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Identification of Genes Induced by Neuregulin in Cultured Myotubes

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The formation of the neuromuscular junction (NMJ) involves a series of inductive interactions between motor neurons and muscle fibers. The neural signals proposed to induce the mRNA expression of acetylcholine receptors in muscle include neuregulin (NRG). In the present study, we have employed RNA fingerprinting by arbitrarily primed PCR analysis to identify the differentially expressed transcripts following NRG treatment in cultured myotubes. Nine partial cDNA fragments were isolated; the mRNA expression of eight of these genes was found to be up-regulated by NRG. The spatial and temporal expression profiles of these NRG-regulated genes in rat tissues during development suggest potential functional roles during the formation of NMJ *in vivo.* Our findings not only allowed the identification of novel genes, but also suggested possible functions for some known genes that are consistent with their potential roles at the NMJ. Furthermore, the identification of G-protein β1 subunit and G-protein-coupled receptor as NRG-regulated genes has provided the first demonstration that activation of the NRG signaling pathway can induce the expression of components in the G-protein signaling cascade.

INTRODUCTION

The formation of the neuromuscular junction (NMJ) involves a series of dynamic changes that occur at both pre- and postsynaptic cells. Anterograde signals from motor neurons act on their target muscle cells to initiate the process of postsynaptic specialization. One of the important processes involved in postsynaptic differentiation is the regulation of the synapse-specific genes at the subsynaptic regions during NMJ formation. There are at least two controlling mechanisms: electrical stimulation of the motor nerves suppresses the expression of genes in the extrasynaptic regions, while one of the nerve-derived factors, acetylcholine receptor-inducing activity (ARIA), up-regulates the synapse-specific genes at the subsynaptic regions (Apel and Sanes, 1995).

ARIA is a 42-kDa protein released from motor neurons which can regulate the transcription of genes encoding acetylcholine receptor (AChR) subunits in muscle fibers (Usdin and Fischbach, 1986). ARIA is the product of the *nrg-1* gene that encodes different proteins, including heregulins (Holmes *et al.,* 1992), glial growth factor (Marchionni *et al.,* 1993), neu differentiation factor (Wen *et al.,* 1992), and sensory- and motor-derived factor (Ho *et al.,* 1995). Different isoforms of these proteins, result by alternative mRNA splicing from a single gene. They are collectively referred to as neuregulins (NRGs; Fischbach and Rosen, 1997); NRG therefore designates transcripts or proteins encoded by the *nrg-1* gene irrespective of species or isoforms (Meier *et al.,* 1998). All members in the NRG family are characterized by a conserved EGF-like domain encoding a peptide that is sufficient for the functional activity of NRGs (Loeb and Fischbach, 1995; Yang *et al.,* 1997).

Several lines of evidence indicate that the regulation of gene expression of postsynaptic proteins by the motor nerve is mediated, at least in part, by NRGs. The expression of NRGs in the motor neurons is confined to the synaptic terminals. Upon release from motor neurons, NRGs are deposited at the basal lamina to trigger the synthesis of synapse-specific genes, such as AChRs and sodium channels (Corfas and Fischbach, 1993; Jo *et al.,* 1995). Recent gene targeting studies support the notion that NRG plays an important role in the induction of synapse-specific gene expression. Mutant mice that lack NRG expression die of cardiovascular malformations by embryonic day 11.5, prior to the stage of NMJ formation as well as muscle development (Meyer and Birchmeier, 1995; Lemke, 1996). A significant reduction of AChRs (~50%) was observed in heterozygous mice, suggesting that NRG is a neural signal that regulates AChR gene transcription *in vivo* (Sanes, 1997).

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Receptors that mediate the actions of NRGs are members of the epidermal growth factor receptor-related (ErbB) family of tyrosine kinases, including ErbB2, ErbB3, and ErbB4. NRGs and the three ErbB receptors have been reported to be localized and enriched at the neuromuscular endplates (Moscoso *et al.*, 1995). Although NRGs were first identified based on their ability to activate ErbB2, they are not direct ligands for ErbB2. The other receptors, ErbB3 and ErbB4, show high affinity to NRGs but ErbB3 has little tyrosine kinase activity. NRG stimulates the tyrosine phosphorylation of ErbB receptors via a ligand-activated receptor homodimerization or heterodimerization mechanism (Carraway and Burden, 1995). The activation of signaling cascades involving kinases such as ras, raf, erk, mitogen-activated protein (MAP) kinase, and phosphatidylinositol 3-kinase leads to changes in the expression of specific genes (Si *et al.*, 1996; Tansey *et al.*, 1996). The detailed molecular mechanism underlying the action of NRG, however, remains to be elucidated.

As part of an effort to dissect the signaling molecules that mediate the actions of NRG, we have employed the technique of RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) to identify candidate genes that are regulated by NRG. Based on this analysis, eight differentially expressed transcripts and one constitutive transcript were identified in C2C12 myotube culture upon treatment with recombinant NRG. Studies on the developmental expression profile of some of these genes suggest potential functions during NMJ formation and provide insight into our understanding of the molecular mechanism involved in synapse formation.

RESULTS

Identification of NRG-Regulated Genes in Myotube Culture by RNA Fingerprinting

To identify the genes that were transcriptionally regulated by NRG, RNA fingerprinting was performed using total RNA collected from C2C12 myotube culture after treatment with recombinant NRG-β1 (4 nM). The functional activity of recombinant NRG was assessed by examining the up-regulation of AChRε and AChRα mRNA expression in C2C12 myotube and primary chick muscle culture, respectively (Fig. 1). Induction of mRNA expression of AChRs in both muscle cultures was observed after treatment with recombinant NRG.

Total RNA prepared from C2C12 myotube culture following treatment with or without recombinant NRG-β1 (4 nM) for 48 h was subjected to the RAP-PCR analysis. RAP-PCR consists mainly of a reverse transcription and two polymerase chain reactions. The first PCR allows the generation of fingerprints from the samples by low-stringency amplification, while the subsequent high-stringency PCR cycles allow the amplification of the fingerprints resulted from the first amplification (for details, see Experimental Methods). RAP-PCR analysis was performed on RNA samples with two dilutions (50

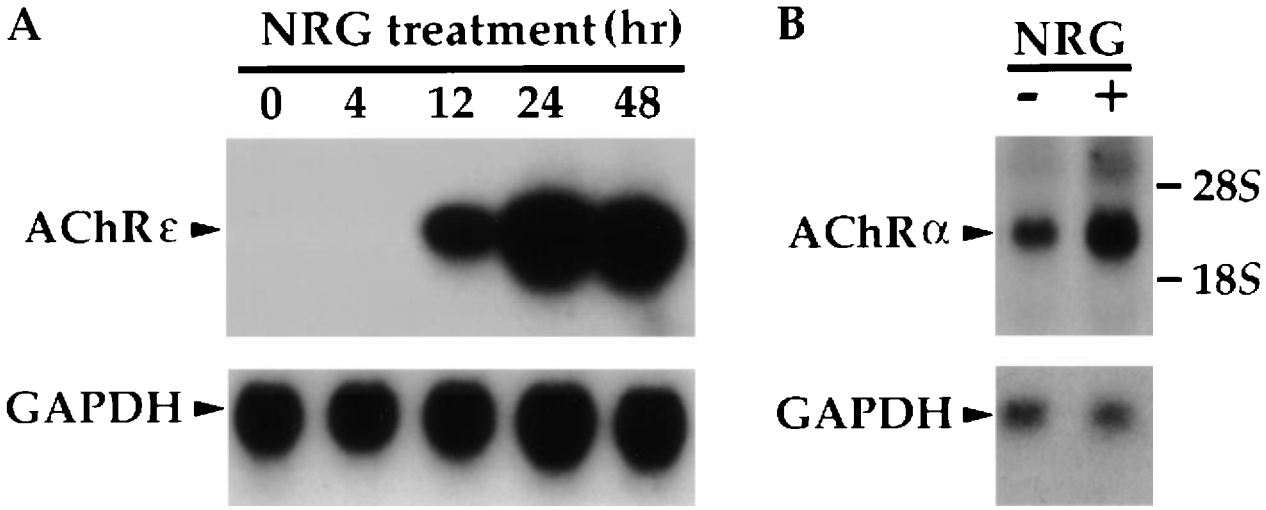

FIG. 1. Recombinant NRG increased the mRNA expression of AChRs in C2C12 myotube and chick primary muscle culture. Total RNAs were collected after treatment with NRG-β1 (0–48 h for cultured C2C12 myotubes; 48 h for chick primary muscle culture). (A) RT-PCR analysis of AChRε expression (top) and GAPDH (bottom) in C2C12 myotubes. (B) Northern blot analysis of AChRα (top) and GAPDH (bottom) in chick primary muscle. Ribosomal RNA bands (18S and 28S) are indicated on the right.

and 150 ng). Only differentially expressed bands reproducibly obtained in both dilutions were used for subsequent analysis. One constitutively expressed fragment (clone 19.4, see below) was selected for further analysis to serve as a control for the equal loading of RNA.

Analysis of Differentially Expressed Transcripts Following NRG Treatment

Based on the results of RAP-PCR analysis, a total of nine cDNA fragments were cloned. Eight of these genes showed differential expression profile following NRG treatment while the constitutively expressed gene served as control. The identity of these cDNA fragments was inferred by comparison with known genes in the database (summarized in Table 1). The identified genes could be classified according to their cellular functions. Two skeletal muscle structural proteins identified include clone 10.15, which shares 100% amino acid identity with rat fibronectin, and clone 13.3, which encodes mouse skeletal muscle β-tropomyosin. Two signaling regulators were identified, including clone 9.2 that shares 100% amino acid identity with rat G-protein β1 subunit ($G\beta_1$) and clone 10.7 that encodes mouse protein tyrosine phosphatase (SHP-2). Clone 11.1, sharing 100% amino acid identity with rat ribophorin I, was also identified. Two membrane-bound receptors were found to be induced by NRG, i.e., clone 7.7 that shares 100% amino acid identity with rat *N*-methyl-D-aspartate (NMDA) receptor glutamate-binding subunit (GBP) and clone 26.1 that encodes a G-protein-coupled receptor (GPCR), RDC-1. Finally, two novel clones, 8.6 and 19.4, which show no homology with known genes, were identified.

TABLE 1
Neuregulin-Regulated Genes Identified by RAP-PCR Analysis

Clone	Expression profile	Gene identity
7.7	Up-regulated	NMDA receptor glutamate binding subunit (GBP)
8.6	Up-regulated	Novel
9.2	Up-regulated	G-protein β subunit ($G\beta_1$)
10.7	Up-regulated	Phosphotyrosine phosphatase (SHP-2)
10.15	Up-regulated	Fibronectin
11.1	Up-regulated	Ribophorin I
13.3	Up-regulated	β-Tropomyosin
19.4	Constitutively expressed	Novel
26.1	Up-regulated	G-protein-coupled receptor, RDC-1

Note. The mRNA expression profile of C2C12 myotubes following neuregulin treatment was obtained using Northern blot analysis. Gene identity of RAP-PCR clones was inferred by comparison of the deduced amino acid sequence of cloned cDNA fragments with known genes as mentioned under Experimental Methods.

Northern Blot Analysis of the Differentially Expressed cDNA Fragments in C2C12 Myotubes

The mRNA expression profile of the cloned cDNA fragments in C2C12 myotube culture was examined by Northern blot analysis. Two transcripts (~11.5 and 9 kb) for clone 10.15 (fibronectin), and two transcripts (~2.2 and ~1.4 kb) for clone 13.3 (skeletal muscle β-tropomyosin), were detected in control C2C12 myotube; these transcripts were prominently up-regulated after NRG treatment (Figs. 2A and 2B).

Northern blot analysis of clone 9.2 ($G\beta_1$) and clone 10.7 (SHP-2) revealed an up-regulation of mRNA expression following NRG treatment. One major transcript of ~3.5 kb for clone 9.2 was detected in C2C12 myotube RNA and ~10-fold induction was observed following NRG treatment (Fig. 2C). For clone 10.7, one major transcript (~7 kb) was detected in C2C12 myotube and was up-regulated by NRG (Fig. 2D). Similarly, the transcript (~2.5 kb) for clone 11.1 (ribophorin I) was detected in C2C12 myotube RNA and induced following NRG treatment (Fig. 3A).

Increased expression of the two membrane receptors was observed following NRG treatment. Multiple transcripts (~7, 4, and 1.8 kb) for clone 7.7 (GBP) were detected in C2C12 myotube; the expression was induced following NRG treatment (Fig. 3B). It is noteworthy that GBP transcript was prominent in the brain. An increase (~twofold) in the mRNA expression for clone 26.1 (RDC-1; transcripts of 3, 2, and 1.5 kb) was detected in C2C12 myotube after 2 days of NRG treatment (Fig. 3C). Prominent expression for RDC-1 could be detected in brain and muscle.

Three transcripts (~10, 4, and 2.3 kb) for the novel clone 8.6 were detected in C2C12 myotube; induction was observed after treatment with NRG for 2 days (Fig. 3D). The expression of this novel gene was also detected in rat adult brain and muscle but not liver. The mRNA expression of another novel clone, 19.4, was not regulated in myotube following NRG treatment (Fig. 4). This novel gene served as a control for the equal loading of RNA.

Northern Blot Analysis of Some of the Differentially Expressed cDNA Fragments in Chick Myotubes

The mRNA expression profiles of several NRG-induced genes identified in C2C12 myotubes were

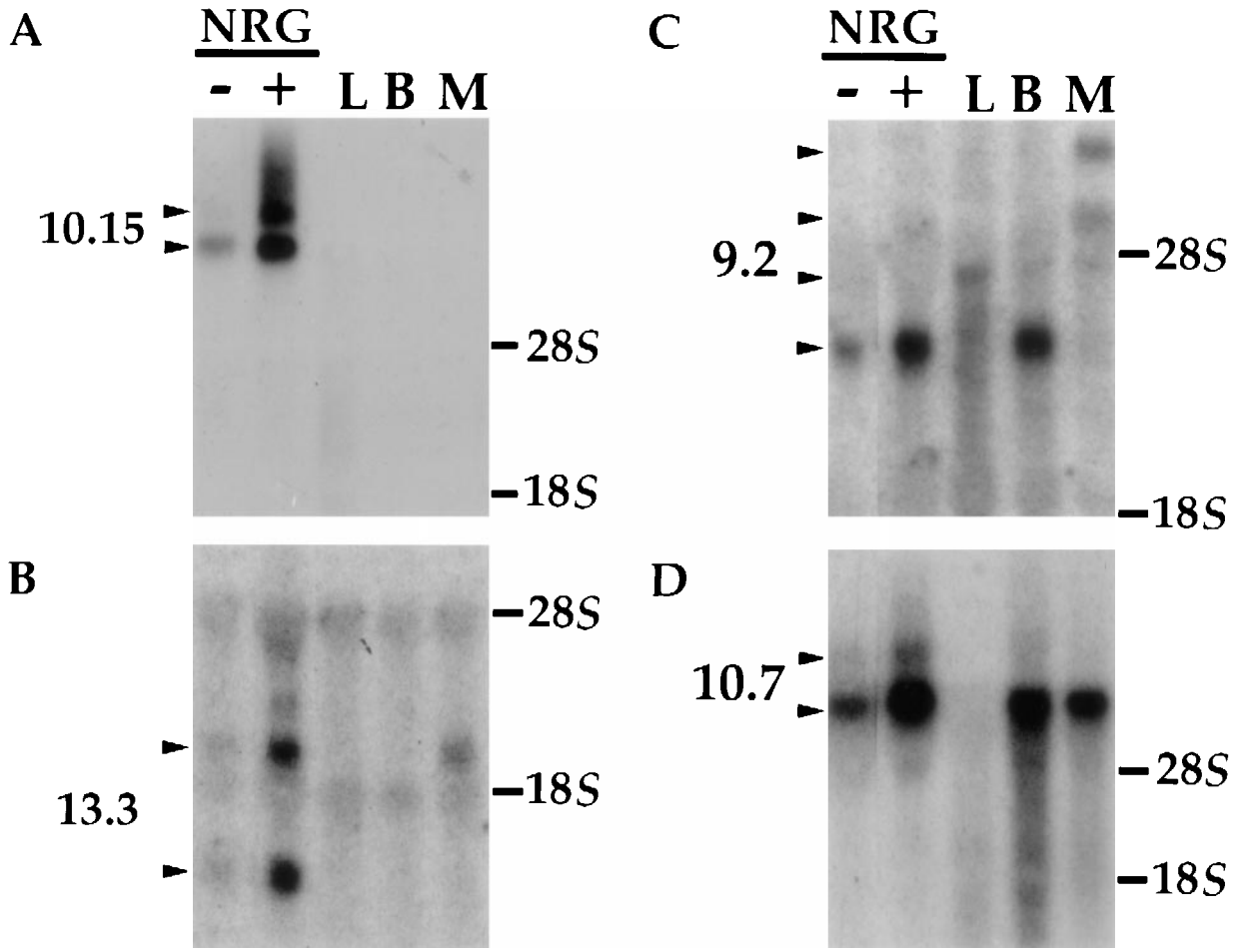

FIG. 2. Northern blot analysis for RAP-PCR clones 10.15, 13.3, 9.2, and 10.7 following NRG treatment of C2C12 myotubes. Clone 10.15 encodes fibronectin (A), clone 13.3 encodes skeletal muscle β-tropomyosin (B), 9.2 encodes $G\beta_1$ subunit (C), and 10.7 encodes SHP-2 (D). L, rat liver; B, brain; M, muscle. Arrowheads indicate the detectable transcripts encoded by the gene. Ribosomal RNA bands (18S and 28S) are indicated on the right.

examined in primary chick muscle culture. The transcripts corresponding to clones 10.7, 10.15, 13.3, and 8.6 could be detected in primary chick muscle culture using mouse cDNA fragments that were cloned based on the RAP-PCR analysis described above. Increased mRNA expression of all four clones following NRG treatment was also observed in primary chick muscle culture (Fig. 5).

Developmental Expression of the NRG-Regulated Genes in Different Rat Tissues

To further investigate the functional roles of the NRG-regulated candidate genes in postsynaptic differentiation events, developmental expression of these genes was examined in rat muscle, brain, and liver. Three transcripts were detected by the partial cDNA fragment encoding β-tropomyosin in rat muscle during development (Fig. 6). Differential regulation of these transcripts was observed: expression of the ~2.2-kb transcript increased along the course of development while the ~1.4-kb transcript increased during the early postnatal stages (P1 and P7) and then down-regulated until adult (Fig. 6). Interestingly, while the ~2.2-kb transcript could not be detected in brain, it could be detected in liver during early embryonic development. Taken together, these findings suggest that the isoforms encoded by β-tropomyosin may play different functional roles in muscle development and/or NMJ formation.

Prominent expression of $G\beta_1$ and SHP-2 was observed in rat muscle during the stages of embryonic

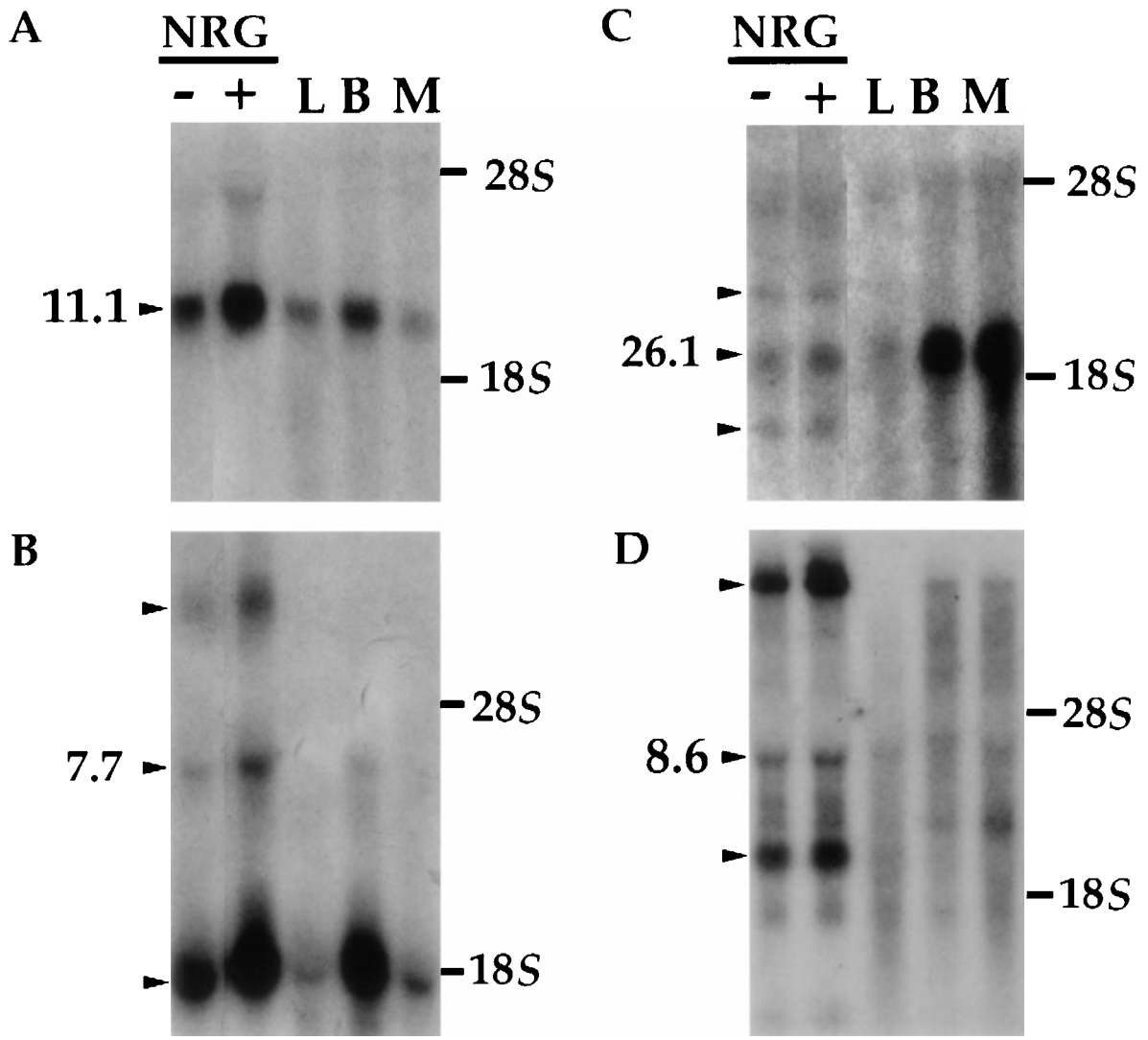

FIG. 3. Northern blot analysis for RAP-PCR clones 11.1, 7.7, 26.1, and 8.6 following NRG treatment of C2C12 myotubes. Clone 11.1 encodes ribophorin I (A), clone 7.7 encodes GBP (B), clone 26.1 encodes RDC-1 (C), and clone 8.6 encodes a novel gene (D). L, rat liver; B, brain; M, muscle. Arrowheads indicate the detectable transcripts encoded by the gene. Ribosomal RNA bands (18S and 28S) are indicated on the right.

development that coincide with the period of NMJ formation. The expression of $G\beta_1$ in muscle decreased after birth and remained at low level until adult (Fig. 7). For SHP-2, mRNA expression was down-regulated from postnatal day 14 to day 21 (corresponding to the period of synapse elimination), was induced at later postnatal stages, and remained at high level in adult muscle (Fig. 8). Interestingly, a similar expression pattern for both genes, i.e., prominent expression during early embryonic development, was observed in both brain and liver (Figs. 7 and 8). On the other hand, the NMDA receptor GBP showed a ubiquitous expression pattern in rat tissues; the level of transcript slightly increased in muscle during early postnatal stages (P1–P14) and the opposite expression profile was observed in liver along the course of development (Fig. 9). It is noteworthy that the expression of GBP was significantly up-regulated in the brain during postnatal stages. The results obtained for GAPDH as a control gene and EtBr-stained gels are also depicted in Fig. 9.

DISCUSSION

In the present study, we have identified genes that are potentially involved in NRG signaling in muscle using

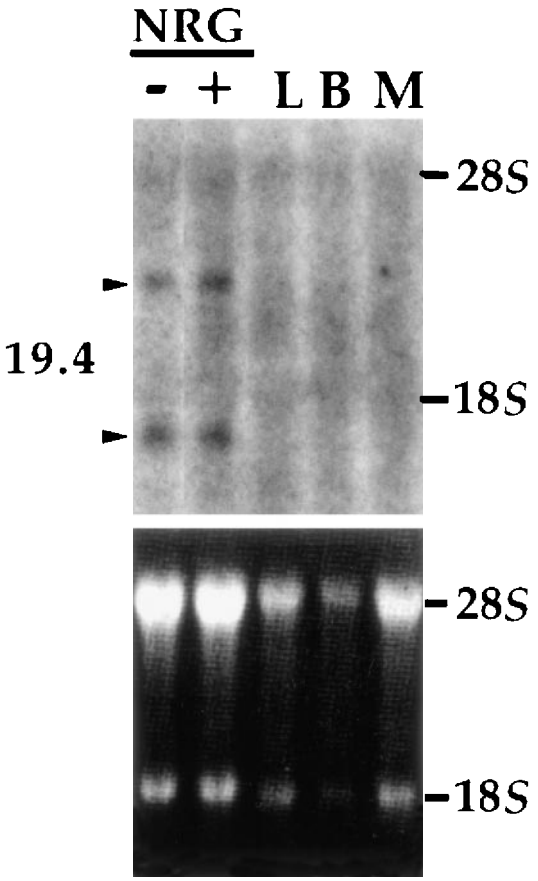

FIG. 4. Clone 19.4 was constitutively expressed in C2C12 myotubes following NRG treatment. Equal loading of RNA for C2C12 myotubes was depicted in the ethidium bromide-stained gel (bottom). L, rat liver; B, brain; M, muscle. Arrowheads indicate the detectable transcripts of clone 19.4. Ribosomal RNA bands (18S and 28S) are indicated on the right.

RAP-PCR analysis. This type of analysis has been used to isolate a number of differentially expressed genes in a variety of experimental systems (reviewed in McClelland *et al.*, 1995; Cheung *et al.*, 1997; Schweitzer *et al.*, 1998). We report here that a total of nine cDNA fragments were cloned and analyzed based on the RNA fingerprints of differentially expressed transcripts following NRG treatment of cultured muscle cells. Two novel genes have been identified in this study. Most importantly, we have provided evidence that the expression of several known genes, such as β-tropomyosin, fibronectin, SHP-2, and GBP, are induced by NRG in cultured myotubes. Furthermore, the identification of $G\beta_1$ and GPCR as NRG-regulated genes has provided the first demonstration that activation of the NRG signaling pathway can induce the expression of components in the G-protein signaling cascade. Taken together, our findings suggest that these NRG-induced genes are involved in NRG signaling and may play important roles in muscle development and NMJ formation.

While not much is known about the signaling cascade downstream of NRG-activated ErbB receptors, some of the signaling molecules are likely to be analogous to those activated by epidermal growth factor, a ligand that also utilizes receptors of the ErbB family. For example, similar to the EGF-activated signaling cascade, recent studies have demonstrated that MAP kinase is activated upon NRG stimulation of target cells (Si *et al.*, 1996; Tansey *et al.*, 1996). The protein tyrosine phosphatase SHP-2, acting upstream of MAP kinase, is a positive mediator of EGF signaling and plays a critical role in EGF-induced responses (Xiao *et al.*, 1994). SHP-2 is able to dephosphorylate the inhibitory phosphotyrosine sites and allows the activation of downstream tyrosine kinases (Feng *et al.*, 1993). Recently, it has been shown that SHP-2 is also required for NRG-stimulated MAP kinase activation and represents a converging point in signaling subsequent to ligand-stimulated activation of the ErbB family of receptors (Deb *et al.*, 1998). Our finding on the NRG-induced expression of SHP-2 in cultured myotubes provides additional evidence that this phosphatase plays an important role in NRG signaling subsequent to the activation of ErbB receptors.

Another signaling molecule, $G\beta_1$, is also up-regulated by NRG in myotube culture. $G\beta_1$ has been demonstrated to stimulate MAP kinase activation when it dimerizes with its γ2 subunit (Lopez-Ilasaca, 1998). Previous studies have revealed that the activation of MAP kinase by Gβγ is mediated by a common signaling pathway shared with receptor tyrosine kinase (RTK; van Biesen *et al.*, 1995; Daub *et al.*, 1996). Cross-talk between the signal transduction pathways of GPCR and RTK, both converging on MAP kinase activation, can result in synergistic interactions. Recent evidence indicates that overexpression of EGF receptor potentiates the MAP kinase activation induced by GPCR ligands (Buist *et al.*, 1998). Our study provides the first demonstration that the activation of the RTK signaling pathway can induce the expression of components in the G-protein signaling pathway, such as $G\beta_1$.

An additional evidence supporting the involvement of GPCR activation in the NRG signaling pathway in muscle is our identification of a GPCR as one of the NRG-regulated genes. RDC-1, an orphan chemokine receptor cloned in the present study, belongs to the superfamily of GPCR (Horuk, 1994). Previous studies suggest that RDC-1 can be activated by calcitonin gene-related peptide (CGRP) and has been proposed to be a potential candidate for the CGRP receptor (Kapas

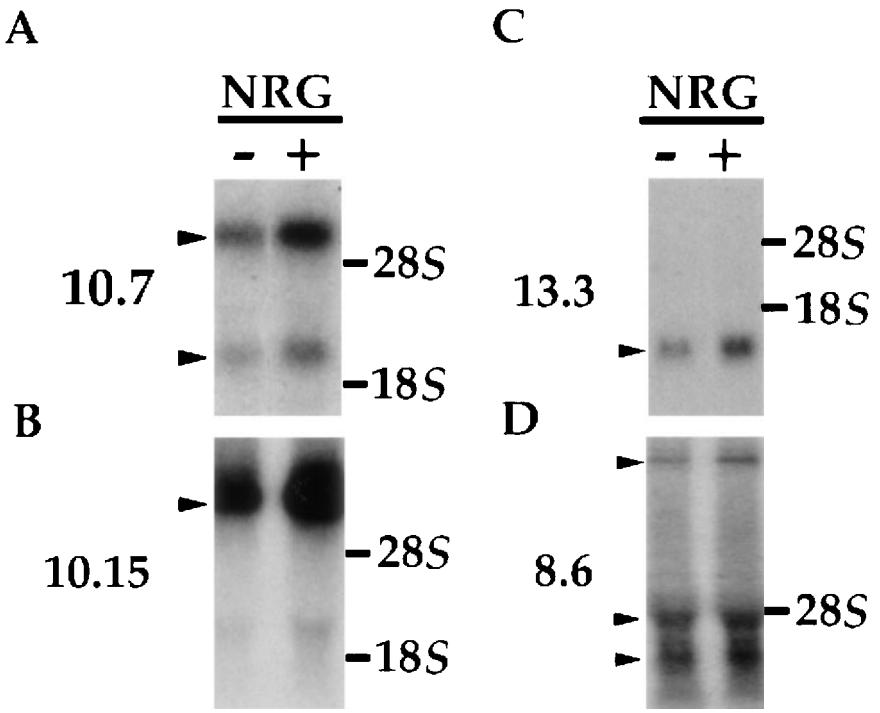

FIG. 5. NRG increases the mRNA expression of (A) clone 10.7, SHP-2; (B) clone 10.15, fibronectin; (C) clone 13.3, skeletal muscle β-tropomyosin; and (D) clone 8.6 in primary chick muscle culture. Northern blot analysis was performed with RNA prepared from primary chick myotubes after NRG treatment. Arrowheads indicate the detectable transcripts of the gene. Ribosomal RNA bands (18S and 28S) are indicated on the right.

and Clark, 1995). The ability of CGRP to stimulate the mRNA expression of AChRs in cultured myotubes (Fontaine *et al.*, 1987) together with the expression profile of this peptide suggests that CGRP is one of the neural factors that is critical in directing the formation of postsynaptic specializations. The identification of RDC-1 as a NRG-regulated gene in our study is of interest in light of a recent observation that CGRP-induced response in muscle is probably mediated by a GPCR that turns on cAMP (Choi *et al.*, 1998). It is possible that RDC-1 may well be the GPCR that mediates the action of CGRP in cultured myotubes. The NRG-induced expression of RDC-1 may provide a potential mechanism to allow for an enhanced response to CGRP and ultimately lead to synergistic cellular responses at the NMJ. Taken together, our finding raises an intriguing possibility that the two neural signals, NRG and CGRP, may indeed collaborate to bring about amplified responses, such as regulation of gene expression, in muscle.

Two membrane-bound and ion-gated channel receptors, AChRs and sodium channels, are well documented to be regulated by NRG in muscle during the formation of NMJ. A recent study demonstrated that NRG can also regulate the composition of neurotransmitter receptor in neuronal synapses in the brain, in a manner analogous to that observed at the NMJ (Ozaki *et al.*, 1997). While one of the NMDA receptor subunits, NR2C, was found to be up-regulated by NRG in the CNS, we have identified another membrane-bound receptor that can be regulated by NRG, i.e., the NMDA receptor GBP. While structurally unrelated to other subunits of glutamate receptor channels, GBP was hypothesized to be a NMDA receptor subunit involved in native NMDA receptor channels (Sato *et al.*, 1995). Our finding supports the hypothesis that NRG may regulate the NMDA receptor subunits in skeletal muscle in a manner similar to that observed in the cerebellum. The expression profile of GBP in CNS during development is consistent with the suggestion that GBP may be involved in synapse differentiation and maintenance (Schweitzer *et al.*, 1998).

It is well accepted that the assembly of a cytoskeleton specialization in postsynaptic muscle cells is an integral part of AChR aggregation and NMJ formation. Two genes encoding for structural proteins, skeletal muscle β-tropomyosin and fibronectin, are among the NRG-regulated genes identified in the present study. It is possible that β-tropomyosin may stabilize the actin filaments in skeletal muscle and that this protein may

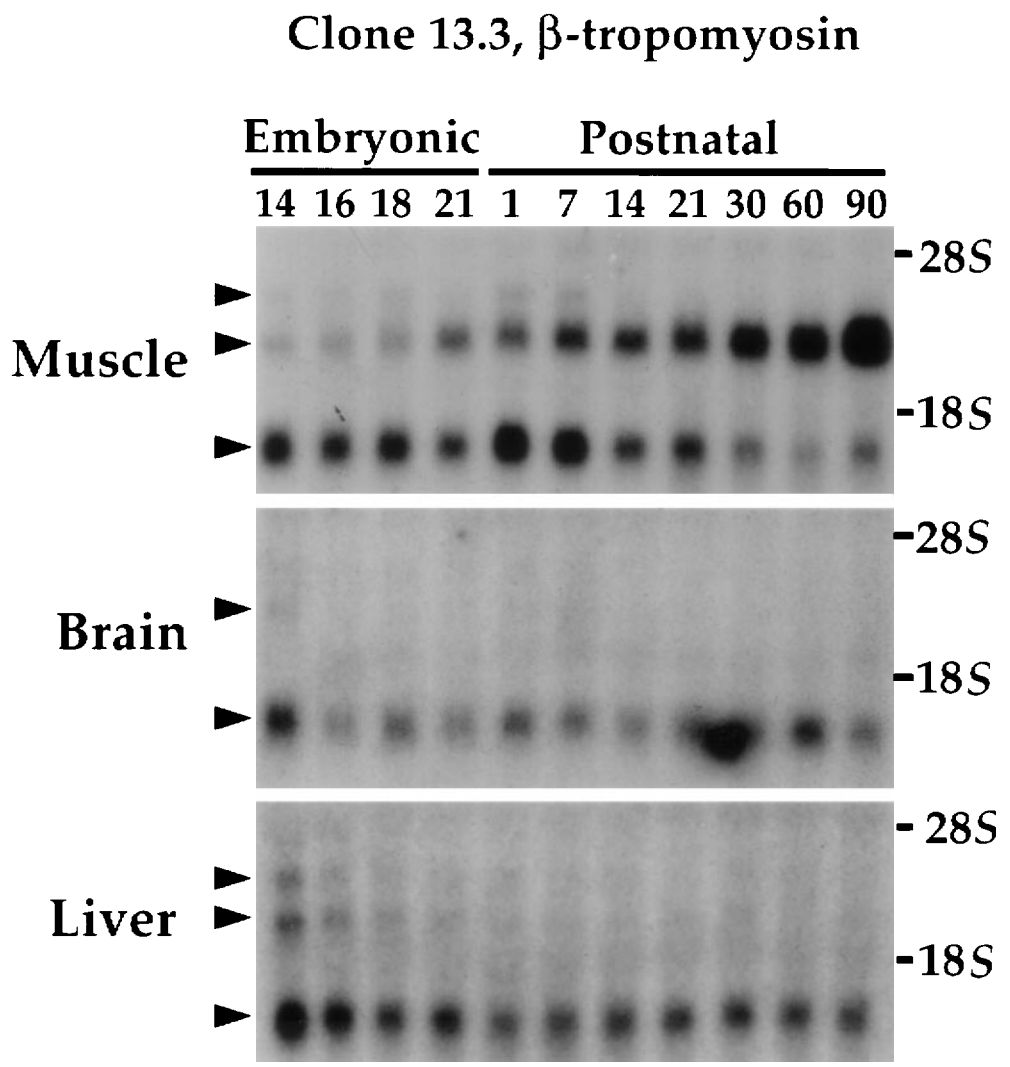

FIG. 6. Northern blot analysis of β-tropomyosin in different rat tissues during development (from embryonic day 14 to postnatal day 90). Two major transcripts (~2.2 and ~1.4 kb) were detected in rat muscle; the larger transcript was muscle specific and prominently induced during muscle development.

actively polymerize the newly formed AChR aggregates on muscle. The putative functional role of tropomyosin in AChR clustering is further supported by the cellular localization of this molecule, i.e., its concentration at the mammalian NMJs. Absence of tropomyosin was previously demonstrated to prevent the formation of new AChR aggregates on muscle (Marazzi *et al.,* 1989). Our finding is therefore consistent with the suggestion that tropomyosin is one of the proteins involved in the insertion of newly formed AChRs into muscle. The other structural protein identified in our study, fibronectin, may have similar functions in enhancing the stabilization of the scaffold of AChRs in muscle. Ribophorin I, one of the NRG-regulated genes, has been demonstrated to enhance protein synthesis by incorporating the newly synthesized protein to the endoplasmic reticulum (Yu *et al.,* 1990). It is possible that ribophorin I is involved in NRG signaling to enhance the synthesis and presentation of AChR subunits and sodium channels on muscle membrane.

RAP-PCR analysis provides a useful approach to identify differentially expressed genes that are involved in many cellular responses. As demonstrated in the present study, this type of analysis can also reveal the unexpected roles for some candidate proteins that are known for their roles in other physiological functions. The panel of NRG-regulated genes identified indicates the complexity of the NRG-mediated signaling cascade that ultimately leads to well-orchestrated responses in muscle cells. Dissecting the precise functions subserved

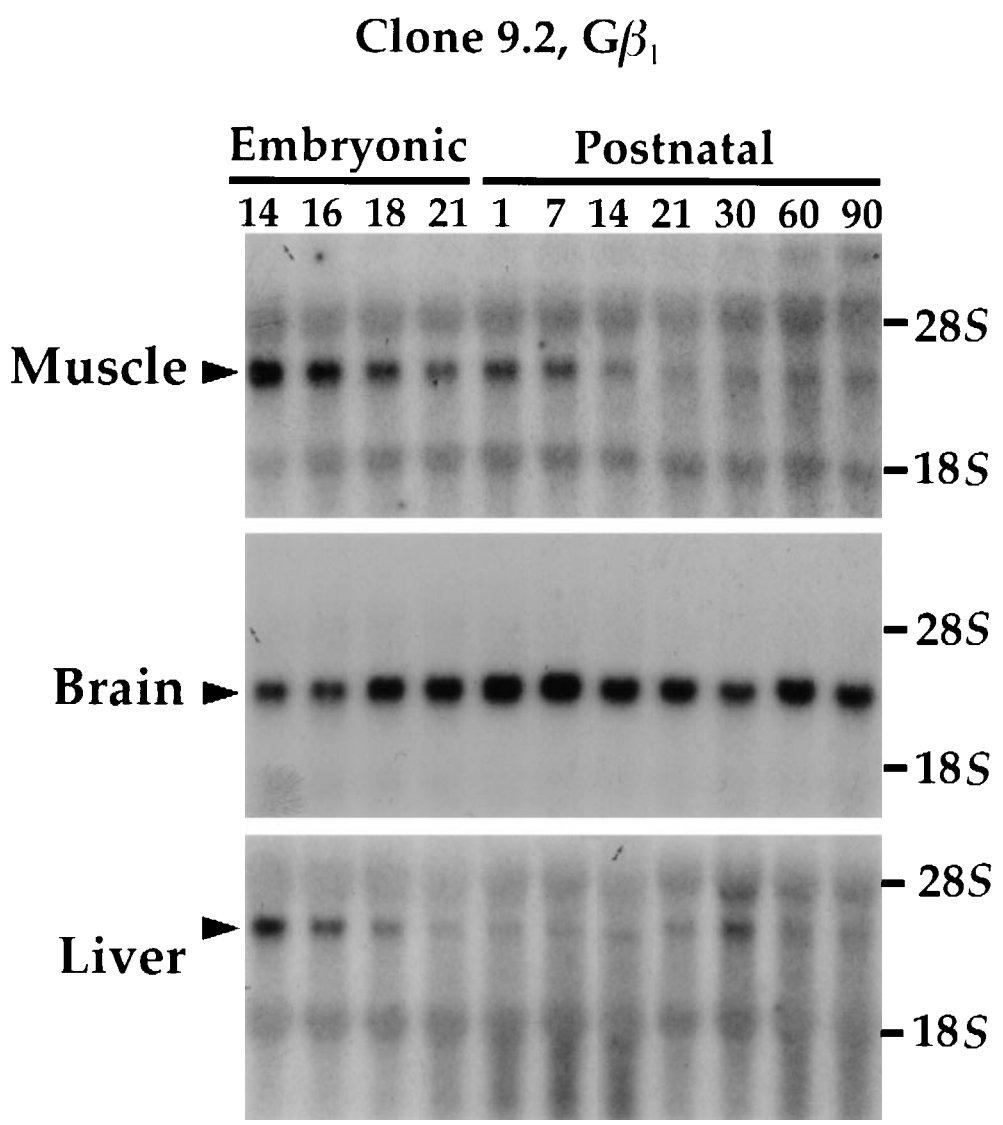

FIG. 7. Northern blot analysis of $G\beta_1$ in different rat tissues during development (from embryonic day 14 to postnatal day 90). $G\beta_1$ mRNA was detected in embryonic muscle, decreased after birth, and remained at low level until adult.

by these genes, and identifying other NRG-regulated genes, should facilitate our understanding of the molecular mechanism underlying NRG-mediated responses at the NMJ.

EXPERIMENTAL METHODS

Recombinant Neuregulin

The EGF-like domain of NRG-β1 was constructed by PCR using a pair of primers flanking S_{136} to K_{205} of the EGF domain of ARIA/NRG (Yang *et al.*, 1997). The cDNA fragment (~210 bp) was subcloned into pGEX vector (Amersham–Pharmacia Biotech, UK). Recombinant NRG-β1 was purified according to the supplier's protocol.

Cell Culture

Mouse C2C12 cells were normally maintained as myoblasts in DMEM supplemented with 10% FBS as previously described (Fu *et al.*, 1997). Differentiation of myoblasts to myotubes was induced by switching the culture medium to DMEM supplemented with 2% HS. Cultured C2C12 myotubes were treated with recombinant NRG-β (4 nM) for 48 h prior to preparation of

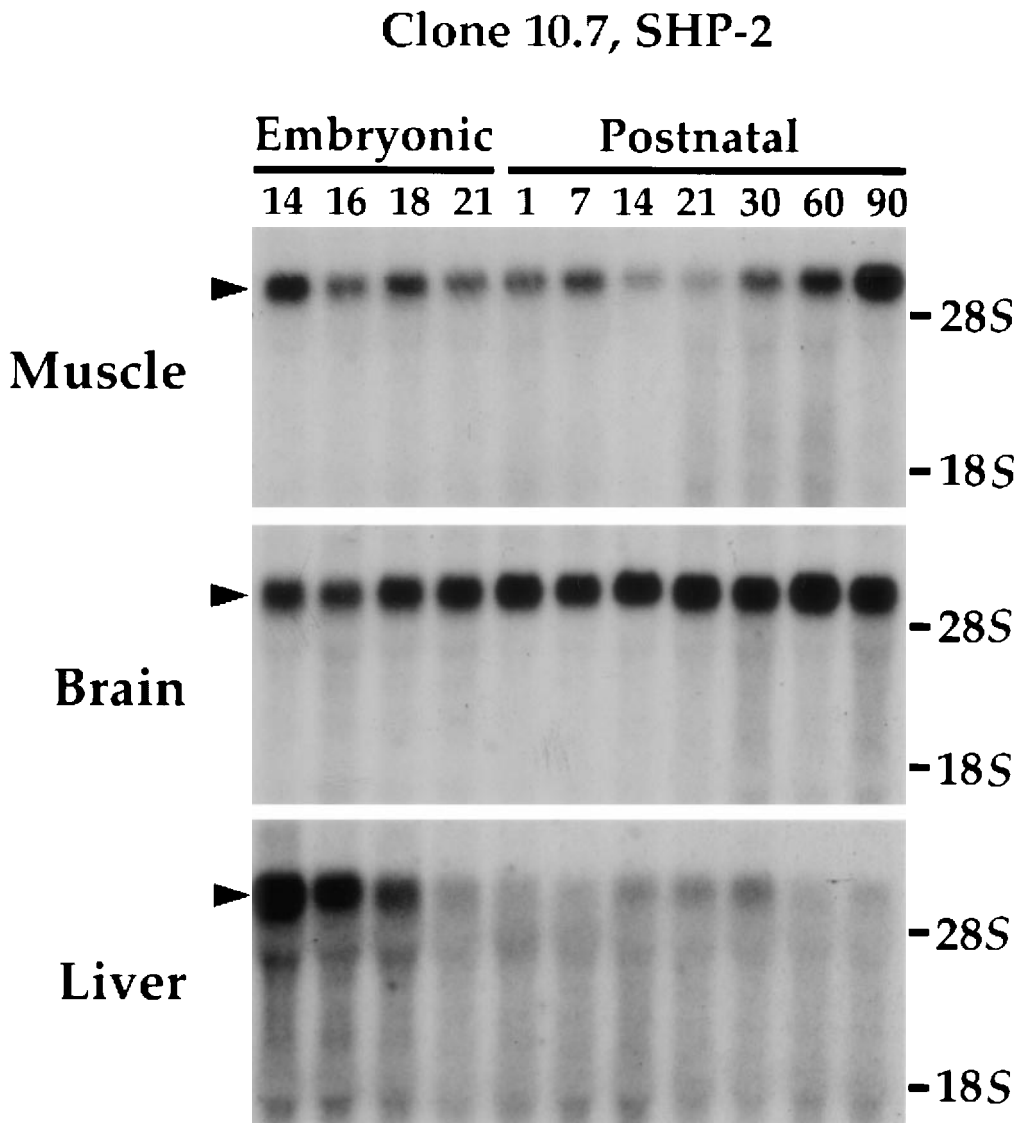

FIG. 8. Northern blot analysis of SHP-2 in different rat tissues during development (from embryonic day 14 to postnatal day 90). SHP-2 was prominently expressed in early embryonic muscle (E14). The expression decreased after birth (P14 to P21) and was up-regulated in the postnatal stages.

RNA. Primary chick muscle cultures were prepared from hindleg muscles of E13 chicks and maintained in MEM supplemented with 10% HS and 2% (v/v) chick embryo extract (Fu *et al.,* 1999). After fusion of myoblasts for 3 days, arabinoside cytosine (10 μM) was added to the muscle culture for 1 day.

Total RNA Extraction and the Northern Blot Analysis

Total RNAs of C2C12 myotubes (control or treated with recombinant NRG-β1) were prepared by guanidinium thiocyanate extraction. The lithium chloride/urea extraction method was used for preparation of total RNAs from rat tissues. Northern blot analysis was performed as previously described (Ip *et al.,* 1995). Nylon filters were hybridized with random-primed ^{32}P-labeled cDNA fragments in phosphate buffer at 65°C, washed at high stringency, and exposed to X-ray film (Fuji) with intensifying screen at −80°C.

Reverse Transcription and Polymerase Chain Reaction

Single-stranded cDNA was prepared from 2 μg of total RNA using Superscript II RNase H$^-$ reverse trans-

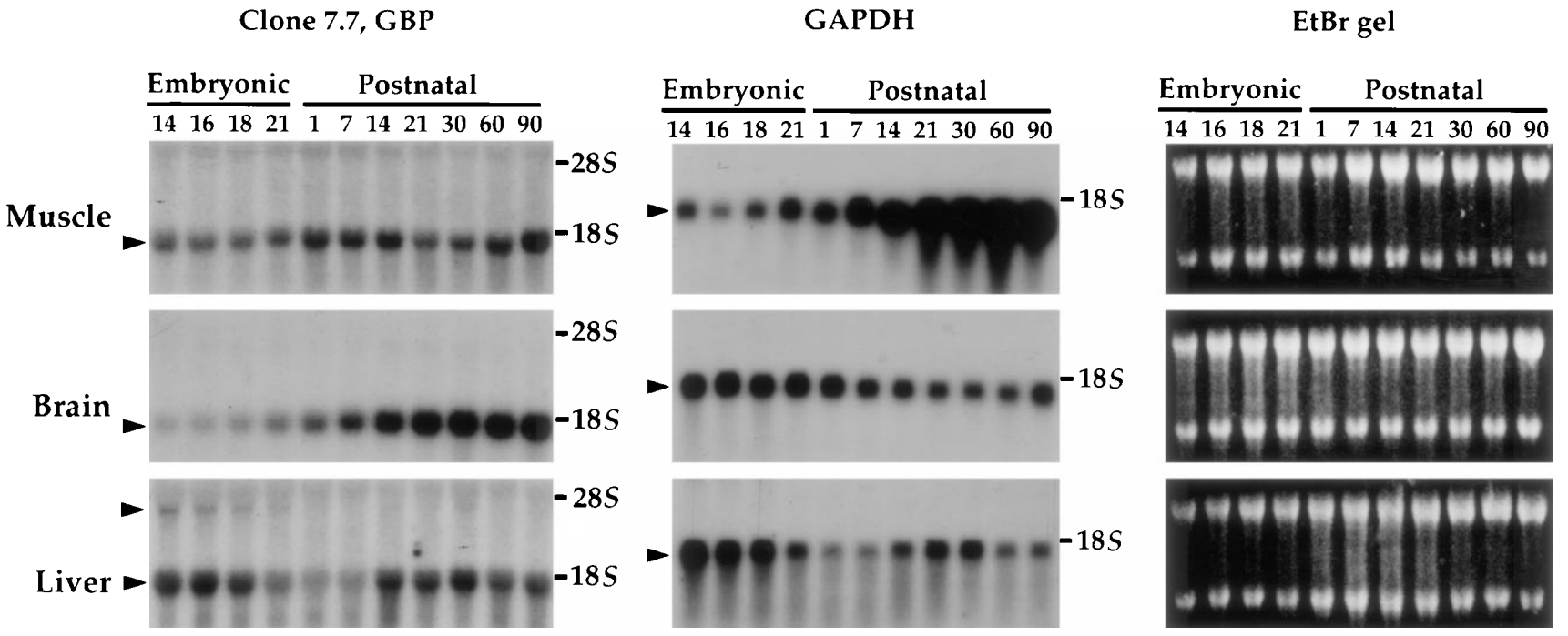

FIG. 9. Northern blot analysis of GBP in different rat tissues during development (from embryonic day 14 to postnatal day 90). An ~1.8-kb transcript of GBP was detected in rat muscle and was up-regulated during early postnatal stages. (Middle) Northern blot analysis with GAPDH as control. (Right) EtBr-stained gels.

criptase (Gibco BRL, NY) according to the supplier's instruction. Amplification of DNA was performed in a thermocycler (Robocycler; Stratagene, CA) with 10% of the reverse transcription product in a total volume of 20 µl using the following parameters: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 25 cycles. PCR products were analyzed by 1% agarose gel electrophoresis. The forward and reverse primers used for amplification of rat AChRϵ cDNA were 5′-GCAATACCATCAACAAG-3′ and 5′-CAGCAGCTCTAATAAAA-3′, respectively.

RNA Fingerprinting by Arbitrary Primed PCR

RAP-PCR was performed as described (Cheung *et al.*, 1997). Briefly, reverse transcription was performed at 37°C for 1 h using total RNA (50 and 250 ng) and a first arbitrary primer (designated RT-primer). Arbitrarily primed PCR mix was prepared by combining 2× *Taq* polymerase buffer, 4 µM $MgCl_2$, 200 µM dNTP, two primers at 1 µM each (i.e., RT-primer and AP-PCR primer), 0.1 U *Taq* polymerase, and 4 µCi [^{32}P]dCTP. The primers used include KS primer and M13-forward primer. AP-PCR consisting of low-stringency amplification followed by 30 normal PCR cycles was performed. During the low-stringency cycle, the reaction was annealed at 35°C for 5 min. After PCR, 4 µl of amplified products was mixed with 18 µl of 95% formamide and heated at 94°C for 2 min, and 2.5 µl of this heated mixture was electrophoresed in denaturing 6% polyacrylamide gel. Differentially expressed PCR bands were excised from the gel and reamplified with the same primers for 20 cycles in the absence of radioactive isotopes. The cDNA fragments cloned in this study were ~300–500 bp.

Cloning of DNA Fragments and Sequence Analysis

Reamplified cDNA was gel-purified with Qiaex (Qiagen, U.S.A.) according to the supplier's instruction. Purified cDNA fragments were subcloned using pCRScript SK(+) cloning kit (Stratagene). Double-stranded sequencing of the plasmids was performed with T3 and T7 primers using T7 DNA polymerase sequencing kit (Amersham–Pharmacia Biotech). Five individual clones from each amplified product were sequenced. The nucleotide sequences were compared with the GenBank and EMBL databases. Deduced amino acid sequences were compared with the CDS translations of the GenBank databases and the amino acid sequences from the PDB, SwissProt, Spudate, and PIR databases. In both cases, the BLAST server provided by the National Center for Biotechnology Information (National Library of Medicine, National Institutes of Health) was used for performing the sequence analysis. TBLASTX was also attempted with the dbEST database at the National Center for Biotechnology Information.

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