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A Cysteine-Rich Form of *Xenopus* Neuregulin Induces the Expression of Acetylcholine Receptors in Cultured Myotubes

Jie F. Yang, Hong Zhou,* Roy C. Y. Choi, Nancy Y. Ip,
H. Benjamin Peng,* and Karl W. K. Tsim

*Department of Biology and Biotechnology Research Institute, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong, China; and *Department of Cell Biology and Anatomy and Curriculum in Neurobiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599*

Neuregulin-1 (NRG-1) has diverse functions in neural development, and one of them is to up regulate the expression of acetylcholine receptors (AChRs) at muscle fibers during the formation of neuromuscular junctions. NRG-1 has two prominent alternative splicing sites at the N-terminus; it could be an immunoglobulin (Ig)-like domain named Ig-NRG-1 or an apolar cysteine-rich domain (CRD) named CRD-NRG-1. cDNAs encoding *Xenopus* CRD-NRG-1 were isolated by cross-hybridization with *Xenopus* Ig-NRG-1 cDNA fragment. The amino acid sequence of *Xenopus* CRD-NRG-1 is 45 to 70% identical to the human, rat, and chick homologs. Similar to Ig-NRG-1, two variation sites within CRD-NRG-1 were identified at the spacer domain with 0 or 43 amino acids inserted and at the C-terminus of the EGF-like domain to derive either α or β isoform. Two transcripts encoding CRD-NRG-1, ~7.5 and ~9.0 kb, were revealed in adult brain and spinal cord, but the expression in muscle was below the detectable level. The recombinant *Xenopus* CRD-NRG-1 when applied onto cultured myotubes was able to induce the tyrosine phosphorylation of ErbB receptors and the expression of AChR. The AChR-inducing activity of CRD-NRG-1 was precipitated by anti-NRG-1 antibody but not by heparin. *In situ* hybridization showed a strong expression of CRD-NRG-1 mRNA in developing brain, spinal cord, and myotomal muscles of *Xenopus* embryo. Similar to the results in other species, both CRD-NRG-1 and Ig-NRG-1 may play a role in the developing *Xenopus* neuromuscular junctions.

INTRODUCTION

During the development of neuromuscular junctions, motor neurons make contact with muscle fibers and direct the formation of postsynaptic specializations (Salpeter, 1987; Hall and Sanes, 1993). These specializations include the aggregation of acetylcholine receptors (AChRs), acetylcholinesterase (AChE), and other synaptic proteins (McMahan, 1990). The increase in postsynaptic AChR density, up to approximately 10,000 receptors / μm^2, is primarily due to the aggregation of AChRs already present in the membrane at the time of nerve–muscle contact as well as an increase in local AChR synthesis (Salpeter, 1987; Hall and Sanes, 1993). In the local synthesis of postsynaptic molecules, mRNAs encoding AChR subunits, AChE, and other synapse-specific proteins are highly concentrated in the synaptic regions (Merlie and Sanes, 1985; Tsim *et al.*, 1992; Moscoso *et al.*, 1995). The motor nerve provides two distinct mechanisms to achieve this striking localization of muscle AChR: (i) it releases factors, such as calcitonin gene-related peptide (Fontaine *et al.*, 1987; Choi *et al.*, 1998), ascorbic acid (Horovitz *et al.*, 1989), and acetylcholine receptor-inducing activity (ARIA; Falls *et al.*, 1990, 1993), that stimulate the synaptic expression of AChR and (ii) nerve-evoked electrical activity represses the synthesis of AChR in the extrasynaptic regions (Laufer and Changeux, 1989; Witzemann *et al.*, 1991).

Fischbach and his colleagues proposed that ARIA, first isolated from chick brain, is released from developing motor nerve terminals, activates its receptor on the postsynaptic muscle membrane, and induces the postsynaptic gene expression of AChR and voltage-gated sodium channel at the neuromuscular junction (Falls *et al.*, 1990; Corfas and Fischbach, 1993; Loeb and Fischbach, 1995; Sandrock *et al.*, 1997). The cDNA encoding ARIA was first isolated from a λgt10 chick brain library

Sequence data from this article have been desposited with the EMBL / GenBank Data Libraries under Accession No. AF142632.

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(Falls *et al.*, 1993). ARIA belongs to a family of proteins, named neuregulin-1 (NRG-1), that have diverse functions in neural development (Jo *et al.*, 1995; Meyer and Birchmeler, 1995). Members of NRG-1 include rat *neu* differentiation factor (NDF; Wen *et al.*, 1994), human heregulin (HRG; Holmes *et al.*, 1992), and bovine glial growth factor (GGF; Marchionni *et al.*, 1993). Different isoforms are produced from the NRG-1 gene (Holmes *et al.*, 1992; Ho *et al.*, 1995; Burden and Yarden, 1997; Meyer *et al.*, 1997). Type I isoforms, originally identified as NDF/HRG/ARIA and now named Ig-NRG-1, contain an immunoglobulin (Ig)-like domain, an epidermal growth factor (EGF)-like domain, a transmembrane domain, and an intracellular C-terminus. Type II isoforms are similar to type I NRG-1, but without transmembrane and intracellular domains, and include a highly hydrophobic domain at the N-terminus. GGF II (Marchionni *et al.*, 1993) and γNRG (Schaefer *et al.*, 1997) are examples for this type. Type III isoforms contain an apolar cysteine-rich domain (CRD), instead of an Ig-like domain as in type I, and are named CRD-NRG-1. Examples of type III isoforms are sensorimotor-derived factor from human (Ho *et al.*, 1995) and CRD-NRG-1/nARIA from chick (Yang *et al.*, 1998b). The EGF-like domains from several members of NRG-1 possess the full biological functions that include the tyrosine phosphorylation of ErbB receptors and the induction of AChR expression (Loeb and Fischbach, 1995; Si *et al.*, 1996; Yang *et al.*, 1997, 1998a). In addition to NRG-1, NRG-2 and NRG-3 have also been identified (Carraway *et al.*, 1997; Chang *et al.*, 1997; Zhang *et al.*, 1997).

Several lines of evidence indicate that Ig-NRG-1 plays an important role in the formation and maintenance of vertebrate neuromuscular junctions (Falls *et al.*, 1993; Corfas *et al.*, 1995; Sandrock *et al.*, 1997). Mutant mice lacking Ig-NRG-1, erbB-2, and erbB-4 genes die in the early embryonic days (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995; Kramer *et al.*, 1996). However, the heterozygous Ig-NRG-1+/− mice have defects in nerve–muscle contacts. These results strongly indicate that Ig-NRG-1, indeed, is required for the induction and maintenance of AChR expression at the neuromuscular junctions (Sandrock *et al.*, 1997). However, the role of CRD-NRG-1 in the formation of neuromuscular junctions has not been fully assessed. Corfas *et al.* (1995) detected a much more intense hybridization signal in chick spinal cord when an EGF-like domain probe of Ig-NRG-1 was used versus an Ig-like domain probe. Their results suggest the existence of Ig-like domain-deficient NRG-1 in the motor neurons. The expression of CRD-NRG-1 in human and rodent is maintained in both adult motor neurons and dorsal root ganglion (Ho *et al.*, 1995). In addition, the expression of CRD-NRG-1 was detected in developing chick visceral motor neurons, and the recombinant chick CRD-NRG-1 could mimic the effect of presynaptic neurons, namely, the sympathetic ganglionic neurons (Yang *et al.*, 1998b). These results suggest that CRD-NRG-1 in motor neurons may mediate the regulation of AChR during synaptogenesis. In order to study the possible roles of CRD-NRG-1 in embryo development and the formation of neuromuscular junctions, we cloned the cDNAs encoding CRD-NRG-1 from embryonic *Xenopus* cDNA libraries and characterized the expression of its transcripts during the early embryo development.

RESULTS

Cloning of Xenopus CRD-NRG-1 cDNAs

Over 2 million individual colonies were screened by cross-hybridization with *Xenopus* Ig-NRG-1 cDNA fragment. Most of the clones identified from the cDNA library screening were Ig-NRG-1 cDNAs. However, one clone, named XA-8, showed a relatively weak signal during the hybridization process. This ~3.5-kb clone did not hybridize with the cDNA encoding Ig-like domain of Ig-NRG-1 (data not shown). The XA-8 was then fully sequenced and analyzed.

XA-8 clone has 2064 bp of open reading frame that encodes a polypeptide of 688 amino acids with a predicted molecular weight (M_r) of 76,962 (Fig. 1A). The first start code (ATG) of XA-8 is at nucleotide 532. The amino acid sequence of XA-8 was analyzed by MacVector software. With the exception of the N-terminal end from M_1 to E_{158}, a great part of the cloned XA-8 is identical to the reported *Xenopus* Ig-NRG-1 sequence (Yang *et al.*, 1998a). This includes EGF-like domain, transmembrane domain, and intracellular domain. Hydropathy analysis revealed that XA-8 has two major hydrophobic regions (Fig. 1B). One region spans the 24 nonpolar amino acids (V_{272} to C_{295}) that are identical to the transmembrane region of *Xenopus* Ig-NRG-1 (Yang *et al.*, 1998a). Another hydrophobic region that is rich in cysteine residues is at the N-terminal end (G_{43} to I_{96}) and is named CRD. The spacer domain, between CRD and EGF-like domain, is rich with serine and threonine residues which are potential sites for O-linked glycosylation. The potential N-glycosylation sites are absent from the sequence. *Xenopus* CRD-NRG-1 lacks the nonpolar amino acids at the very N-terminus that could serve as a signal peptide. The dibasic residues K_{270}–R_{271} adjacent to the transmembrane region are conserved compared with other NRG-1 family members. Thus,

XA-8 shares a similar structure with other CRD-NRG-1s that have an apolar CRD followed by a spacer domain, an EGF-like domain, a transmembrane region, and a long intracellular domain (Figs. 1A and 1B).

In addition, more clones were isolated from the library by PCR with primers specific for CRD-NRG-1. The cDNA sequences of individual clones were sequenced and they encoded for various isoforms of the CRD-NRG-1 family with variation at two sites: (i) the spacer domain with either 0- or 43-amino-acid insertion, namely SP0 or SP43; (ii) the C-terminus of EGF-like domain to give rise to either the α or the β isoform (Fig. 1C). These splicing isoforms are identical to the reported *Xenopus* Ig-NRG-1 (Yang *et al.*, 1998a).

Sequence Comparison with NRG-1 Family

Xenopus CRD-NRG-1 is highly homologous to human, rat, and chick counterparts. The amino acid sequence of *Xenopus* CRD-NRG-$1_{SP43\beta2}$ is ~45% identical overall to that of human (Ho *et al.*, 1995), ~63% to that of rat (Bermingham-McDonoghh *et al.*, 1997), and ~70% to that of chick (Yang *et al.*, 1998b; see Fig. 1A). The apolar CRD is highly conserved; there is over 85% amino acid identity among human, rat, chick, and *Xenopus* proteins. The eight cysteines and their spacing within the CRD are highly conserved among all species. This indicates that the CRD may play some important roles in the functions of CRD-NRG-1. In the spacer domain between CRD and EGF-like domain, *Xenopus* CRD-NRG-1 sequence is distinct from those of the other two species (Fig. 1A).

Relationship between Xenopus Ig-NRG-1 and CRD-NRG-1

In *Xenopus* NRG-1, the amino acid sequence of CRD-NRG-1 is partly identical to that of Ig-NRG-1. They are identical in the spacer domain and the C-terminus (from P_{159} onward; Fig. 1A). The N-terminus is different in having either an Ig-like domain insertion or an apolar CRD insertion. There is no obvious sequence relationship between the Ig-like and the CRD of the two types of NRG-1s, and their relationship is illustrated in Fig. 2A. This sequence variation could indicate that they might be derived from the same gene by alternative RNA splicing.

In order to determine the genomic structure of Ig-NRG-1 and CRD-NRG-1, genomic DNA from *Xenopus* tissue was isolated. After digestion with various endonucleases, the genomic DNAs were applied onto an agarose gel for Southern blot analysis. Three cDNA fragments were used as probes (Fig. 2A): a CRD-NRG-1-specific fragment (C_{54}–G_{108}), an Ig-NRG-1-specific fragment (K_4–D_{125}; see Fig. 1 of Yang *et al.*, 1998a), and a common fragment of NRG-1 (C_{214}–I_{278}). Several enzyme digests resulted in a single and identical band that was recognized by the three different individual probes (Fig. 2B). This suggests that Ig-NRG-1 and CRD-NRG-1 could be derived from alternative splicing of a single NRG-1 gene.

Functional Expression of Xenopus CRD-NRG-1

Similar to Ig-NRG-1, the recombinant *Xenopus* CRD-NRG-1 when expressed in HEK 293 cells induced the expression of AChR α-subunit mRNA by threefold in cultured chick myotubes (Fig. 3). In order to study the function of the novel apolar CRD, different domains of CRD-NRG-1 cDNAs were tagged with human Ig Fc cDNA and subcloned into mammalian expression vector. The chimeric cDNA construct was transfected into HEK 293 cells, and the NRG-1–Fc fusion protein was purified by protein G column. The Fc-tagged NRG-1 was recognized by anti-NRG-1 and anti-human Fc antibodies at ~60 kDa (data not shown). The pnlacZ plasmid containing the AChR α-subunit promoter was transfected into cultured myotubes, and they were treated with NRG-1–Fc recombinant proteins. Figure 4A shows the induced β-galactosidase in differently treated myotubes. The induction potency of CRD-NRG-1 was slightly lower than that of Ig-NRG-1. The EGF-like domain alone showed similar ability in the activation of AChR α-subunit promoter compared to CRD together with EGF-like domain. However, the CRD alone contained no AChR-inducing activity. In addition, the induction activity of AChR α-subunit promoter by NRG-1 was dose-dependent. The difference in AChR-inducing activity of CRD-NRG-1, as described here, was not due to the existence of the Fc tag. Figure 4B shows an equal potency in AChR α-subunit induction between CRD-EGF and CRD-EGF–Fc. Although we did not quantify the potency of different CRD-NRG-1 isoforms, the α and β variants at the EGF-like domain of *Xenopus* Ig-NRG-1 exhibited similar AChR-inducing activity in cultured myotubes (see Fig. 4 of Yang *et al.*, 1998a). On the other hand, the result in phosphorylation assay of ErbB-3 receptor was similar to that of the AChR induction assay. All constructs of NRG-1, except the CRD, induced the tyrosine phosphorylation of ErbB-3 in cultured C2C12 myotubes (Fig. 4C). This further supports the role of AChR-inducing activity of CRD-NRG-1 at the neuromuscular junctions.

In order to determine the nature of CRD, the conditioned medium of either Ig-NRG-1 or CRD-NRG-1 cDNA-transfected cells was treated with heparin and

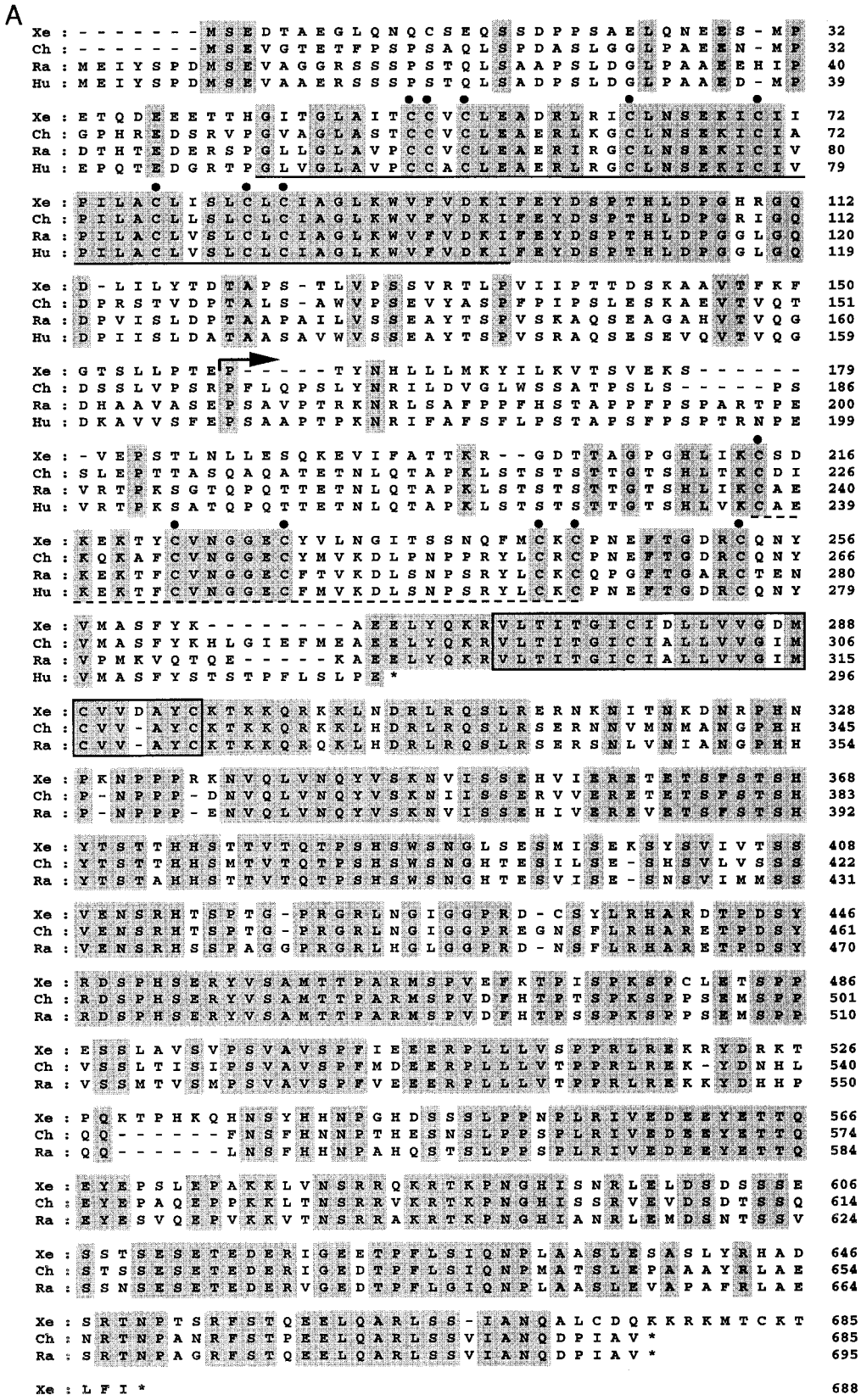
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FIGURE 1

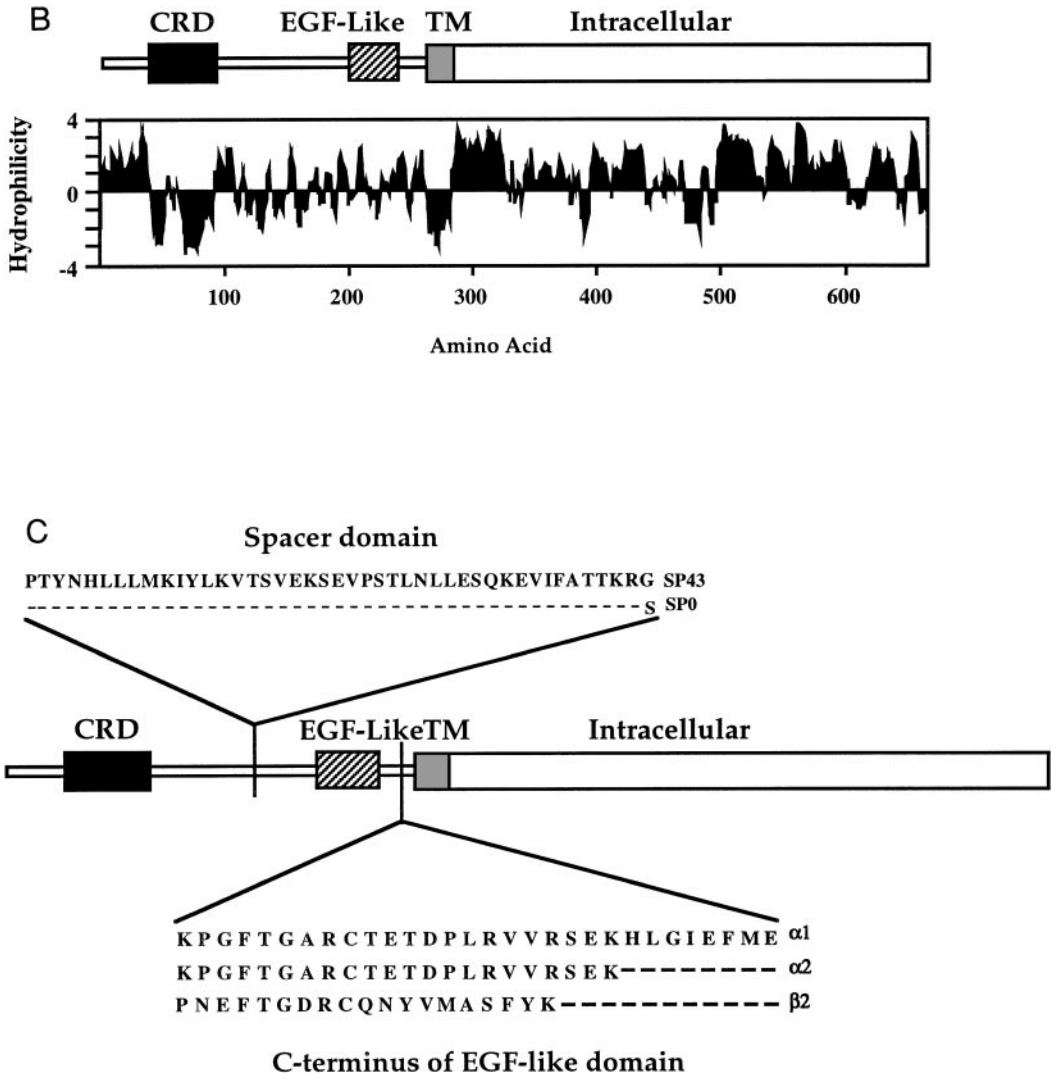


FIG. 1. Isolation of *Xenopus* CRD-NRG-1. (A) Alignment of *Xenopus* CRD-NRG-1 to members of the family. The sequence is derived from XA-8 that encodes for CRD-NRG-$1_{SP43\beta2}$. CRD (underlined), EGF-like domain (dotted line), and hydrophobic domain (boxed) are shown. Cysteines within CRD and EGF-like domains are dotted, and stop codons are represented by an asterisk. The C-terminus after E_{158} is identical between CRD-NRG-1 and Ig-NRG-1 as indicated by an arrow. *Xenopus* CRD-NRG-$1_{SP43\beta2}$ is chosen to compare with chick (Ch; Yang *et al.*, 1998b), rat (Ra; Bermingham-McDonogh *et al.*, 1997), and human (Hu; Ho *et al.*, 1995) proteins. Identical residues among all species are shaded. The amino acid sequence of *Xenopus* CRD-NRG-1 is ~45% identical overall to that of human, ~63% to that of rat, and ~70% to that of chick protein. (B) Hydrophilicity profile of deduced *Xenopus* CRD-NRG-1. The method of Kyte and Doolittle (1982) was used with a window size of 7 residues. Negative values indicate increasing hydrophobicity. Amino acid numbers are shown below the profile. The predicted structure of CRD-NRG-1 is shown above the profile for reference. (C) Various isoforms of *Xenopus* CRD-NRG-1. Two splicing sites were detected in CRD-NRG-1: (i) the spacer domain with either 0- or 43-amino-acid insertion, namely SP0 or SP43, respectively; (ii) the C-terminus of EGF-like domain to give rise to α1, α2, or β2 isoform. All these cDNA clones were isolated from the same cDNA library that was used for hybridization screening.

anti-NRG-1 antibody. These two agents were shown to bind Ig-NRG-1 (Pun *et al.*, 1997). Similar to Ig-NRG-1, the anti-NRG-1 antibody precipitated the AChR-inducing activity that derived from CRD-NRG-1 cDNA-transfected cells (Fig. 5A). The NRG-1-induced β-galactosidase activity was reduced by over 90% after precipitation with the antibody. However, the CRD-NRG-1 activity was insensitive to the heparin precipitation, and that was in contrast to the property of Ig-NRG-1. The binding of Ig-NRG-1 to heparin is due to the Ig-like domain alone. Both CRD-Fc and Ig-Fc recombinant fusion proteins were treated with either heparin-

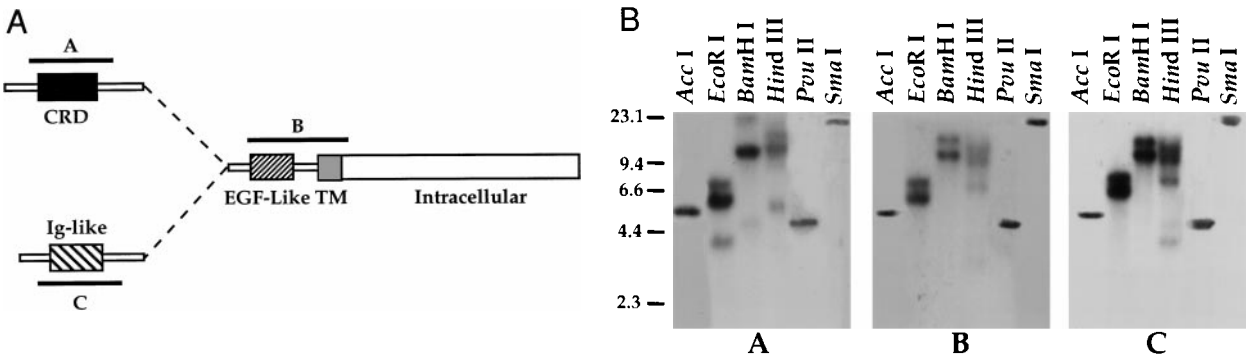


FIG. 2. CRD-NRG-1 and Ig-NRG-1 are generated by alternative splicing of a single gene product. (A) Different specific probes (A, B, and C) as indicated by solid bars were used in Southern blot analysis of *Xenopus* genomic DNA. (B) DNA restriction endonucleases used to digest genomic DNA are shown at the top of each lane. The specific probes (A, B, and C) are indicated on the bottom. Molecular size markers are in kb.

agarose or protein G agarose. Ig–Fc at ~55 kDa, but not CRD–Fc, could be effectively precipitated by heparin-agarose (Fig. 5B). Protein G precipitated both fusion proteins, and that served as a control. Thus, the CRD properties differ from those of the Ig-like domain of NRG-1.

mRNA Expression and In Situ Hybridization of CRD-NRG-1

Northern blot analysis using cDNA encoding the CRD to probe for corresponding transcripts in different *Xenopus* tissues provided results comparable to those obtained from other species. Transcripts encoding CRD-NRG-1 were detected in RNA isolated from adult brain and spinal cord, while the level of expression was too low to be detected in adult muscle (Fig. 6A). The lengths of the corresponding transcripts were ~7.5 and ~9.0 kb higher than that of the single Ig-NRG-1 transcript (~6.5 kb) detected in *Xenopus* (Yang *et al.*, 1998a). The intensity of the transcripts was similar in RNA isolated from brain and spinal cord (Fig. 6A).

By using primers flanking the carboxyl-terminus of EGF-like domain of *Xenopus* CRD-NRG-1, RT-PCR was performed on various *Xenopus* tissues. All tissues, except adult muscle, gave three major PCR products with sizes of 218, 194, and 185 bp; they represented the partial sequence at the C-terminus of the EGF-like domain of *Xenopus* NRG-1 α1, α2, and β2 (Fig. 6B). Although CRD-NRG-1 mRNA was too low to be detected in adult *Xenopus* muscle, RT-PCR analysis revealed a weak signal of β2 isoforms of CRD-NRG-1. In contrast, the α1, α2, and β2 isoforms were detected in embryonic muscles. In order to determine the splicing variants at the spacer domain, CRD-NRG-1-specific primers were used for RT-PCR analysis in various tissues. Two PCR products of 127 and 256 bp were revealed, and they represented the splicing variants of CRD-NRG-1 at the known spacer. These CRD-NRG-1 variants comprise a 0-amino-acid insertion and a 43-amino-acid insertion, namely CRD-NRG-1_{SP0} and CRD-NRG-1_{SP43}, respectively (see Fig. 1C). CRD-NRG-1_{SP43} was the only species found in the brain, spinal cord, and muscle of adult *Xenopus* (Fig. 6C). CRD-NRG-1_{SP0} was detected in embryonic muscle while the whole embryo contained both CRD-NRG-1_{SP0} and CRD-NRG-1_{SP43} transcripts. Thus, the source of CRD-NRG-1 at the adult neuromuscular junction could be derived from both motor neuron and muscle.

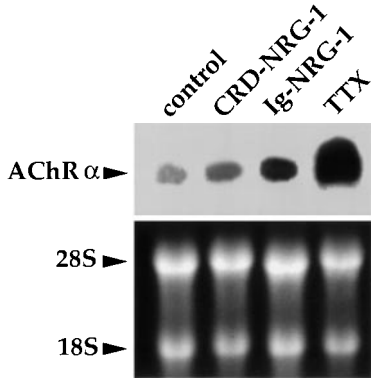


FIG. 3. CRD-NRG-1 induces the up regulation of AChR α subunit in cultured myotubes. Four-day-old chick myotubes (5 ml) were treated with 0.5 ml conditioned medium from CRD-NRG-1 or Ig-NRG-1 cDNA-transfected cells for overnight. Tetrodotoxin (TTX; 1 μM) served as a positive control and untreated myotubes as background control (control). Total RNA was isolated from the cells and 10 μg of RNA was subjected to a 1% formaldehyde–agarose gel. The membrane was probed with AChR α-subunit cDNA (~1.2 kb), and a transcript of ~3.2 kb was detected. Bottom shows the ribosomal RNA staining with 18S and 28S as markers.

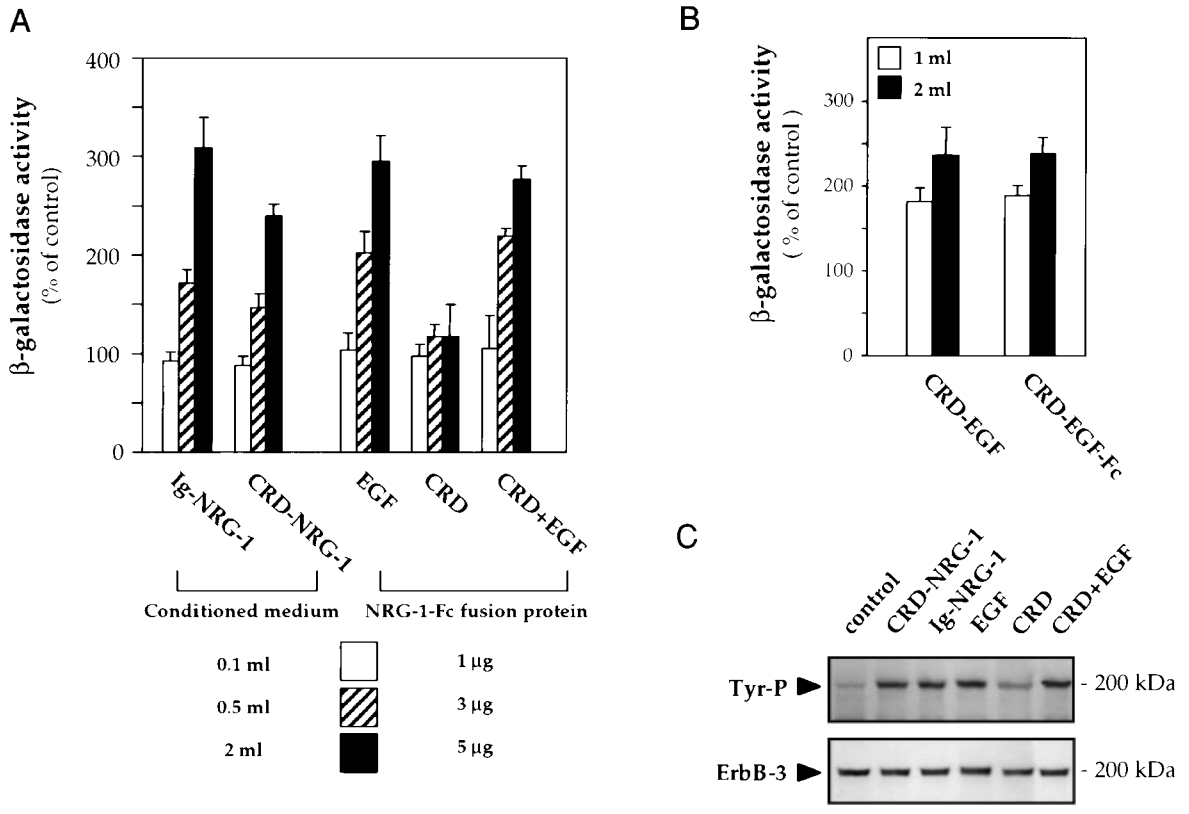


FIG. 4. CRD-NRG-1 induces the expression of AChR α-subunit promoter and tyrosine phosphorylation of ErbB-3. (A) The pnlacZ plasmid containing the 850-bp chick AChR α-subunit promoter tagged with β-galactosidase gene was transfected into 2-day-old chick myotube cultures by using calcium phosphate precipitation. Two days after transfection, the conditioned medium or the purified Fc-tagged NRG-1 was applied onto 1 ml cultured myotubes. Two days after the treatment, the induced β-galactosidase activity was determined. Values are expressed in % of control (no NRG-1 treatment as 100%) and are in means ± SEM, $n = 4$. (B) CRD-EGF and CRD-EGF–Fc recombinant proteins were applied onto pnlacZ-transfected myotubes. The induced β-galactosidase activity was determined as in (A). Values are expressed in % of control (no NRG-1 treatment as 100%) and are in means ± SEM, $n = 4$. (C) The C2C12 myotubes were treated with conditioned medium as above for 30 min. Control was untreated myotubes. The treated cells were lysed in RIPA buffer. The ErbB-3 receptor was immunoprecipitated with 1:1000 dilution of a rabbit antibody (C17) against ErbB-3. The immunoprecipitated proteins were collected on protein G agarose beads and fractionated by 7.5% SDS–PAGE. Peroxidase-conjugated anti-tyrosine phosphorylation antibodies RC 20 (top) were used. The same filter was washed and probed with anti-ErbB-3 receptor antibody to confirm the identity of the recognized band (bottom). A 200-kDa molecular marker is indicated.

To understand the spatial relationship of *Xenopus* CRD-NRG-1 expression in development, whole-mount *in situ* hybridization was conducted at different developing stages of *Xenopus* embryos. Specific probes for either Ig-like domain or CRD were used (see Fig. 2A). Comparing the results from two probes, the expression profiles were very similar (Fig. 7). The CRD-NRG-1 mRNA is highly expressed in the developing nervous system. During neurulation, it is restricted to the developing nervous system. The increasing signal can be observed in stages 23 to 26 in the brain and spinal cord (Fig. 7). Within the central nervous system, CRD-NRG-1 mRNA was abundant in the brain, the eye, and the spinal cord (Figs. 7B–7D). For the control, embryos at similar stages were hybridized with probe synthesized in the sense direction and processed in the identical fashion. The control embryo showed no labeling (Figs. 7G and 7G′). In a higher magnification of the whole-mount *in situ* of developing embryo, the mRNA expression of CRD-NRG-1 was found in muscle nuclei and the junction between myotomes where the future neuromuscular junctions are located (Fig. 8A). In addition, the expression of CRD-NRG-1 in the brain and eye of the embryo was clearly demonstrated in Fig. 8B.

DISCUSSION

Here, we isolated the cDNAs encoding *Xenopus* CRD-NRG-1 and demonstrated that the CRD-NRG-1 was

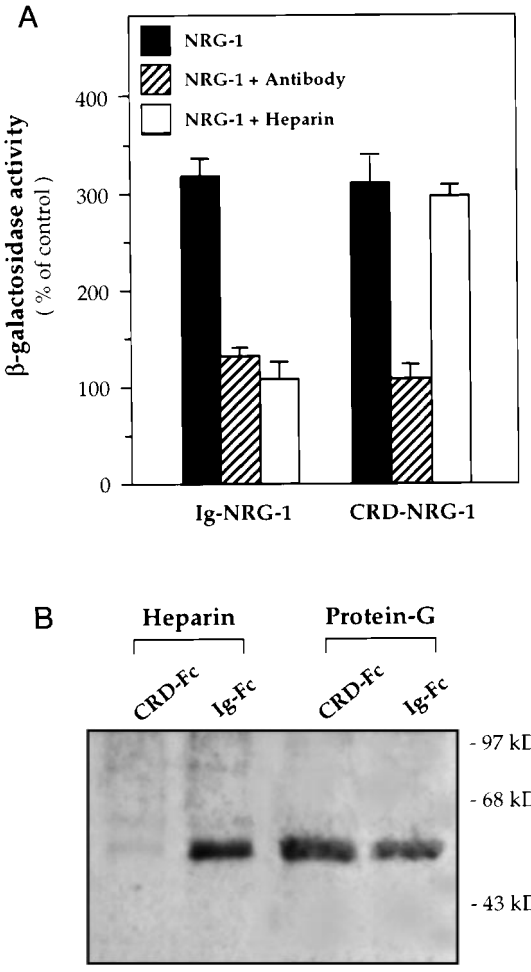


FIG. 5. The Ig-like domain binds to heparin but not CRD. (A) The AChR-inducing activity of CRD-NRG-1 is precipitated by anti-NRG-1 antibody but not by heparin. The conditioned medium of CRD-NRG-1 or Ig-NRG-1 cDNA-transfected cells was treated with heparin-agarose beads (50 μl / ml) or anti-NRG-1 antiserum (1:500 dilution) for 30 min before being applied onto cultured myotubes. The untreated conditioned medium served as a control. The treated conditioned medium was applied onto the cultured myotubes for 2 days, and the induced β-galactosidase activity was determined. Values are expressed in % of control and are in means ± SEM, $n = 4$. (B) Ig-like domain binds to heparin. CRD and Ig-like domains were tagged with Fc. The conditioned medium from the transfected cells was treated with heparin-agarose beads (50 μl / ml) or protein G beads (50 μl / ml) for 30 min. The beads were boiled for 10% gel electrophoresis. The Fc-tagged proteins at ~55 kDa were recognized by peroxidase-conjugated anti-Fc antibody. Protein G served as a control.

able to induce the expression of AChR in cultured myotubes. The cDNA sequence of *Xenopus* CRD-NRG-1 is in good agreement with previous reports of other species (Ho *et al.*, 1995; Yang *et al.*, 1998b). In *Xenopus*, CRD-NRG-1 and Ig-NRG-1 are derived from a single gene. They have identical spacer domains, EGF-like domains, transmembrane regions, and intracellular domains. Moreover, the splicing variants at the spacer domain and at the C-terminus of the EGF-like domain could be revealed in both forms of NRG-1. The only difference between the two NRG-1s is at the 5′end with either an Ig-like domain in Ig-NRG-1 or an apolar CRD in CRD-NRG-1. Compared to other CRD-NRG-1s, the apolar CRD is highly conserved. The eight cysteines and their spacing within the CRD are highly conserved among all species. This indicates that the CRD may play some important roles in the function of CRD-NRG-1.

Several lines of evidence are against the possibility that CRD-NRG-1 plays a role in the formation of neuromuscular junctions. In heterozygous knockout

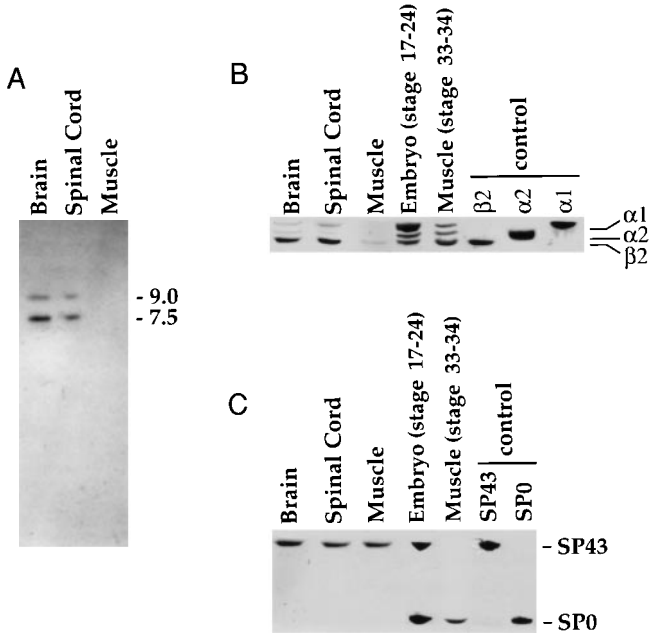


FIG. 6. Northern blot and RT-PCR analysis of *Xenopus* CRD-NRG-1 mRNA distribution. (A) RNAs (40 μg) isolated from adult *Xenopus* brain, spinal cord, and muscle were electrophoresed on a 1% agarose gel and blotted onto a nitrocellulose paper. The blot was probed with cDNA encoding CRD of CRD-NRG-1 under conditions of high stringency. Two transcripts with sizes of ~7.5 and ~9.0 kb, as indicated, can be detected in the brain and spinal cord. (B) Reverse transcription was used to prepare first-strand cDNA from total RNAs. The cDNAs were first amplified by PCR with CRD-NRG-1-specific primers (S-2 and AS-1), then with primers flanking the EGF-like domain (S-1 and AS-1). The PCR products were revealed on a 12% acrylamide gel and stained with ethidium bromide. The α1, α2, and β2 isoforms were detected in RNA isolated from adult *Xenopus* brain and spinal cord, stage 17–24 embryo, and stage 33–34 embryonic muscle. Adult muscle expressed a low level of β2 isoform. (C) The cDNAs were amplified with primers (S-2 and AS-2) flanking the spacer domain of CRD-NRG-1. The PCR products were revealed as in (B). CRD-NRG-1_{SP43} is expressed in adult *Xenopus* brain, spinal cord, and muscle. Embryonic muscle expressed CRD-NRG-1_{SP0} isoform only. Embryos from stage 17 to 24 expressed both CRD-NRG-1_{SP43} and CRD-NRG-1_{SP0}.

Ig-NRG-1-deficient mice, Sandrock *et al.* (1997) showed that the postsynaptic AChR density and the synaptic transmission were reduced in Ig-NRG-1-deficient mice. Their result indicates that although Ig-NRG-1 may represent a small fraction (10 to 20%) of the total NRG-1 in spinal motor neurons, Ig-NRG-1 could be the key AChR-inducing factor during the formation of neuromuscular junctions. The Ig-like domain of Ig-NRG-1 was shown to bind heparin *in vitro* (Goodearl *et al.*, 1995; Pun *et al.*, 1997), which could retain Ig-NRG-1 at the synaptic basal lamina. This binding could explain why the AChR-inducing activity persists at denervated neuromuscular junctions after the presynaptic nerve terminals have degenerated (Jo *et al.*, 1995). In addition, the expression of motor neuron Ig-NRG-1 was selectively increased by the muscle-derived neurotrophic factors (Loeb and Fischbach, 1997). On the other hand, there is evidence to support CRD-NRG-1 playing a role in developing neuromuscular junctions. First, although its potency is slightly smaller than that of Ig-NRG-1, CRD-NRG-1 contains the full AChR-inducing activity and the ability to phosphorylate ErbB-3 receptors in cultured myotubes. Second, the secretory pathway of CRD-NRG-1 could be very similar to that of Ig-NRG-1. A membrane-bound precursor of NRG-1 could be required before the release of biologically active EGF-like fragment as proposed by Loeb and Fischbach (1995). Whether the binding of Ig-like domain of Ig-NRG-1 to heparin within the synaptic basal lamina is a prerequisite for its functions at the neuromuscular junctions is not known. The binding of NRG-1 to the synaptic basal lamina may not be necessary for AChR-inducing activity. In addition, CRD of CRD-NRG-1 may bind to other members of the synaptic basal lamina. Third, the restricted and high level of CRD-NRG-1 expression in motor neurons further supports the hypothesis. The predominant NRG expression in motor neurons and sensory neurons is CRD-NRG-1 (Ho *et al.*, 1995; Yang *et al.*, 1998b). The CRD-NRG-1 mRNA expression is reduced in both motor and sensory neurons following axotomy, but the expression returns to normal levels after reinnervation of the target tissues (Bermingham-McDonogh *et al.*, 1997). Last, the expression profiles of Ig-NRG-1 and CRD-NRG-1 are very similar in developing *Xenopus* embryos. These expression profiles suggest that CRD-NRG-1 could play a role in directing the postsynaptic AChR up regulation during the formation of neuromuscular junctions.

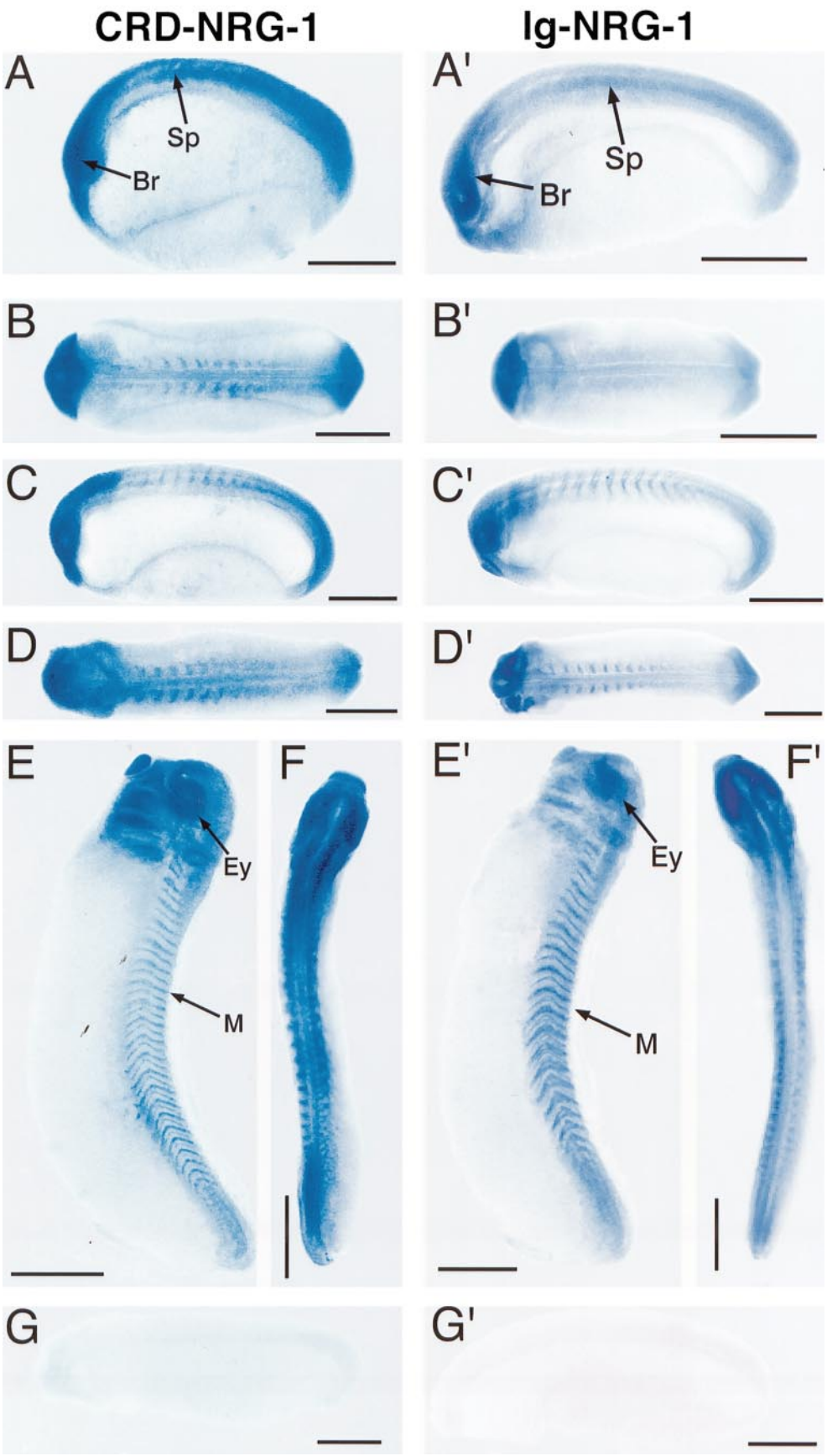
In the developing neuromuscular junctions, spinal cord and muscle express both Ig-NRG-1 and CRD-NRG-1 mRNAs. The expression of CRD-NRG-1 mRNA in adult *Xenopus* muscle is reduced to a level which could not be detected by Northern blot analysis, while the spinal cord still retains a high level of expression. This expression profile of CRD-NRG-1, however, is different from that of Ig-NRG-1 in *Xenopus* (Yang *et al.*, 1998a). *Xenopus* muscle expresses a significant amount of Ig-NRG-1 mRNA as well as various isoforms from very early stage of embryo development to adult muscle. The expression level and the isoform profile of Ig-NRG-1 in chick muscle also change during development, denervation, and nerve regeneration (Ng *et al.*, 1997). The situation of CRD-NRG-1 mRNA expression is completely different. A transient high expression of CRD-NRG-1 in developing *Xenopus* muscle is consistent with the time of the formation of neuromuscular junctions. The level of expression of CRD-NRG-1 drops tremendously in adult muscle, while the spinal cord remains the major contributor of CRD-NRG-1 at the adult neuromuscular junctions. Indeed, the motor neurons in chick express predominantly CRD-NRG-1 instead of Ig-NRG-1 as proposed by Sandrock *et al.* (1997). Whether CRD-NRG-1 at neuromuscular junctions could induce the postsynaptic specializations is still lacking a direct demonstration.

NRG-1 was also shown to induce the postsynaptic specializations during synaptogenesis of neuron-to-neuron synapses. The recombinant CRD-NRG-1 increased the magnitude of ACh-evoked macroscopic currents in synaptically naïve sympathetic neurons, and it mimicked the effects of presynaptic input. CRD-NRG-1 also increased the expression of neuronal nicotinic AChR mRNAs encoding α3, α5, α7, and β4 subunits in synaptically naïve sympathetic neurons (Yang *et al.*, 1998b). In addition, Ig-NRG-1 β isoform, but not the α isoform, increased at least 100-fold the expression of NR2C subunit of *N*-methyl-D-aspartate receptor in cultured cerebellar granular neurons (Ozaki *et al.*, 1997). NRG-1 also promoted the survival of and neurite extension from cultured retinal neurons in a dose-dependent manner while the expression of NRG-1 and ErbB receptors was developmentally regulated in the retinal neurons (Bermingham-McDonoghen *et al.*, 1996). The expression profiles of both Ig-NRG-1 and CRD-NRG-1 in *Xenopus* as well as in other species also support the notion (Corfas *et al.*, 1995; Sandrock *et al.*, 1995; Yang *et al.*, 1998b).

EXPERIMENTAL METHODS

Screening cDNA libraries

An embryonic stage E40–45 cDNA library in Lambda ZAP II (Stratagene, La Jolla, CA) was screened by using procedures described in Sambrook *et al.* (1989). In brief, about 1×10^6 recombinant phages from the amplified


CRD-NRG-1
Ig-NRG-1
A
Sp
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A'
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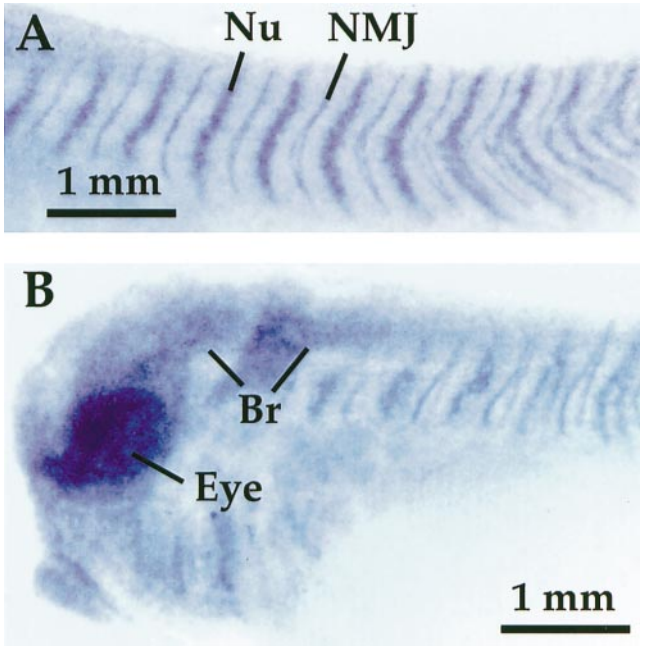

FIG. 8. Expression of CRD-NRG-1 mRNA in embryonic muscle, brain, and eye. CRD-specific probe was used as in Fig. 7. A stage 40 embryo was used for hybridization. (A) The mRNA was revealed in muscle nuclei (Nu) and the junction between myotomes where the neuromuscular junction is located (NMJ). (B) Eye and brain (Br) contained CRD-NRG-1 mRNA.

library were screened with random primed ^{32}P-labeled *Xenopus* Ig-NRG-1 cDNA (~0.9 kb from nucleotide 170 to 955; Yang *et al.*, 1998a) that had a specific activity of about 1×10^9 cpm/μg of DNA. Hybridization was carried out overnight at 42°C in 30% formamide, 5× SSC, 5× Denhardt's reagent, 0.1% SDS, 0.l mg/ml denatured salmon sperm DNA to which about 5×10^5 cpm/ml radioactive probe had been added. Filters were washed twice for 30 min in 2× SSC, 0.2% SDS at room temperature and then twice for 30 min at 55°C. Three rounds of screening were done to ensure positive clones. All cDNA clones were sequenced by the dideoxy chain termination method using Sequenase (USB, Cleveland, OH). The internal sequences of these cDNAs were sequenced using synthetic oligonucleotides. Both strands of the cDNA clones were sequenced at least twice and the DNA sequences were aligned using the MacVector (Kodak, New Haven, CT) software package.

Functional Expression of Xenopus Neuregulin

cDNAs encoding CRD, EGF-like domain, Ig-like domain, or CRD plus EGF-like domain of *Xenopus* NRG-1 were tagged with human Ig Fc. In brief, an artificial leader sequence was inserted into a mammalian expression vector, pcDNA I (Invitrogen, La Jolla, CA). The downstream of the leader sequence had an *Eco*RI cloning site followed by an ~0.6-kb cDNA fragment encoding the Fc region of human Ig G1 (Yang *et al.*, 1997, 1998a). The fragments of *Xenopus* CRD-NRG-1 were constructed by PCR with primers having an artificial *Eco*RI site at both ends. The PCR was carried out for 30 cycles in standard reaction mixture containing *Xenopus* CRD-NRG-1 cDNAs, 2.5 units *Vent* DNA polymerase, and 0.3 μg primers. The DNA fragment was subcloned into *Eco*RI site of the modified pcDNA I vector. The modified pcDNA I containing Fc-tagged NRG-1 cDNA was transfected into human embryonic kidney fibroblast (HEK) 293 cells by calcium phosphate precipitation (Sambrook *et al.*, 1989; Pun and Tsim, 1995, 1997). The Fc-tagged fusion protein was purified by protein G column as described before (Yang *et al.*, 1997). The set of primers used for amplifying the EGF-like domain (G_{209}–K_{270}) was 5′-CGG AAT TCC GGT CCA CCT TTA TTA AG-3′ (sense; 1154–1170) and 5′-TG GAA TTC TTT TTG GTA CAA CTC-3′ (antisense; 1339–1323). The set of primers used for amplifying the CRD (C_{54}–G_{108}) was 5′-CGG AAT TCC TGT CTT GAG GCA GAC-3′ (sense; 689–703) and 5′-TG GAA TTC GCC AGG GTC CAA ATG-3′ (antisense; 853–839). The set of primers used for amplifying the CRD plus the EGF-like domain (C_{45}–K_{270}) was 5′-CGG AAT TCC TGT CTT GAG GCA GAC-3′ (sense; 689–703) and 5′-TG GAA TTC TTT TTG GTA CAA CTC-3′ (antisense; 1339–1323). The set of primers used for amplifying the Ig-like domain of Ig-NRG-1 (E_{49}–T_{128}) was 5′-GAG GGA AAA AAG-3′ (sense; 304–316) and 5′-GGT CAC GGT GTC A-3′ (antisense 554–542).

FIG. 7. Expression of NRG-1 mRNA during embryo development. Localization of CRD-NRG-1 and Ig-NRG-1 mRNAs was compared by whole-mount *in situ* hybridization. Specific probes either for CRD or for Ig-like domain were used as shown in Fig. 2A. (A, A′) A stage 22 embryo. The mRNA was located in spinal cord (Sp) and brain (Br). (B, B′) A stage 22 embryo but with a dorsal view. (C, C′) A stage 25 embryo with lateral view. (D, D′) A stage 25 embryo with dorsal view. (E, E′) Lateral view of a stage 32 embryo. Prominent labeling was found in myotomes (M) and eye (Ey). (F, F′) Dorsal view of a stage 32 embryo. (G, G′) Stage 25 embryo hybridized with the sense probes. Scale bar, 0.5 mm.

RNA Isolation and RT-PCR

Total RNA from the collected tissues was prepared using the LiCl method (Chirgwin *et al.*, 1979). Total RNA from cultured myotubes was isolated using the Micro RNA Isolation Kit (Stratagene). RNA concentration and purity were determined by ultraviolet absorbance at 260 nm. In RT-PCR analysis, 5 μg of total RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Grand Island, NY) by random oligonucleotide priming in a 20-μl reaction. One-fifth of the reverse transcription product was used as a template in PCR analysis with primers described below. PCR was carried out for 30 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min in a 25-μl volume containing 0.8 mM dNTPs, 1× PCR buffer, and 0.625 U of *Taq* polymerase (GIBCO BRL). PCR products were analyzed in a 12% polyacrylamide gel (Sambrook *et al.*, 1989) and directly cloned into pCR II vector (Invitrogen). The identity of the cloned PCR products was confirmed by DNA sequencing. The PCR primers were designed according to *Xenopus* CRD-NRG-1 cDNA sequence. A set of primers flanking the EGF-like domain, S-1, 5′-GGT CAC CTT ATT AAG TG-3′ (sense; 1154–1170), and AS-1, 5′-TTT TTG GTA CAA CTC CT-3′ (antisense; 1339–1323), and a set of primers flanking the spacer domain, S-2, 5′-AG GCT GCA GTT ACA TTC-3′ (sense; 957–973), and AS-2, 5′-ACA CTC TCC TCC ATT GAC ACA-3′ (antisense; 1213–1193), were used.

Northern Blot and Southern Blot Analysis

RNA samples were fractionated on a 1% formaldehyde gel. Ethidium bromide was used to assess the equivalency of loading among different samples. After electrophoresis, samples were transferred to a charged nylon membrane (Hybond-N; Amersham, UK) and were UV cross-linked. Blots were hybridized with a CRD-NRG-1-specific cDNA fragment that encodes the CRD (from nucleotide 180 to 972) or an ~1.2-kb chick AChR α-subunit cDNA (Tsim *et al.*, 1992; Pun *et al.*, 1997). Probes were labeled with [α-^{32}P]dCTP, and the hybridization was performed at 42°C overnight in 40% deionized formamide, 5× Denhardt's solution, 0.5% SDS, 5× SSC, 10% dextran sulfate, and 0.1 mg/ml denatured salmon sperm DNA. After hybridization, the filters were washed twice with 2× SSC and 0.1% SDS at room temperature for 30 min each and then twice with 0.1× SSC with 0.1% SDS at 55°C for 30 min each. The washed filters were exposed to X-ray film with double intensifying screens at −80°C.

In Southern blot analysis, genomic DNA was isolated from *Xenopus* muscle by CTAB buffer (2% hexadecyltrimethyl ammonium bromide, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0) and 0.1 mg/ml protease K. After incubation at 60°C for 3–5 h, the lysate was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) twice. The aqueous phase was taken out and 2/3 volume of isopropanol was added to precipitate the genomic DNA. The air-dried DNA pellet was then dissolved in Tris–EDTA buffer. The genomic DNA (30 μg) was digested to completion with the restriction enzymes indicated in Fig. 2. DNA fragments were separated on a 0.8% agarose gel, transferred to nylon membranes, and probed with ^{32}P-labeled cDNAs encoding different domains of NRG-1. Hybridization conditions were as described for the isolation of cDNAs. Filters were exposed to X-ray films overnight.

Cell Culture

Primary chick myotube cultures were prepared from the hindlimb muscles dissected from E11 chick embryos according to a modified protocol (Wallace, 1989; Pun and Tsim, 1997). Muscle cells were cultured in MEM supplement with 10% heat-inactivated horse serum, 2% chick embryo extract, 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The C2C12 myoblasts were maintained in DMEM supplemented with 10% heat-inactivated horse serum, 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated at 37°C in a 5% CO_2 humidified incubator, and the medium was replaced every 3 to 4 days. The C2C12 myoblasts were induced to fuse at confluent stages by reducing the serum to 2% (Si *et al.*, 1996; Yang *et al.*, 1998a). In AChR α-subunit induction assay, the chick myotubes were treated with NRG-1 overnight, and total RNA was collected from the treated chick myotubes. In the phosphorylation studies, fused C2C12 myotubes were treated with NRG-1 for 30 min, and then cells were collected for immunoprecipitation assay.

Immunochemical and Precipitation Analysis

In the phosphorylation studies, C2C12 myotubes were treated with purified recombinant NRG-1 for 30 min. The treated cells were lysed in RIPA buffer (PBS, pH 7.4, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM orthovanadate, 1 mM aprotinin). The ErbB-3 receptors were immunoprecipitated by an antibody against ErbB-3 (C17; Santa Cruz Biotech, Santa Cruz, CA) in 1:1000 dilution. The immunoprecipitated proteins were collected on protein G-agarose beads (Boehringer Mannheim, Indianapolis, IN) and fraction-

ated by 7.5% SDS–PAGE (Yang *et al.*, 1997). Electrophoresed proteins were transferred onto nitrocellulose membrane. The membrane was blocked with 2.5% dry milk containing 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, for 1 h at 37°C, and incubated with horseradish peroxidase-conjugated anti-tyrosine phosphorylation antibody RC 20 (Transduction Laboratories, Lexington, KY) diluted 1 in 1000. Immunoreactivity was detected by ECL Western Blot System (Amersham, UK) in accordance with the instructions from the supplier.

In the precipitation of NRG-1, the conditioned medium from transfected HEK293 cells was precipitated for 30 min by 1:500 dilution of anti-NRG-1 antibody (Transduction Laboratories; Yang *et al.*, 1998a) and by protein G- or heparin-agarose beads (Sigma, St. Louis, MO). The treated conditioned medium was tested for AChR-inducing activity in the AChR α-subunit promoter induction assay, while the precipitated proteins in the beads were analyzed by immunoblotting.

Induction of AChR α-Subunit Promoter

The pnlacZ plasmid containing 850-bp chick AChR α-subunit promoter tagged with β-galactosidase gene was described in Sanes *et al.* (1991) and provided by Dr. Joshua Sanes from the Washington University School of Medicine. The cDNA was transfected into 2-day-old chick myotube cultures by using calcium phosphate precipitation (Sambrook *et al.*, 1989; Pun *et al.*, 1997). Two days after transfection, the myotubes were treated with either purified Fc-tagged NRG-1s or the conditioned medium from NRG-1 cDNA-transfected cells for 36–48 h. The β-galactosidase activity was measured following Sambrook *et al.* (1989). In brief, the cells from one 100-mm plate were lysed in 100 ml 0.25 M Tris–HCl (pH 7.8). The cells were thawed and frozen between dry ice/ethanol and 37°C several times. The cell extract was assayed in a solution containing 1 mM $MgCl_2$, 45 mM β-mercaptoethanol, 0.9 mg/ml *o*-nitrophenyl-β-D- galactopyranoside and 0.66 M sodium phosphate (pH 7.5). The assay mixture was incubated at 37°C for 30 min and stopped by adding 500 μl 1 M Na_2CO_3. The activity was determined by reading the optical density at 420 nm.

In Situ Hybridization

Whole-mount *in situ* hybridization was conducted as described (Harland, 1991; Yang *et al.*, 1998a). Albino *Xenopus* embryos were collected at different stages and fixed in MEMFA (Mops 0.1 M, pH 7.4, EGTA 2 mM, $MgSO_4$ 1 mM, formaldehyde 3.7%) for 2 h and stored in absolute methanol at −20°C. Before hybridization, embryos were rehydrated by 5-min washes in 75% methanol:25% diethyl pyrocarbonate (DEPC)-treated water, 50% methanol:50% DEPC water, and 25% methanol:75% PBS–Tween (1× PBS+0.1% Tween); rinsed twice for 5 min each in 0.1 M triethanolamine (Sigma); and rinsed in 0.1 M triethanolamine with 12 ml acetic anhydride (Sigma) for 5 min, and then another 12.5-ml acetic anhydride was added. Embryos were refixed in 4% paraformaldehyde for 20 min, washed 5 × 5 min in PBS–Tween, and replaced with hybridization buffer (50% formamide, 5× SSC, 1 mg/ml *Torula* RNA, 100 μg/ml heparin, 1× Denhardt's, 0.1% Tween 20, 0.1% Chaps, 10 mM EDTA). This prehybridization step was carried overnight at 60°C. The solution was then replaced with 0.5 ml probe solution (hybridization buffer with 0.1 μg/ml probe) and the incubation was carried out at 60°C overnight. The digoxigenin-labeled *Xenopus* NRG-1 cRNA probe was synthesized in the antisense direction using SP6 RNA polymerase with the Riboprobe In Vitro Transcription kit according to manufacturer's protocol (Promega, Madison, WI). Digoxigenin-11–UTP was purchased from Boehringer Mannheim. After hybridization, the embryos were placed into hybridization buffer without probe for 10 min at 60°C, washed two times in 2× SSC for 20 min each at 37°C and once in 2× SSC with RNase A (20 μg/ml) and RNase T1 (10 units/ml) for 30 min at 37°C, and rinsed in 2× SSC and 0.2× SSC for 30 min at 60°C. They were then washed four times in maleic acid buffer (MAB; 100 mM maleic acid, 150 mM NaCl, pH 7.5) at room temperature and in MAB with 20% fetal bovine serum (FBS) at room temperature for 1 h. The solution was replaced with fresh MAB containing 20% FBS and a 1:2000 dilution of anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim), and the incubation was carried out overnight at 4°C on a rocking platform. Embryos were washed at least five times, 1 h each at room temperature with MAB to remove excess antibody. For chromogenic reaction, embryos were washed twice with alkaline phosphatase buffer (AP buffer; 100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 5 mM levamisol) and then treated with AP buffer containing 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate 4-toluidine salt. The chromogenic reaction was stopped when satisfactory signal-to-background ratio was achieved by replacing the AP buffer with MEMFA. Before microscopic examination, the embryos were cleared with a 2:1 mixture of benzyl benzoate:benzyl alcohol in a glass dish. For the control, another set of embryos was hybridized with digoxigenin-labeled *Xenopus* NRG-1

cRNA probe synthesized in the sense direction using T7 RNA polymerase and processed in the same fashion.

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