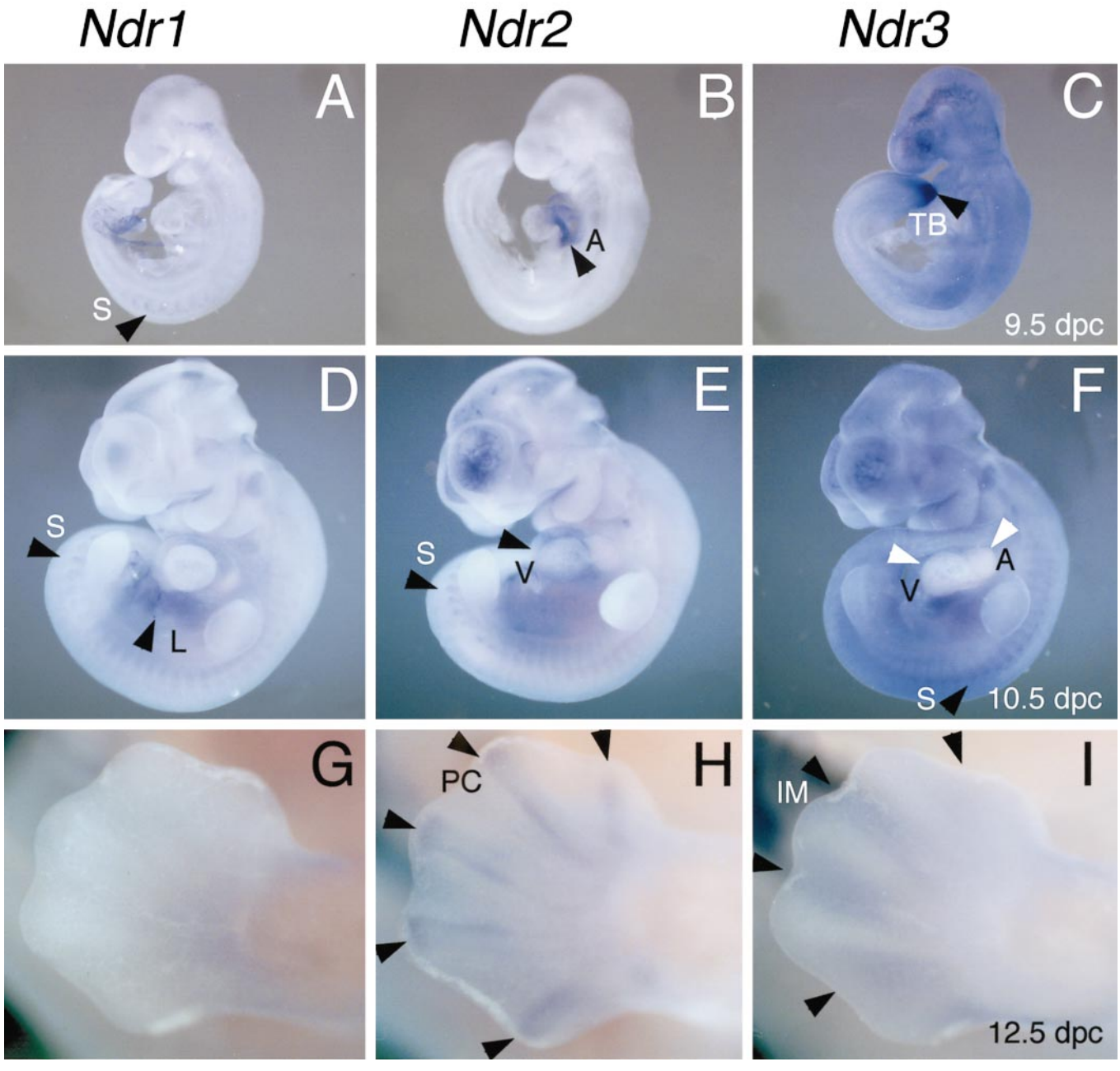
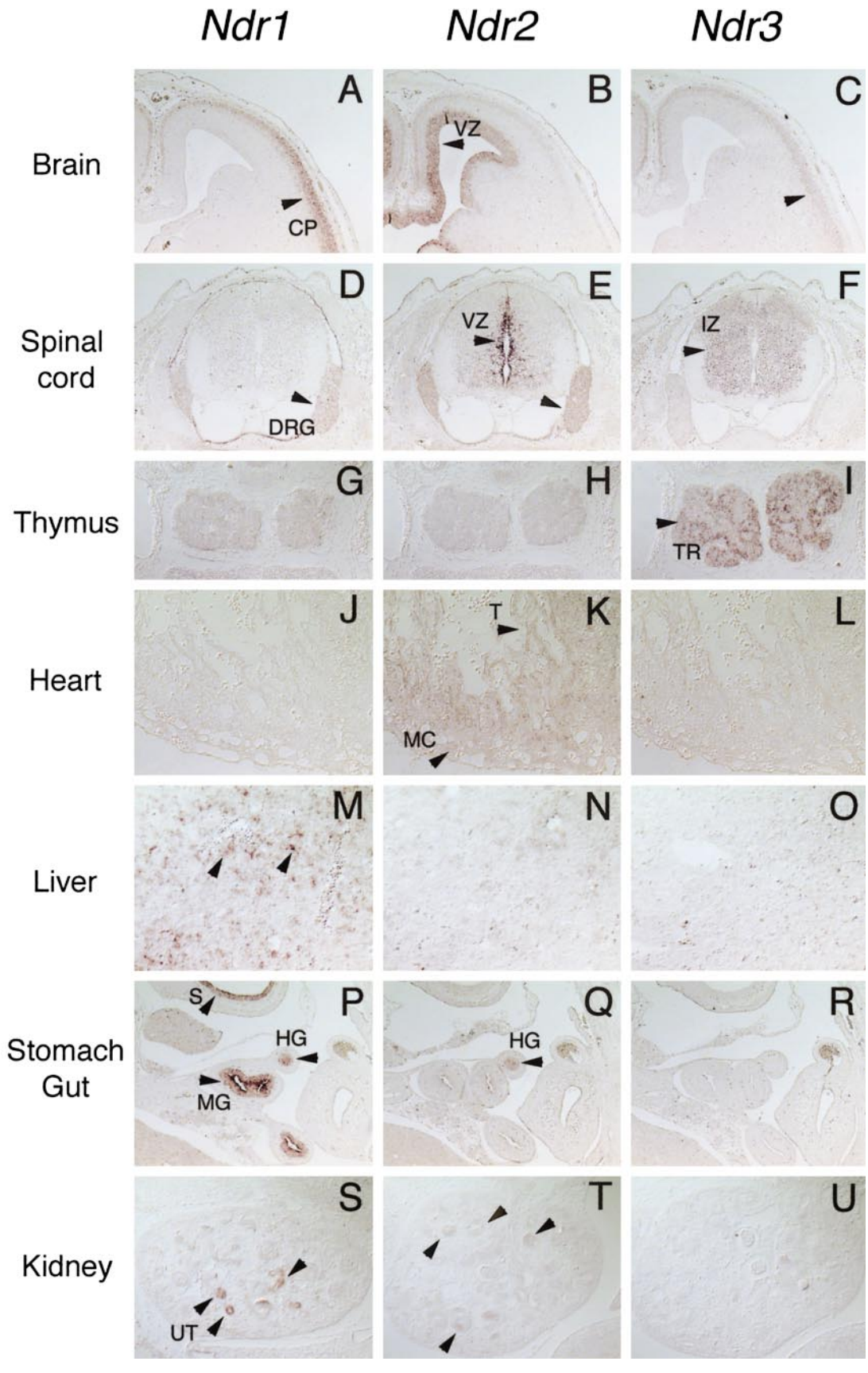


FIG. 3. Whole-mount *in situ* hybridization of *Ndr1, Ndr2* and *Ndr3* transcripts in 9.5, 10.5 and 12.5 day wild type embryos. (A–C) 9.5 dpc mouse embryos hybridized with *Ndr1, Ndr2* and *Ndr3* probes, respectively. The most prominent sites of expression at this stage were the somite for *Ndr1* (A), the atrium (A) for *Ndr2* (B) and the tail bud (TB) for *Ndr3* (C) as indicated by black arrowhead. (D–F) 10.5 dpc mouse embryos. *Ndr1* expression in somites (S) and liver (L) (D), *Ndr2* expression in the somite and ventricle (V) (E), and *Ndr3* expression in the somite (F) are indicated by black arrowheads. Note that *Ndr3* expression is absent from the heart tissues (white arrowhead). (G–I) Forelimb bud of 12.5 dpc embryo. *Ndr1* is only barely expressed (G); *Ndr2* is conspicuously expressed in the perichondrium of the phalanges (PC) (H, arrowheads), while *Ndr3* is expressed in the interdigit mesenchyme (IM) (I, arrowheads).

FIG. 4. *In situ* hybridization of histological sections of 14.5 dpc embryos. The sites of expression of *Ndr1, Ndr2* or *Ndr3* are indicated by arrowheads. (A–C) Sections through the cerebellum. *Ndr1* and *Ndr3* are expressed in the cortical plate (CP), while *Ndr2* is expressed in the ventricular zone (VZ). (D–F) Sections through the spinal cord and the dorsal root ganglia (DRG). *Ndr1* and *Ndr2* are expressed in the dorsal root ganglia; *Ndr2*, in the ventricular zone; *Ndr3* in all cells in the spinal cord from ventricular zone to the intermediate zone (IZ). (G–I) Sections through the thymus rudiments where only *Ndr3* is expressed. (J–L) Sections through the heart ventricle. *Ndr2* is expressed in the myocardium (MC) and the trabeculae (T). (M–O) Sections through the liver. Only *Ndr1* is expressed. (P–R) Sections through stomach and gut. *Ndr1* is expressed along the endodermal epithelia of the caudal alimentary tract, from stomach (S), through midgut (MG) to hindgut (HG), while *Ndr2* is expressed only in the hindgut. (S–U) Sections through the kidney. Both *Ndr1* and *Ndr2* are expressed in the urinary tubules, but *Ndr1* expression seems to be stronger in the nascent tubules just inside of the cortex (arrowheads).


Ndr1
Ndr2
Ndr3
Brain
Spinal cord
Thymus
Heart
Liver
Stomach Gut
Kidney
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
CP
VZ
DRG
IZ
TR
T
MC
S
HG
MG
UT

(Fig. 4P). These epithelia form mucous epithelia in later development. *Ndr1/Drg1* expression observed in normal adult colon (5) is thus initiated at the embryonic stage. *Ndr2* was expressed moderately in the hindgut, while *Ndr3* was not expressed at all (Figs. 4Q and R).

In developing kidney, it has been shown that *Ndr1* is expressed in the convoluted urinary tubules after N-myc expression ceases in the tubule (4), and as a result strong *Ndr1* expression is observed in some, probably nascent, population of the tubules as shown in Fig. 4S. In contrast, *Ndr2* was expressed moderately and rather uniformly through the tubules (Fig. 4T), while *Ndr3* expression was not detected in the kidney at this stage (Fig. 4U).

DISCUSSION

The same *Ndr1/RTP/Drg1* gene has been identified in different contexts, indicating the breadth of its regulation. It is repressed by N-myc and c-myc (4). The second group of observations is that expression of *Ndr1/RTP/Drg1* is down-regulated under oncogenic state of the cells (5–9, 16) and is reactivated when differentiation is induced by chemicals such as retinoic acid (6, 16), which may be related to the first observation. The third is that expression of *Ndr1/RTP/Drg1* is activated by various chemicals which may induce a stress condition on the cells: for example, homocysteine and tunicamycin in human umbilical vascular cells (10), and nickel compounds in several cell lines (11).

Our recent analysis of the *Ndr1* gene indicated that repression by N-myc and c-myc is through the activity of the promoter (4), but response to some chemicals is mediated by an upstream-located enhancer (our unpublished observation). Thus, transformation-related regulation and chemical-induced regulation are probably separate processes. It is noted that the same chemical does not always induce *Ndr1* expression, but the response is highly cell specific ((11), our unpublished data), suggesting that the response is mediated by complex intracellular events. In the survey of a few conditions of chemical stimuli which induce *Ndr1* expression, *Ndr2* and *Ndr3* were not induced in the same cells (our unpublished observation).

In this work, we compared expression specificity of *Ndr1, Ndr2* and *Ndr3* during mouse embryogenesis. *Ndr3* expression is activated already at 9.5 dpc, and expressed widely in early stages. *Ndr2* expression is significantly activated around 12 dpc, and *Ndr1* around 14 dpc (Fig. 2). In addition to this difference in the temporal regulation, they are different in the tissue spectra of expression. In some tissues, only one of these genes is expressed, e.g., liver (*Ndr1*), heart (*Ndr2*) and thymus rudiment (*Ndr3*). In other tissues, three genes showed partial overlap in the nervous system, gut and kidney (Fig. 4).

An interesting observation is that at 12.5 dpc when the phalangeal bones are beginning to form, *Ndr2* was expressed in the perichondrium while *Ndr3* was expressed in the interdigit mesenchyme of the limbs (Fig. 3). These are tissues under regulation by TGFβ-family molecules; in particular, the interdigit mesenchyme is eventually lost by apoptosis induced by BMP subfamily molecules (17, 18). It will be interesting to learn how the possibly related functions of *Ndr2* and *Ndr3* are differentially utilized in these processes.

In this regard, protein localization in a cell is an important issue. When endogenous Ndr1/RTP/Drg1 protein or epitope-tagged exogenous Ndr1/RTP/Drg1 protein in cultured cells were immunohistologically analyzed, the proteins were usually found in the cytoplasm (4–6, 9), but under certain conditions, Ndr1/RTP/Drg1 protein was accumulated in the nucleus (5, 6). This raises a strong possibility that Ndr1 functions as a signaling molecule shuttling between the cytoplasm and the nucleus.

A remarkable feature of the *Ndr1* gene family is that homologues are found in a wide variety of multicellular organisms, nematode (ZK1073.1, accession number Z68135), fruit fly (accession number AAD38579) and sunflower (*sf21* (19)), arguing for an important and conserved biological function of *Ndr1/RTP/Drg1* and its homologues. In addition, another gene distantly related to *Ndr1/RTP/Drg1* was identified as the one expressed predominantly in postnatal rat brain and termed *Bdm1* (20). *Ndr1/RTP/Drg1, Ndr2* and *Ndr3* may represent a large gene family which bears important differentiation-related functions. Actual function of Ndr family proteins in a cell is yet to be identified, but we feel it likely that this family of putative signaling molecules plays an important role in the cellular process, such as establishing and maintaining cell differentiation, counteracting malignant transformation, and protecting cells from chemical stress.

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