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A Novel Synapse-Associated Noncoding RNA

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Synaptic nuclei of innervated muscle transcribe acetylcholine receptor (AChR) genes at a much higher level than extrasynaptic nuclei. To isolate candidate synaptic regulatory molecules responsible for the unique transcriptional potential of synaptic nuclei, we have taken a subtractive hybridization approach. Here, we report the cloning and characterization of a novel synapse-associated RNA, 7H4. 7H4 is expressed selectively in the endplate zone of skeletal muscle and is upregulated during early postnatal development and after denervation. Interestingly, the 7H4 gene has no introns, and yet two different-size RNAs with identical polyadenylated 3′ ends are generated. Most intriguingly, the nucleotide sequence does not contain any significant open reading frames, suggesting that 7H4 may function as a noncoding RNA.

Neurons can change the targets they innervate by altering the genetic program of the postsynaptic cell. The best-studied example of such transynaptic regulation of gene expression occurs at the developing neuromuscular junction, where the genes coding for the acetylcholine receptor (AChR) subunits are subject to neurotrophic control. The surface expression of the AChR changes markedly during muscle development and synapse formation; these changes occur by both the redistribution of AChRs and the regulation of AChR synthesis (see reference 58 for a review). Embryonic AChRs, comprised of α, β, δ, and γ subunits, are synthesized throughout the syncytial myofiber and are found diffusely over the entire muscle surface. Upon innervation, the muscle becomes electrically active, which represses extrasynaptic AChR synthesis; at the synapse, adult-type receptors, which contain ε subunits in place of γ, accumulate to high density. If the nerve is cut, receptors (γ containing) are again synthesized extrasynaptically—the molecular basis of denervation supersensitivity.

Previously (38), we defined three properties of AChR gene regulation to account for the aforementioned changes in receptor synthesis during synaptogenesis (see reference 30 for a review): (i) developmental (AChR genes are transcribed by nuclei throughout the syncytial myofiber in a time- and tissue-specific manner as part of the myogenic program), (ii) activity mediated (AChR genes are repressed in extrajunctional nuclei by the electrical activity of the contracting muscle), and (iii) synaptic (junctional nuclei continue to transcribe AChR genes at high levels as a result of trophic support from the nerve).

We have focused our studies on this third feature of AChR gene regulation, the notion of "privileged" synaptic nuclei that preferentially transcribe AChR genes (see reference 13 for a review). The first suggestion of such differential transcription came from experiments that showed AChR α- and δ-subunit RNAs to be more abundant in synapse-rich than synapse-free regions of adult muscle (42). Since then, in situ hybridization has confirmed and extended these results, showing α, β, δ, and ε RNAs enriched at the endplate (7, 23, 24, 28, 48). Recently, studies of transgenic mice have proven that the concentration of AChR RNA at the synapse is due to enhanced transcription of AChR genes by junctional nuclei. In these experiments, promoters of the α subunit (35), the α and ε subunits (29, 55), or the δ subunit (33, 60) were analyzed for the ability to direct transcription of a reporter gene. In each case, differential transcription was observed; the α promoter demonstrated only slightly enhanced synaptic expression of the reporter, while the δ, and especially the ε, promoters directed precise synapse-specific transcription.

Several studies indicate that the unique expression pattern of the ε gene demonstrates stringent nerve-induced, synapse-specific transcription (7, 27, 38, 67–69). Unlike the other subunits, ε mRNA does not appear in significant amounts until after innervation and is then found only near synaptic nuclei. Even after denervation, ε RNA remains synaptically localized and its levels do not change significantly, suggesting that endplate nuclei are permanently imprinted. There appears to be a stable signal from the motor nerve, perhaps deposited in the synaptic basal lamina, that induces and maintains preferential transcription of the ε (and α, β, and δ) genes at synaptic nuclei.

The nature of this nerve-derived signal, and the postsynaptic regulatory factors that transduce it, remains largely unknown. Most progress has been made presynaptically with the identification of two putative regulatory factors: calcitonin gene-related peptide (44) and AChR-inducing activity (22). But the molecules responsible for the unique transcriptional potential of synaptic nuclei have yet to be described.

We hypothesized that a molecule expressed only at the endplate regulates the localized transcription of AChR genes. Here we report the use of subtractive hybridization to identify candidate synaptic regulators. We describe a subtractive cloning strategy that resulted in the >1,000-fold enrichment of a cDNA, 7H4, that represents a novel synapse-associated RNA. We show that 7H4 expression is muscle specific, differentiation dependent, developmentally regulated, and neurally controlled. Finally, we describe the organization of the 7H4 gene and its unique nucleotide sequence. Intriguingly, the 7H4 gene has no introns, and its sequence does not contain any significant open reading frames (ORFs). We suggest that the product of the 7H4 gene is a noncoding RNA and discuss its possible function in light of the recent discoveries of noncoding RNAs with potent regulatory properties.

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MATERIALS AND METHODS

Subtractive cloning. We used a modified version of the directional phagemid subtraction procedure (49, 52). In brief, cDNA libraries were constructed (as described in reference 17) from synaptic and extrasynaptic diaphragm RNA and from sciatic nerve trunk RNA. Synaptic (target) cDNAs were cloned in a coding orientation into a polylinker-modified version of pEMSVscribe (16). Extrasynaptic and sciatic nerve (driver) cDNAs were cloned in an anticoding orientation into pSKII+ (Stratagene). After conversion to single-stranded DNA and biotinylation of driver DNA, 50 ng of synaptic library DNA was hybridized with 20 μg of extrasynaptic library DNA and 10 μg of sciatic nerve library DNA for 2 days. After removal of biotinylated DNA with avidin beads (62), the hybridization and subtraction was repeated; we estimate that a C_0t of 2,700 mol · s/liter was achieved (56). The remaining DNA was made double stranded and introduced into bacteria by electroporation; control experiments with nonspecific driver yielded 10^6 colonies, while the experiment described yielded 10^4. Since plasmids lacking inserts will remain unsubtracted, half (5,000) of the clones were analyzed by hybridization to an oligonucleotide (70) to determine which plasmids contained cDNAs; only 375 clones were insert positive. After eliminating 29 clones that hybridized to an extrasynaptic cDNA probe, we prepared DNA from the remaining 346. Twenty-seven had unrecoverable plasmids, 21 were derived from the driver libraries, and 24 were target derived but without insert. The remaining 274 synaptic library clones were then screened on synapse-containing and synapse-free Northern (RNA) blots.

Cell cultures. RN22 (47), R2 (64), L6 (71), and BC3H1 (57) cells were cultured as described previously, with modifications for muscle cell lines as detailed elsewhere (15, 25).

Animal dissections. Denervations and diaphragm dissections were performed as described previously (41, 42). Animals were anesthetized and sacrificed, and relevant tissues were immediately dissected, frozen in liquid nitrogen, and then stored at −80°C.

RNA preparation and Northern blot analysis. Total RNA was prepared by the method of Chomczynski and Sacchi (14), and poly(A)$^+$ RNA was purified by single selection on oligo(dT)-cellulose (3). RNA was fractionated on formaldehyde agarose gels and transferred to nylon membranes (Nytran; Schleicher & Schuell) by standard procedures (54). Hybridization was carried out in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS)–10× Denhardt's solution–10% dextran sulfate–0.1 mg of salmon testis DNA per ml–0.1 mg of yeast RNA per ml at 68°C for 12 to 24 h. Washes were in 1× SSC–0.5% SDS (three times for 5 min each at 20°C and three times for 60 min each at 65°C). Mouse RNA blots (Fig. 3b and 4b) were washed at 50°C, although subsequent blots washed at high stringency (65°C, 1× SSC) gave comparable results (data not shown). All blots were stripped and reprobed with actin to demonstrate equal loading of RNA, and band intensity was quantitated with a phosphorImager (Molecular Dynamics).

Random-primed, [α-^{32}P]dCTP-labeled probes (Prime-It II kit; Stratagene) were generated from the following cDNAs: ε, a 2.1-kb cDNA isolated from a rat diaphragm library (17, 65); β, a 1.7-kb rat cDNA (28); and actin, 0.7-kb mouse cDNA (61). For Fig. 2b, the 7H4 probe is the 1,257-bp cDNA isolated by subtractive hybridization (nucleotides 3900 to 5156; Fig. 5). For Fig. 3 and 4, the 7H4 probe is a 990-bp cDNA (nucleotides 3684 to 4673; Fig. 5) that was isolated by screening a random-primed rat muscle library (32) with a 5′ fragment of the original 1,257-bp 7H4 cDNA.

cDNA and genomic cloning and sequencing. Rat cDNA and genomic libraries were screened by standard procedures (54), using hybridization conditions and high-stringency washing (65°C, 1× SSC) as described above for Northern analysis. We screened three different cDNA libraries: a dT-primed diaphragm library (17), a random-primed hindlimb muscle library (32), and a dT- and random-primed chest muscle library (5′-Stretch Plus; Clontech). A 5′ SLIC-RACE (single-stranded ligation of cDNAs–rapid amplification of cDNA ends) (21) kit was used exactly as recommended by the manufacturer (Clontech). The rat gene was cloned by screening 4.5 × 10^5 plaques of a genomic library (63) with a 3,609-bp cDNA probe (nucleotides 1553 to 5161; Fig. 5). The one positive plaque obtained had a 16-kb insert containing the entire 7H4 gene.

Positive plaques were purified, and DNA was prepared; 5′ RACE products were subcloned into a T/A plasmid vector (Invitrogen). Clones were sequenced with a Taq DyeDeoxy kit and a 373A DNA sequencer (Applied Biosystems, Inc.) or a Sequenase kit (U.S. Biochemical) manually. Sequence was analyzed with GeneWorks software (Intelligenetics) and checked for homologies by searching the National Center for Biotechnology Information database with the BLAST algorithm (1). ORF analysis shown in Fig. 6b was done with the program DNA Strider.

Southern blot analysis. Rat and human genomic DNA was prepared and digested with *Eco*RI and *Bam*HI. Digested DNA (10 μg per lane) was fractionated on an agarose gel and transferred to a nylon membrane (GeneScreen Plus; Dupont) by standard procedures (54). For Fig. 7a, the 5′ probe was a 246-bp PCR-generated fragment (nucleotides 76 to 321; Fig. 5) and the 3′ probe was a 233-bp *Bsa*I-*Eco*RV fragment (nucleotides 4052 to 4284; Fig. 5). For Fig. 7b, the probe was a 600-bp restriction fragment (nucleotides 3684 to 4284; Fig. 5). Hybridization conditions and high-stringency washing (65°C, 1× SSC) were as described above for Northern analysis.

Nucleotide sequence accession numbers. Sequence data reported in this paper have been submitted to the Genome Sequence DataBase (Los Alamos National Laboratory, Los Alamos, N.Mex.) under accession numbers L33721 (rat 7H4 gene) and L33722 (rat 7H4 cDNA).

RESULTS

Subtractive cloning strategy. We began by taking advantage of the simple anatomy of the rat hemidiaphragm, a broad flat muscle with a clearly defined central endplate zone that underlies the phrenic nerve (Fig. 1). Diaphragms were dissected into synapse-containing and synapse-free zones by cutting along the intramuscular nerve; RNA prepared from these fractions and analyzed by Northern blots showed distinct differences in the amounts of AChR transcripts present (see also reference 42). As shown in Fig. 2a, ε-subunit RNA is found exclusively in the synaptic fraction, whereas actin RNA is distributed equally. Because of the crude nature of the dissection, the so-called synaptic fraction is still ≈99% extrajunctional muscle; hence, the β-subunit RNA, which is ≈50-fold concentrated at endplate nuclei but also present extrasynaptically (28), shows only a slight synaptic enrichment in this assay. Our aim was to use such synapse-containing and synapse-free Northern blots as a means of identifying RNAs whose distribution is more like that of ε than that of β RNA.

Our strategy to facilitate this aim was to generate an enriched collection of cDNAs that represent other putative synaptic RNAs. cDNAs prepared from the synaptic fraction of diaphragm were enriched for endplate-specific clones by subtractive hybridization to cDNAs prepared from the extrasyn-

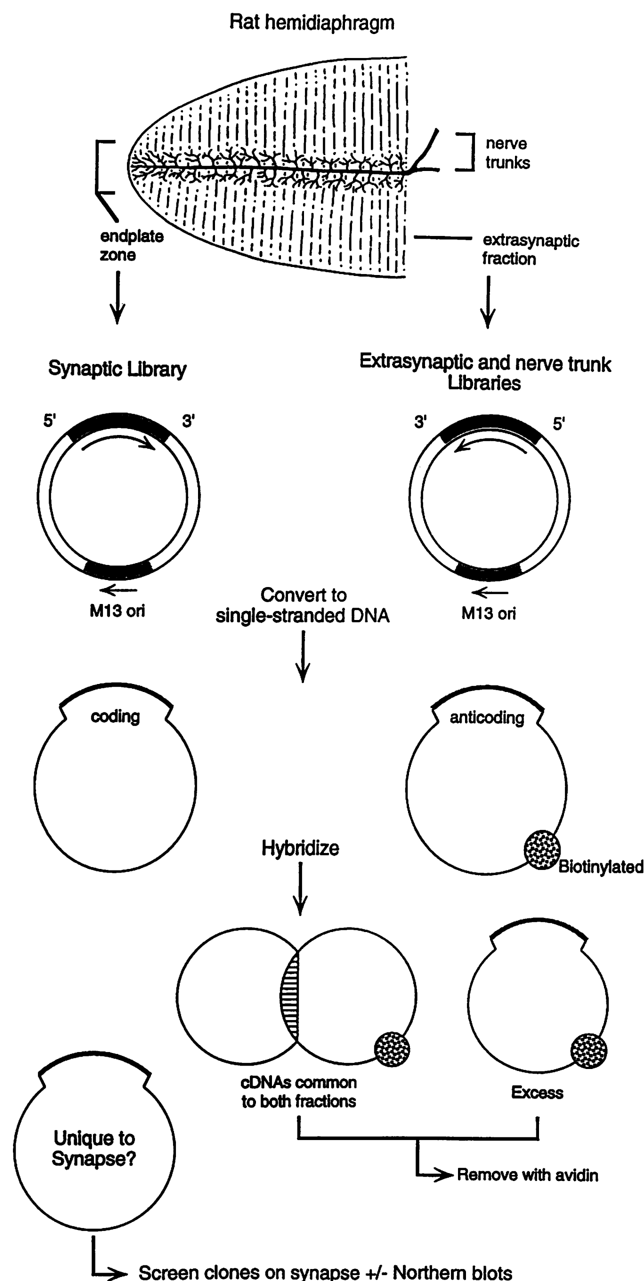

FIG. 1. Subtractive cloning strategy. Diaphragms were dissected into synapse-containing and synapse-free zones; RNA prepared from these fractions was used for Northern blot analysis and cDNA library construction. These cDNA libraries were then used in the subtractive cloning strategy (see text for details). ori, origin of replication.

aptic fraction. We also used cDNAs prepared from nerve trunks to remove clones derived from cells of the intramuscular nerve (fibroblasts and Schwann cells). We used the directional phagemid subtraction procedure of Rubenstein et al. (49, 52). As diagrammed in Fig. 1, target cDNAs (prepared from the synaptic fraction) were cloned in one orientation, while driver cDNAs (from the extrasynaptic fraction and nerve trunks) were cloned in an opposite orientation. Following conversion of these libraries to single-stranded DNA, we mixed vast excesses of driver cDNAs to the synaptic cDNAs.

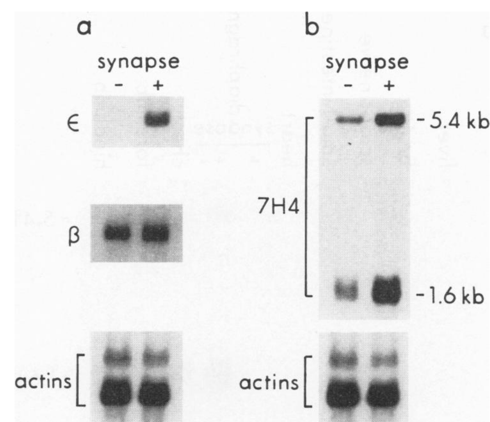

FIG. 2. Northern blot analysis of synapse-containing and synapse-free RNA to determine the distribution of AChR ε- and β-subunit mRNAs and 7H4 transcripts. (a) ε mRNA is found exclusively in the synaptic fraction, while β mRNA is only slightly enriched; actin mRNAs are distributed equally. Eight micrograms of poly(A)$^+$ RNA was loaded for the ε blot (18-h exposure with an intensifying screen); 5 μg of poly(A)$^+$ RNA was loaded for the β blot (23-h exposure). (b) Both 7H4 RNAs (5.4 and 1.6 kb) are enriched threefold in the synaptic fraction. Eight micrograms of μg poly(A)$^+$ RNA was loaded (18-h exposure with an intensifying screen); actin content is shown to indicate loading.

After removing hybrids representing molecules found in both fractions, we were left with a highly enriched, rigorously selected collection of putative synaptic RNAs. The library prepared from the synaptic fraction contained ≈10^6 independent clones; after two rounds of subtractive hybridization and several screening procedures, the number of candidate clones was reduced to 274 (see Materials and Methods for details). Clones from the subtracted library were then assayed for synapse-specific expression by Northern blot analysis; only one cDNA (clone 7H4) reproducibly demonstrated synapse-enriched expression. The other 273 clones represented RNAs of a very low abundance class; 66 cDNAs gave no detectable signal on synapse-containing and synapse-free Northern blots, while 207 cDNAs showed low level expression that was equivalent between the synapse-containing and synapse-free fractions (data not shown).

Synapse-enriched expression of 7H4. Clone 7H4 (named because of its grid position in a 96-well plate) was found only once in the collection of 274 and is extremely rare (≈1 in 10^6) in an unsubtracted synaptic library (data not shown). Therefore, the subtractive procedure specifically enriched this rare cDNA by >1,000-fold. As shown in Fig. 2b, 7H4 hybridizes to two RNAs (1.6 and 5.4 kb) that are both more abundant in endplate-containing than endplate-free regions of adult rat diaphragm. The amount of synaptic enrichment is greater than that seen for the β subunit but less than that for the ε subunit (Fig. 2a). In three separate preparations of endplate-containing and endplate-free RNAs from adult muscle, we observed synaptic enrichment of both 7H4 RNAs ranging from threefold (Fig. 2b) to sevenfold (Fig. 3a). Moreover, we have found 7H4 RNA enriched at the endplate zone of muscles from 2-week-old rats (data not shown), suggesting that 7H4 is synaptically localized from the onset of its expression (see Fig. 4a).

Muscle-specific and differentiation-dependent expression of 7H4. Many proteins concentrated at the endplate are also expressed in nonmuscle tissue (30). To determine whether this is true of 7H4 expression, Northern blotting was performed

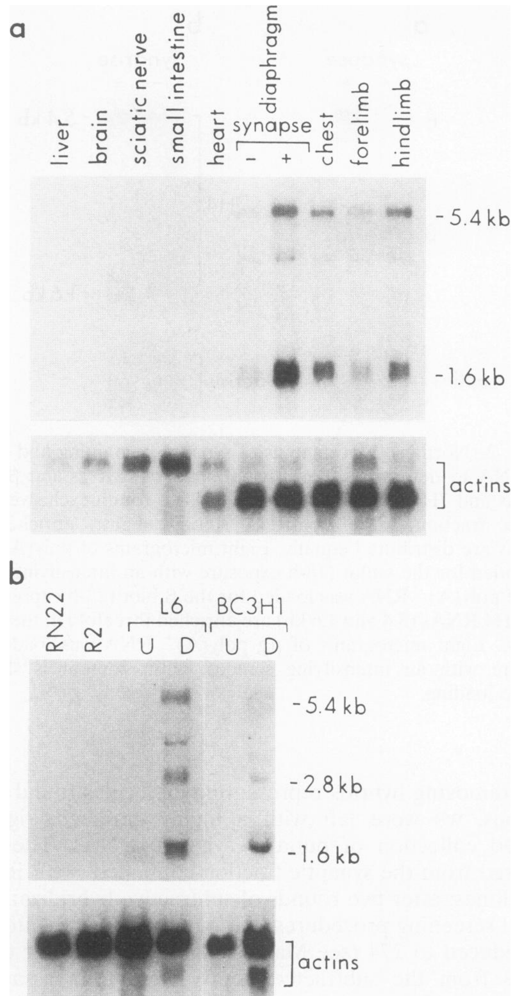

FIG. 3. Northern blot analysis of 7H4 expression in tissues and cell lines. (a) Total RNA from 10 different tissues of adult rats. Chest, forelimb, and hindlimb are muscle samples dissected indiscriminately with regard to the presence or absence of endplates. 7H4 expression is restricted to skeletal muscle and is enriched sevenfold (synaptic versus extrasynaptic) in the diaphragm. The minor band below the 5.4-kb band is a gel artifact that sometimes appears with total RNA samples. For this blot and the blot in panel b, 10 μg of RNA was loaded (8-day exposure with an intensifying screen); actin content is shown to indicate loading. (b) Total RNA from four different clonal cell lines: RN22 (rat Schwann cell line), R2 (rat fibroblast line), L6 (rat skeletal muscle line), and BC3H1 (mouse nonfusing, muscle-like line). L6 and BC3H1 were harvested in both their undifferentiated (U) and differentiated (D) states. 7H4 expression is restricted to differentiated L6 and BC3H1 cells; note the presence of a 2.8-kb RNA that appears only in these two cell lines and during early postnatal development (see Fig. 4a).

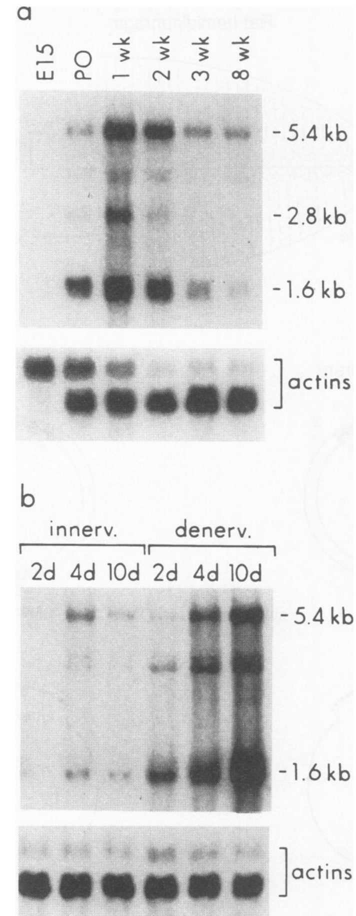

FIG. 4. Northern blot analysis of 7H4 expression during development and after denervation. (a) Total RNA from skeletal muscle (dissected from the chest and hindlimb) at various times during rat development. E15 is embryonic day 15, and P0 is the day of birth. 7H4 begins to be expressed at birth, peaks 1 week after birth, and then falls fivefold over the next few weeks to adult levels. Liver, brain, and heart dissected at the same time points (E15 to 8 weeks) shows no 7H4 expression (data not shown). For this blot and the blot in panel b, 10 μg of RNA was loaded; actin content is shown to indicate loading. The blot was exposed for 2 days with an intensifying screen. (b) The hindlimbs of three mice were denervated on day zero; 2, 4, and 10 days (2d, 4d, and 10d) after sciatic nerve transection, the muscles from the denervated and contralateral innervated limbs were dissected, and total RNA was extracted. Denervation induces 7H4 RNA 5-fold (lane 2d) to 28-fold (lane 10d). The blot was exposed for 8 days with an intensifying screen.

with RNA from several different tissues dissected from adult rats. 7H4 is expressed in all muscles tested (from the chest, forelimb, and hindlimb, as well as diaphragm), but no expression is seen in liver, brain, sciatic nerve, small intestine, heart, lung, spleen, thymus, kidney, or testis (Fig. 3a and data not shown). This Northern analysis of 14 adult tissues indicates that 7H4 expression is restricted to skeletal muscle. Furthermore, we have not found 7H4 RNA in the liver, brain, and heart during development, suggesting that expression of 7H4 is skeletal muscle specific throughout embryonic and postnatal life (Fig. 4a and data not shown).

The absence of 7H4 RNA from sciatic nerve (Fig. 3a) suggests that the enhanced synaptic expression is derived from muscle, not from cells associated with the nerve terminal (Schwann cells and fibroblasts). To explore this further, 7H4 expression was analyzed in cultured cells by Northern blotting. We prepared RNA from two different muscle cell lines, L6 and BC3H1, by harvesting cells either as undifferentiated myoblasts or as differentiated myotubes (in the case of the nonfusing BC3H1 line from mouse, cells differentiate but do not form myotubes [57]). We also extracted RNA from a Schwann cell line, RN22, and a fibroblast line, R2. Figure 3b demonstrates that 7H4 is not expressed in Schwann cells, fibroblasts, or

undifferentiated muscle cell lines but is expressed in differentiated muscle cells. This result establishes that 7H4 expression is skeletal muscle specific and differentiation dependent; it also strongly suggests that expression of 7H4 in the synaptic fraction is derived from endplate zone nuclei.

Developmentally regulated and neurally controlled expression of 7H4. Given the unique expression pattern of the ε-subunit gene during synaptogenesis (see the introduction), we were curious as to the time course of expression of 7H4 during neuromuscular development. We prepared RNA from chest and hindlimb muscle dissected from rat embryos, newborns, and pups of several different ages and examined 7H4 expression by Northern analysis. As shown in Fig. 4a, 7H4 begins to be expressed at birth, peaks 1 week after birth, and then falls fivefold over the next few weeks to adult levels. This pattern closely parallels, but interestingly slightly precedes, the time course of the ε-subunit RNA (38, 67). In addition to the two 7H4 RNAs seen in adult muscle (1.6 and 5.4 kb), a third transcript of 2.8 kb is transiently expressed during early postnatal life; this transcript is also observed in differentiated muscle cell lines (Fig. 3b).

We also analyzed 7H4 expression after motor nerve transection, since denervation is known to upregulate the AChR genes (26, 41). We denervated mouse hindlimbs and then extracted RNA from denervated and contralateral innervated limbs 2, 4, and 10 days after sciatic nerve transection. Figure 4b demonstrates that denervation induces 7H4 RNA 5-fold by day 2 and up to 28-fold by day 10. These data suggest that, like expression of the AChR genes, 7H4 expression is controlled by the nerve.

Genomic organization and sequence analysis of 7H4. We began our molecular analysis of 7H4 by cloning a full-length cDNA for the 1.6-kb RNA. To achieve this, we screened dT- and random-primed libraries with a 5′ fragment of the original 7H4 cDNA (the 1,257-bp dT-primed cDNA isolated from the subtracted library); seven independent overlapping clones representing 1,478 bp were obtained and sequenced. Using the 5′ SLIC-RACE technique (21), we obtained a single-band amplification product that extended the sequence of the 5′-end 89 bp. Given the juxtaposition of the 5′-most nucleotide in 3′ RACE cDNAs sequenced (Fig. 5, nucleotides in boldface at positions 3596 to 3598), we concluded that the composite 1,567-bp cDNA represents a full-length copy of the 1.6-kb polyadenylated RNA. We then used a similar "walking" strategy to isolate a full-length cDNA for the 5.4-kb RNA; 16 independent overlapping cDNAs were obtained and sequenced. Again, we used the 5′ SLIC-RACE method and obtained a single-band amplification product that extended the sequence of the 5′-end 58 bp (Fig. 5, nucleotides in boldface at positions 1, 2, and 10). Therefore, we concluded that this 5,161-bp composite cDNA represents a full-length copy of the 5.4-kb polyadenylated RNA. Finally, we isolated and sequenced the entire rat gene, which was contained in a single λ clone.

The analysis of the gene and both cDNAs revealed two unexpected features of 7H4. First, all sequences from the gene, the 5.2-kb cDNA, and the 1.6-kb cDNA are exactly colinear with no discontinuities (Fig. 5 and 6a). Although the 7H4 gene has no introns, two different-size RNAs with identical polyadenylated 3′ ends are generated. We have mapped these two transcripts by Northern analysis; probes from the 3′ end hybridize to both the 5.4- and 1.6-kb RNAs (Fig. 2b, 3, and 4), while probes 5′ of the 1.6-kb cDNA hybridize only to the 5.4-kb RNA (data not shown). Since the colinearity of all the sequences makes alternative splicing and polyadenylation mechanisms unlikely, we suspect either that there is an internal transcriptional start site or that the 1.6-kb RNA is generated from the 3′ end of the 5.4-kb transcript by endonucleolytic cleavage (the 5′ 3.8-kb fragment is presumably unstable because of its unprotected 3′ end). Such cleavage has been demonstrated for the insulin-like growth factor II RNA (39, 45). Since we have not identified cDNAs corresponding to the 2.8-kb transcript (Fig. 3b and 4a), we do not know how it is generated.

The second surprising feature of 7H4's sequence is the lack of any significant ORFs because of the large number (241) of stop codons distributed in all three reading frames (Fig. 6b). The longest ATG-initiated ORF occurs in frame 3 (nucleotides 1920 to 2231); it is only 312 bp long, has a poor consensus sequence for translation initiation (36), and has no homology to any known sequence. Indeed, the sequence of 7H4 is unrelated to any in the database. Only three short regions of homology are found, and each is to a previously described repetitive element (Fig. 5). The first is a 60-bp TG dinucleotide repeat (nucleotides 327 to 387), the second is a 161-bp B1 element (46) (nucleotides 2480 to 2641), and the third is a 61-bp TA-rich repeat (nucleotides 4313 to 4374); these three elements occur only once and are well separated from each other. The lack of a significant ORF together with the lack of homology to known coding sequence argues against any of the RNA species being translated and suggests that 7H4 may function as a noncoding RNA.

Despite these novel characteristics, 7H4 has many traits of a typical structural gene. Genomic Southern analysis indicates that 7H4 is unique in the rat and human genomes and is encoded by a single-copy gene (Fig. 7). Sequence of the rat gene 5′ to the cDNA contains a TATA homology (−40 to −33, with respect to the first nucleotide of the cDNA), consistent with a polymerase II (Pol II)-transcribed gene (19); it also contains two E boxes (−133 to −128 and −61 to −56), elements found to be important in muscle-specific gene regulation (reference 20 and data not shown). Sequence 3′ to the cDNA does not encode the poly(A) tails found just 3′ of two consensus polyadenylation signals, indicating that the RNAs are polyadenylated posttranscriptionally, another feature of Pol II transcripts (Fig. 5 and 6a and data not shown). Moreover, the lack of direct repeats flanking the gene indicates that 7H4 is not a processed pseudogene. Most importantly, the sequence of 7H4 is highly conserved among rats, mice, and humans, as indicated by cross-hybridization (Fig. 3b, 4b, and 7b), suggesting functional evolutionary conservation.

DISCUSSION

The principal aim of our study was to clone synaptic RNAs by subtractive hybridization as a means of identifying factors that regulate AChR genes at the muscle endplate. The results reported in this paper describe a unique synapse-associated RNA, 7H4, that fits many of the criteria for such a factor.

Regulated expression of 7H4: similarities and differences with AChR subunit genes. The overall expression pattern of 7H4 (muscle endplate associated, differentiation dependent, developmentally regulated, and neurally controlled) is remarkably similar to that of the AChR subunit genes; however, some interesting contrasts can be drawn.

Several studies indicate that there are significant differences among the adult subunit genes in their degrees of synapse-specific transcription (29, 33, 35, 55, 60). The α, β, and δ genes are not completely suppressed by activity and therefore show variable amounts of leaky extrasynaptic expression; these differences are revealed on Northern blots of synapse-containing and synapse-free RNA. Only ε shows exclusive synaptic

TTTTGGCAA**G**TGCTTCTTCACGGCCGACGACTCCACCCAGAGAACACACCGGTGACTTCTTCGTTCCTTGGCCAC 75
GTTTGTCCTACCTGCAGGTGCCCTCTTCCCCAGCAATCCACCTCTGCAGACTTCTCCCGCAGCACGGGGCTTTTA
GGCCTCCAACCTAGCCCCTTTATACCAACCTCCCTGGTGCCACTCCTCAGACCTACTCTCAGAGAATCAGCTCCC
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ATGGTGCCACATGTGTAGCGGTTGCTCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
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ACACTGTCTGAGGAAAGGTGGAGGTGCTAAAATACTGATGCATTCATTTAATGGCCTTAGTTCCTTTGTTCTTAA
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TTGAGAAGATGAGATGCTATAGTGATGGGCTAGAACTCCATTAAACGTGGAGCTGAGCTCATTAGCAAAACAAAA 750
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TAGCAAATCCTCAATATCCTATAAATAAGTTAAATAAATAAATAAATAATAAAAATGAATCAAGAAAGCATTGGT
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GGGTTTTGTTTGCTTCTTTTGTTTTGTTTTTAGAGACAGGCTTTCCCTGTGTAGCATTGGCCATCCTGGAACTCA
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GATGCTCGAACACTCCAAGGACTTCACACACTAGAGGCTGCAGTCACTTCCCAAGGGAGCGGCCCCCTGCTCTGG
CTGGTCAAACGGAACCAAGTCCGTCTTCCTGAGAGGTTTGGTCCCCTTCAACCAGCTACAGCAGGGCTGGCAAAG
CCCAATCTTTGGAGAAAGAGAAGAGAAGAGAAATTATCTGCTACCATCTTGCTTATGAAAAGCAGTTCTGGTTTT
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ACAGAAGCATAAGTAAGGCCACTGACAATGGAAATACAGCACAACTCAACGGAGTTGCAGGCTGAAACATGCTCG
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ATGAATATATATTATATATATATACTGAATTTGCCTGTGACGTTCTACATTCTCCATGAAGCCCAGAGCTTTGTG
TACACTGAAATTAAAGTACACAGAAGGAGGTTACCCAACTCCCCATAGCTGGTACCTATGATTAAGGTAAAGCCC 4500
TGTCTTCCTGTTTGAAACCTTCCTGTTTTGTTTATTAGTTATCATGTGAACTCATAACTAGCCTCGGTCAAATAG
CCTCAGTCACCTCTGTTAAAACCTTAATTGCCTTTTCATCAGTACATAGAAATAGTGACCTGATCAATCACATCA
TTGTTCTAAGACAATGTGTGTAAATAACCAACCATAAGCATTCAGGGAATGCATAGCTTGCTATTATGACAATAA
TGGTGTCATGCCCAGAGGCATGGTCCCTTGAGGAGGAAGAACAAATTTTAAAAGATAAAATTGCCAAAATAAATG
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CAGCATCGAGCTTGCTCTATAGCCATAGAGCCTTCTGGGTGTGTGAGCATGTTTTCTAGATGCAACCAGAAGGAA
ACGTGGCTGCAGTTGGCTGCCTGTGAAAATAATGTACGAAGGTCTAAAATTAGGACAGGGTGCACAATGAGGTTT
TTCTCTTTGACAATGCTTTTAAAACCAAATTGGTATTCAATAAACTTTTCCCTAAT*AAATG* 5161

FIG. 5. Sequence of 7H4. Boldface type indicates the nucleotide found at the 5′ end of a cDNA generated by the 5′ RACE technique. Two 5′ RACE clones ended at the first T shown. The three underlined regions are those homologous to known repetitive elements. The double-underlined sequences represent poly(A) addition signals. The asterisks indicate the positions of poly(A) tails found in several independent dT-primed cDNAs. The sequence shown is based on double-stranded analysis of two or more independent overlapping clones. Genomic sequence 5′ and 3′ to the cDNA is not shown.

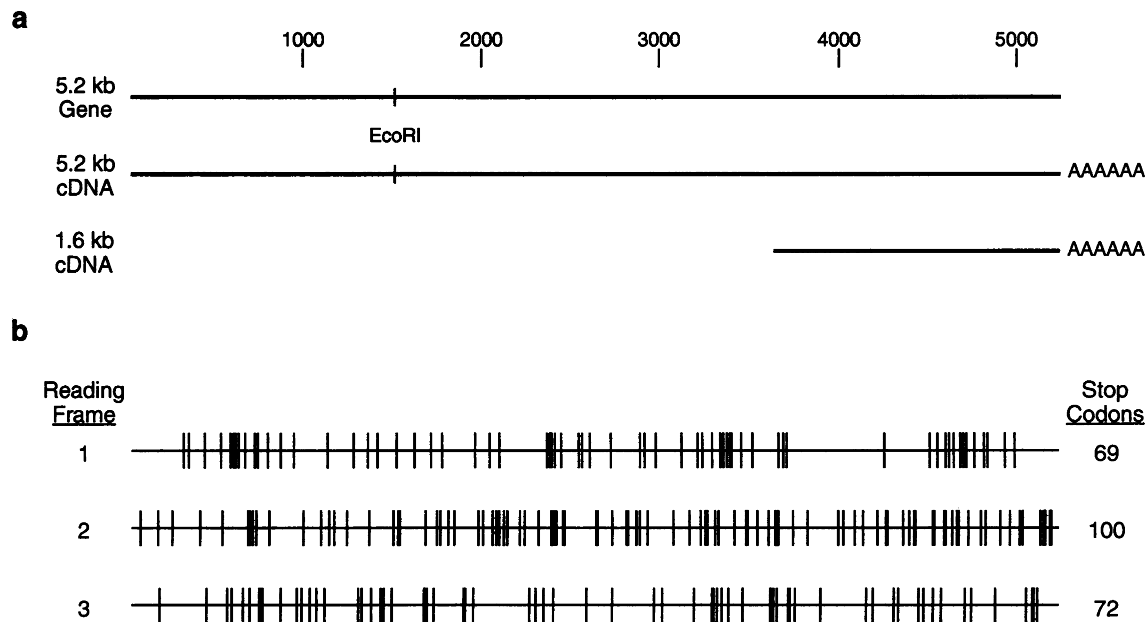

FIG. 6. Genomic and cDNA organization and ORF analysis of 7H4. (a) All sequences from the gene, the 5.2-kb cDNA, and the 1.6-kb cDNA are exactly colinear with no discontinuities. Poly(A) tails in both cDNAs are not encoded by the gene. (b) Results of a search for possible ORFs. Each tick mark represents a translation stop codon; the tally on the right indicates the total number of stop codons in each reading frame.

expression in this assay; of the other subunits, δ demonstrates the most synaptic versus extrasynaptic enrichment, and α and β show the least (Fig. 2a) (42, 65). The degree of synaptic enrichment of 7H4 is most similar to that of the δ subunit in this continuum.

AChR genes, which are transcribed only in skeletal muscle, are induced when myoblasts differentiate into myotubes (12). 7H4 also exhibits this induction when L6 and BC3H1 cells are allowed to differentiate in culture (Fig. 3b), indicating that 7H4 expression follows an intrinsic program of myogenesis that does not require the nerve. However, the level of 7H4 RNA in cultured cells is quite low compared with the α- and β-subunit mRNA levels (12) and is more reminiscent of ε-subunit mRNA levels (38), suggesting that the nerve is necessary for high level expression of 7H4 and ε (see below).

The time course of 7H4 expression during neuromuscular development is also reminiscent of the ε subunit. Although α, β, δ, and γ genes are expressed in fetal muscle (43), the ε gene is not transcribed until after birth, peaking 2 weeks postnatally before dropping to adult levels (38, 67). Similarly, 7H4 RNA is absent from embryonic day 15 (E15) muscle, accumulates to peak levels 1 week after birth, and then falls gradually (Fig. 4a). Since motor neurons begin to establish synaptic contacts with muscle at E14 to E15 in rats (18), it is probably that, like ε, 7H4 expression is nerve induced. Given its robust, but transient, postnatal expression, 7H4 may be involved in some aspect of synapse elimination or endplate maturation. The fact that 7H4 expression precedes that of ε by 1 week suggests a possible role in the γ-to-ε subunit switch.

Our observation that 7H4 RNA increases after denervation may seem paradoxical for a nerve-induced transcript, especially since ε RNA levels are relatively unaffected by nerve transection (38, 68). However, recent studies of transgenic mice bearing ε-promoter/reporter constructs indicate that activity-dependent elements are present in the ε gene and that these elements respond to denervation by increasing transcription (29). Perhaps *cis* elements in the 7H4 gene are more responsive than those of the ε gene to factors that increase with inactivity, thus explaining 7H4's upregulation after denervation.

Novelties of 7H4's genomic organization and nucleotide sequence: a noncoding cleaved RNA? Both the intronless genomic organization and apparently noncoding nucleotide sequence of 7H4 are unusual. Although Pol II-transcribed genes that lack introns have been reported, the generation of multiple different sized RNAs from such a simple genomic structure may be unique to 7H4. The colinearity of the 7H4 gene with cDNAs for the 1.6- and 5.4-kb RNAs precludes alternative splicing and polyadenylation mechanisms in generating these different-size RNAs; only two other possibilities can account for this RNA diversity. The first of these, internal transcriptional start sites, is a plausible model, but we are unaware of any intronless Pol II-transcribed genes that contain such disparate multiple internal start points. Future studies to determine if the 2.8- or 1.6-kb transcripts are modified by a methylated cap, which is indicative of Pol II transcription (59), should resolve this issue.

The second possibility, endonucleolytic cleavage, does have precedent: endonucleolysis of an insulin-like growth factor II transcript has been demonstrated by two groups (39, 45). The cleavage is site specific, and sequences surrounding the point of endonucleolysis can confer cleavage susceptibility to a hybrid RNA (40). The mechanism and physiological role of this unique posttranscriptional modification are unknown, but it is clear that initiation of RNA degradation can involve cleavage (5). We presume that if the 5.4-kb 7H4 RNA is cleaved, the 5′ 3.8-kb product is rapidly degraded, since we do not detect such a species on Northern blots; this would not be surprising given that RNAs with unprotected 3′ ends are often subject to degradation (53).

Rigorously proving that 7H4 RNA is untranslated will be difficult (see reference 6, for example), but the two lines of evidence reported here are very suggestive. First, the ORF analysis is remarkable for the abundance and distribution of

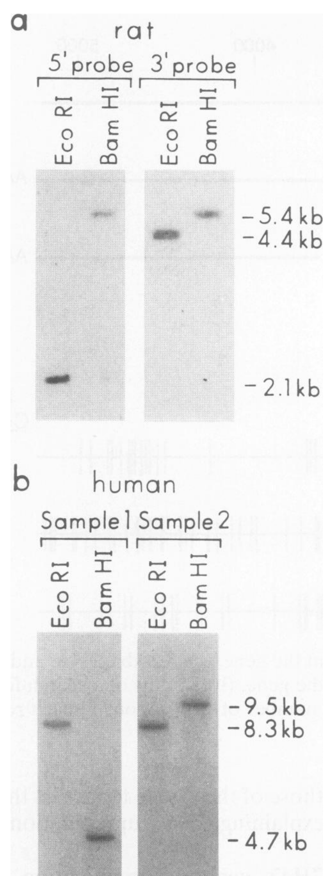

FIG. 7. Genomic Southern analysis of 7H4. (a) Rat liver genomic DNA was digested with *Eco*RI or *Bam*HI; the restriction fragments were analyzed by Southern blotting using probes from the 5′ and 3′ ends of the 5.2-kb cDNA. This blot indicates that the rat 7H4 gene lies on a 5.4-kb *Bam*HI fragment. (b) Human genomic DNA from two different individuals (placenta [sample 1] and peripheral blood lymphoblasts [sample 2]) was digested with *Eco*RI or *Bam*HI; the restriction fragments were analyzed by Southern blotting using a probe from the 3′ end of the 5.2-kb cDNA. This blot indicates that the human 7H4 gene contains a polymorphic *Bam*HI site.

translation stop codons in all three reading frames; the longest ATG-initiated ORF is only 312 bp (see Results). It is formally possible that a non-methionine-initiated protein is synthesized (see reference 36 for a review); however, the longest non-ATG-initiated ORF is only 537 bp. Second, there is no homology of 7H4 to any known coding sequence; thus, if 7H4 RNA is translated, the protein product would be novel and very small, especially for such a large RNA. These two characteristics of 7H4, multiple stop codons and lack of protein-coding sequence homology, show marked similarity to two other putative noncoding Pol II transcripts, H19 and XIST (see below).

7H4: a synaptic riboregulator? 7H4's endplate-associated, neurally controlled expression is consistent with it being a potential synaptic regulator. Our expectation was that it would be a protein in some known second-messenger pathway, and the finding that it appears to be a noncoding transcript was surprising. Recently, however, much interest has been generated in the potential regulatory properties of two mammalian noncoding RNAs, H19 (6) and XIST (9, 10); both are products of single-copy genes that are monoallelically expressed (4, 8). Dubbed "riboregulators" (51), these noncoding transcripts, or 3′ untranslated regions of coding mRNAs (50, 51), may act in *trans* to control gene expression, cell growth, and cellular differentiation (11, 31, 34, 50, 51). 7H4 is similar to H19 and XIST in that all three are polyadenylated and are therefore assumed to be products of RNA Pol II (Pol II transcription has been proven for the H19 gene [6]). As such, these three RNAs (along with a subset of small nuclear RNAs) are the only examples in mammals of noncoding Pol II transcripts. The 7H4 gene differs from H19 and XIST in that it has no introns and its expression is highly tissue specific. Recently, another noncoding RNA, expressed at the *His-1* locus, has been cloned from transformed mouse cell lines, although it is unclear if this RNA is expressed in vivo (2).

As yet, the mechanisms by which this unique class of noncoding RNAs exert their regulatory effects are unknown. Indeed, it has been difficult to study their function, especially in the case of H19, whose ectopic expression in transgenic mice causes prenatal lethality (11). Several mechanistic models have been proposed (51). Antisense inhibition is clearly a possibility, but no targets have been identified; in *Caenorhabditis elegans*, however, such an antisense regulatory RNA, *lin-4*, and its target, *lin-14*, have recently been reported (37, 66). Alternatively, riboregulators may bind proteins and thereby inactivate, sequester, or titrate critical regulatory factors. Finally, these RNAs may possess catalytic activity, as do self-splicing introns and ribozymes, and cleave target RNAs.

We conclude with the reminder that 7H4 was cloned on the basis of its restricted synaptic location. For the majority of cloned eukaryotic genes, some information regarding the proteins they encode is used in their isolation. Thus, in our effort to isolate putative synaptic regulators by means of their localization, we may have unveiled a novel RNA-mediated mechanism for the transynaptic regulation of gene expression.

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