

## Syne-1, A Dystrophin- and Klarsicht-related Protein Associated with Synaptic Nuclei at the Neuromuscular Junction\*

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**We describe a novel protein, Syne-1, that is associated with nuclear envelopes in skeletal, cardiac, and smooth muscle cells. Syne-1 contains multiple spectrin repeats similar to those found in dystrophin and utrophin, as well as a domain homologous to the carboxyl-terminal of Klarsicht, a protein associated with nuclei and required for a subset of nuclear migrations in *Drosophila*. In adult skeletal muscle fibers, levels of Syne-1 are highest in the nuclei that lie beneath the postsynaptic membrane at the neuromuscular junction. These nuclei are transcriptionally specialized, expressing genes for synaptic components at higher levels than extrasynaptic nuclei in the same cytoplasm. Syne-1 is the first protein found to be selectively associated with synaptic nuclei. Syne-1 becomes concentrated in synaptic nuclei postnatally. It remains synaptically enriched following denervation or degeneration/regeneration, and is also present at high levels in the central nuclei of dystrophic myotubes. The location and structure of Syne-1 suggest that it may participate in the migration of myonuclei in myotubes and/or their anchoring at the postsynaptic apparatus. Finally, we identify a homologous gene, *syne-2*, that is expressed in an overlapping but distinct pattern.**

Skeletal muscle fibers are syncytial; in most mammalian skeletal muscles, each fiber contains several hundred myonuclei. Of these, a few are located beneath the postsynaptic membrane at the neuromuscular junction (NMJ).<sup>1</sup> Synaptic nuclei are specialized in several respects. First, multiple nuclei (generally 3–8) are invariably associated with synaptic sites. Because <1% of the muscle fiber surface is synaptic, one would expect only a minority of synaptic sites to be associated with even a single nucleus if nuclear distribution were random. Second, most nuclei are well separated from their neighbors, but synaptic nuclei occur in tight clusters. Third, synaptic nuclei are larger and rounder than extrasynaptic nuclei (1–3).

Finally, synaptic nuclei are transcriptionally specialized; they express genes for several synaptic proteins, including subunits of the acetylcholine receptor (AChR), at levels far higher than those of extrasynaptic nuclei in the same cytoplasm (4–6). As a result, mRNAs for synaptic proteins are concentrated in synaptic areas, allowing local synthesis of synaptic constituents. This local synthesis has been of considerable interest to neurobiologists, because it contributes to postsynaptic differentiation, and because it may serve as a model for central synapses, in which some components of dendritic spines are thought to be synthesized within the spine itself (7).

The formation of the postsynaptic apparatus, including the accumulation and specialization of synaptic nuclei, is controlled by the nerve. One critical nerve-derived signal is the proteoglycan agrin which is required for all aspects of postsynaptic differentiation, including transcriptional specialization of synaptic nuclei (8–10). A critical component of the agrin receptor is the muscle-specific tyrosine kinase (MuSK), which is concentrated in the postsynaptic membrane (11). Agrin activates MuSK, and postsynaptic differentiation fails in mutant mice lacking MuSK (12–14). Little is known, however, about how activation of MuSK leads to postsynaptic differentiation, or how agrin interacts with other signals such as neuregulins, which have been implicated in induction of AChR gene expression in synaptic nuclei (15).

With the aims of identifying novel components of the postsynaptic apparatus and gaining insight into mechanisms of postsynaptic differentiation, we used the yeast two-hybrid system to seek proteins that bind to the cytoplasmic domain of MuSK. In this paper, we describe one protein identified in this screen, synaptic nuclear envelope-1 (Syne-1), and its homologue, Syne-2. Remarkably, Syne-1 is selectively associated with synaptic nuclei. Its location and structure raise the possibility that Syne-1 might be involved in the formation or maintenance of nuclear aggregates at the neuromuscular junction.

### EXPERIMENTAL PROCEDURES

**Yeast Two-hybrid Screen**—Two-hybrid screening was performed in the HF7c yeast strain, which harbors two reporter genes, *HIS3* and *β-gal*, under the control of GAL4-binding sites. We generated a “bait” vector by fusing the intracellular domain of rat MuSK (11) to the DNA-binding domain of GAL4. This vector was used to screen 4 × 10<sup>6</sup> clones from a library in which cDNAs from embryonic day (E) 17 mouse embryos were fused to the GAL4-activation domain of vector pGAD10 (CLONTECH). Sixty-one positive colonies were detected based on β-galactosidase activity and growth in the absence of histidine. Plasmids containing positive cDNAs were rescued by *trp* selection in HB101 bacteria and sequenced. Twenty were discarded because they contained open reading frames that were very short or in the wrong orientation. The remaining 41 positives represented 20 different gene products.

**Isolation of *syne* cDNAs**—One 2.1-kb cDNA isolated from the yeast 2-hybrid library, called C-15, had an open reading frame over its entire sequence. To obtain additional sequence, nested PCR primers corre-

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<sup>1</sup> The abbreviations used are: NMJ, neuromuscular junction; AChR, acetylcholine receptor; DGC, dystrophin-glycoprotein complex; MuSK, muscle-specific kinase; RT-PCR, reverse-transcription polymerase chain reaction; kb, kilobase(s); aa, amino acid(s).

sponding to C-15 and vector sequences were used to amplify cDNAs from the yeast 2-hybrid library and from a C2 muscle cell line plasmid library (provided by David Glass, Regeneron Pharmaceuticals). Other clones were obtained by RT-PCR of poly(A) selected RNA from E17 embryos and muscle of postnatal day (P) 2 mice, and by screening a mouse heart  $\lambda$  gt11 library (CLONTECH). Clones were sequenced in their entirety, and the identity of each nucleotide was confirmed by sequencing two or more independently obtained clones. Sequence motifs were detected using PFAM and SMART programs (16).

**RNA Analysis**—Poly(A)<sup>+</sup> RNA prepared from mouse tissues and cells was fractionated on formaldehyde-agarose gels and transferred to GeneScreen Plus membrane (Dupont). A Northern blot containing RNA samples from multiple human tissues was purchased from CLONTECH. Blots were probed with <sup>32</sup>P-labeled cDNAs (random primed DNA labeling kit; Roche Molecular Biochemicals).

For PCR, cDNA samples from fetal and adult human tissues (Multiple Tissue cDNA panels; CLONTECH) were amplified using primers corresponding to human *syne-1* and *syne-2* sequences. Sequences are: *syne-1* (forward), 5'-CTGGAGTCTGCAATGTCCAGAGC; *syne-1* (reverse), 5'-GCAGGTTAACAAGCCAGGAAGGC; *syne-2* (forward), 5'-CTCCTCTCAGGAAGAGGACGAGG; *syne-2* (reverse), 5'-CTGTGGGT-TGCCATTCAGGACTCG. Glycerolaldehyde-3-phosphate dehydrogenase primers, supplied by CLONTECH, were used as a positive control.

**Cell Culture and Transfection**—The quail fibroblast cell line QT-6 and the mouse muscle cell line Sol 8 were maintained as described by Apel *et al.* (17) and Chu *et al.* (18), respectively. Cells were transfected by the calcium phosphate method (19), as modified by Phillips *et al.* (20). QT-6 cells were transfected at approximately 75% confluency 1 day after plating, then harvested 2 days after transfection for immunoprecipitation experiments. For immunofluorescence staining, QT-6 cells were plated onto glass coverslips (13 mm diameter) in 35-mm tissue culture dishes, transfected 1 day later, and fixed and stained 2 days after transfection. Sol 8 cells were plated onto gelatin-coated glass coverslips and transfected 1 day later. For studies of myoblasts, cells were plated at 50–100,000 cells per culture, then fixed and stained 2–3 days later. To study myotubes, 400,000 cells were plated, then serum levels were reduced (from 10% fetal calf serum to 2% horse serum) 2 days later to promote the fusion of myoblasts to myotubes. Cultures were fixed and stained 2–3 days later, when myotubes were abundant. In some experiments, a recombinant carboxyl-terminal fragment of agrin (21) was added to myotubes 4–8 h prior to fixation to induce AChR clustering.

**Generation of Antibodies**—A segment of mouse *syne-1B* corresponding to amino acids 948–1322 was expressed in bacteria. Purified protein was obtained by preparative gel electrophoresis of inclusion bodies and used to immunize two rabbits. Serum from both rabbits showed immunoreactivity to the antigen, but not to an unrelated recombinant protein generated in the same vector, when analyzed by immunoblotting. For some experiments, antibodies were affinity purified from the serum by absorption to and elution from recombinant *syne-1* that had been immobilized on nitrocellulose.

**Immunoprecipitation**—Transfected QT-6 cells were lysed and solubilized in saline containing 0.1% Nonidet P-40, and the soluble extracts were subjected to immunoprecipitation with antibodies to the FLAG epitope (M2, Sigma). FLAG-tagged and associated proteins were isolated by binding to protein G-Sepharose (Amersham Pharmacia Biotech), then subjected to electrophoresis and immunoblotted with antibodies to MuSK (11) and the FLAG epitope.

**Histology**—For routine immunohistochemical staining, tissues were frozen unfixed in liquid nitrogen-cooled isopentane and sectioned in a cryostat at 8  $\mu$ m. Cross-sections of muscle (tibialis anterior except as noted) were incubated with anti-Syne-1 for 2–4 h, then rinsed with phosphate-buffered saline. Sections were then incubated for 1 h with a mixture of fluorescein-conjugated goat anti-rabbit IgG, rhodamine-conjugated  $\alpha$ -bungarotoxin, and the DNA binding dye 4',6-diamidino-2-phenylindole. Slides were then rinsed with phosphate-buffered saline, mounted with *para*-phenylenediamine to retard fading, and viewed with a compound fluorescent microscope. Muscles were denervated by cutting the sciatic nerve in anesthetized mice in one leg with the contralateral leg serving as a control.

For confocal microscopy, sternomastoid muscles were dissected, fixed in 1% paraformaldehyde in phosphate-buffered saline for 20 min, equilibrated with sucrose, frozen, and sectioned longitudinally at 40  $\mu$ m. Sections were stained as described above and viewed with an Olympus confocal microscope.

QT-6 cells on glass coverslips were fixed and immunostained as described by Apel *et al.* (17). Sol 8 cells on glass coverslips were pro-

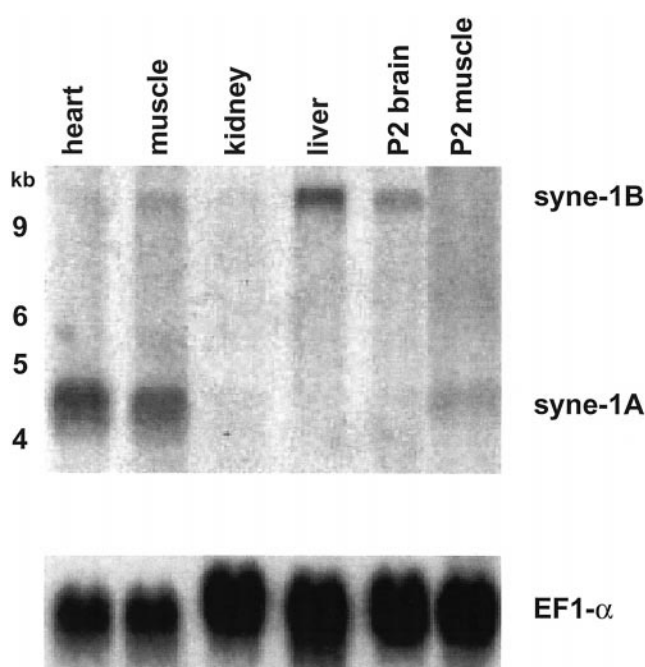


FIG. 1. Northern analysis of *syne-1* expression in tissues from neonatal (P2) and adult mice. A ~10-kb RNA, presumably encoding Syne-1B, is detectable in multiple tissues. A ~4.7-kb RNA, presumably encoding Syne-1A, is detectable only in cardiac and skeletal muscle. The blot was re-probed with cDNA encoding EF1 $\alpha$  to ensure equal loading of lanes.

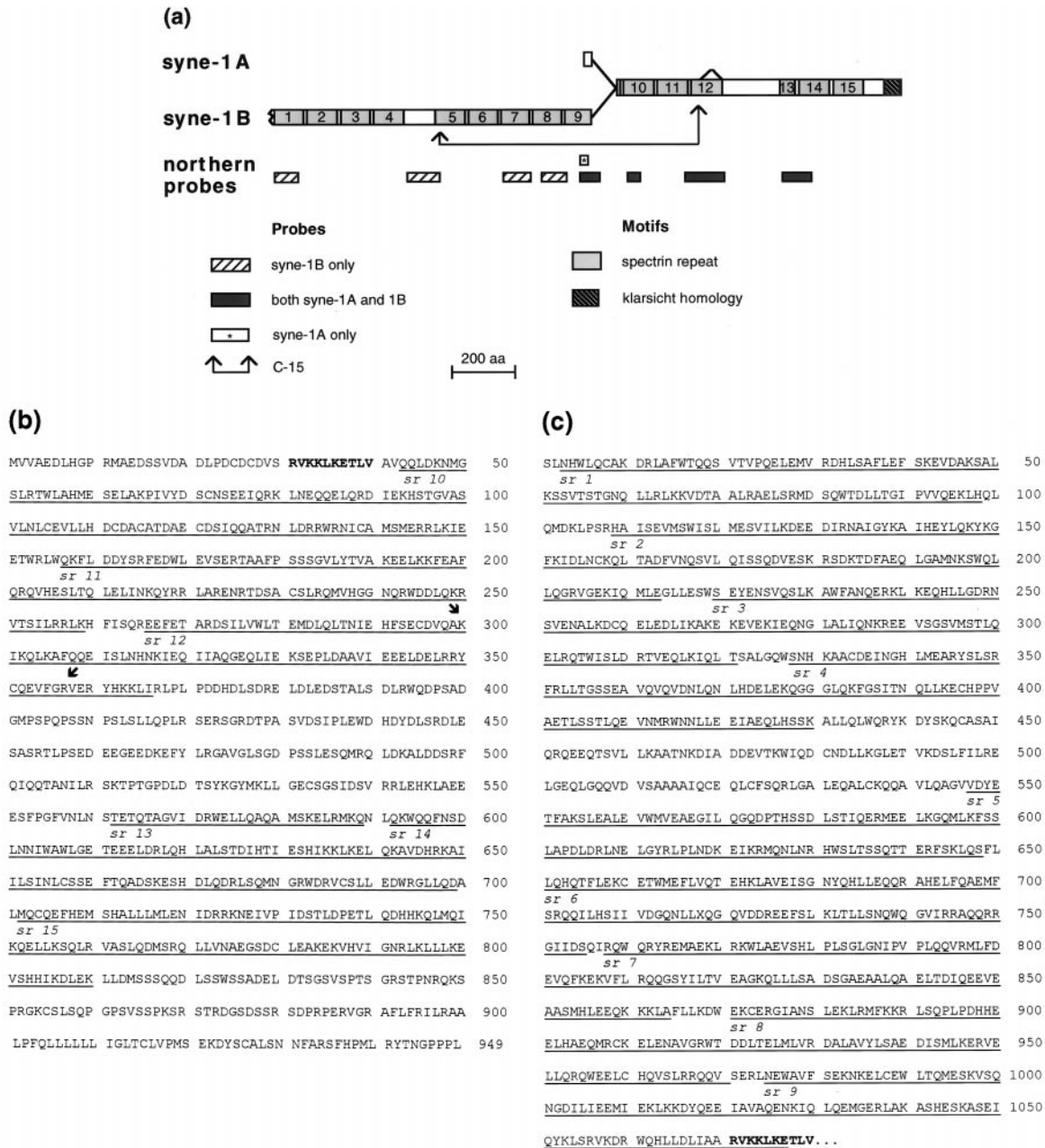
cessed in the same manner, except that rinsing solution and fixative were warmed to 37 °C before being added to the cells.

## RESULTS

**A Screen for Components of the Postsynaptic Apparatus**—To identify novel components of the postsynaptic apparatus at the NMJ, we used the yeast two-hybrid system. A bait was generated by fusing the DNA-binding domain of GAL4 to the cytoplasmic domain of MuSK, a receptor tyrosine kinase that is highly concentrated in the postsynaptic membrane (11) from the earliest stages of synaptic development into adulthood (17). The bait was used to screen a library prepared from fetal (E17) mice. Clones obtained in this screen were re-tested with each of four unrelated baits: the GAL4 DNA-binding domain alone, or the domain fused to lamin C, p53, or the intracellular domain of the Kv2.1 potassium channel. All positives interacted only with the MuSK-GAL 4 fusion demonstrating specificity of binding to the MuSK cytoplasmic domain (data not shown). In this paper, we focus on the gene initially identified by cDNA C-15 and later renamed synaptic nuclear envelope-1 (*syne-1*) for reasons described below.

**Differential Distribution and Cloning of Two Syne-1 RNAs**—Proteins capable of interacting in yeast might not actually be coexpressed *in vivo*. Because our aim was to identify components of the NMJ, we first used Northern analysis to ask whether genes identified in our screen were expressed in skeletal muscle. RNA was prepared from several adult tissues and from postnatal day (P) 2 limb muscle and brain. C-15 hybridized to an RNA of ~4.7 kb in skeletal and cardiac muscles, but in no other tissues tested. A ~10-kb C-15 reactive RNA was also present in numerous tissues (Fig. 1 and see below).

To determine which sequences corresponded to each RNA species, we probed Northern blots with short segments of the 2.1-kb C-15 cDNA. As shown in Fig. 2a, C-15 contained common sequences at its 3' end and ~10-kb specific sequences at its 5' end. This pattern suggested that C-15 was derived from the ~10-kb RNA, and that the two RNAs have distinct 5'



**FIG. 2. Primary sequence of Syne-1.** *a*, structure of Syne-1A and -1B, as determined by Northern analysis. Northern blots of RNA from cardiac or skeletal muscle were probed with sequences whose positions are indicated. Probes hybridized to ~4.7 and/or ~10 kb as shown. This analysis revealed that Syne-1A and -1B have common 3' sequences but distinct 5' sequences. *b*, primary sequence of Syne-1A, encoded by the ~4.7-kb RNA. *c*, partial sequence of Syne-1B, encoded by the ~10-kb RNA. Positions of spectrin repeats (*sr*) are indicated by numbered boxes *a*, and underlining in *b* and *c*. The first 10 aa common to Syne-1A and -1B are shown in **bold** in *b* and *c*. *Arrowheads* in *b* indicate sequence deleted in an alternatively spliced form encoded by one cDNA. Genbank accession numbers for mouse Syne-1A and Syne-1B are AF281869 and AF281870, respectively.

termini, which might arise either by alternative splicing or from separate promoters. To obtain ~4.7-kb specific sequence, we used the 5'-most common sequences to probe cDNA libraries from embryos, skeletal muscle, and heart. One cDNA obtained from this screen contained 233 base pairs of ~4.7-kb specific sequence. Other cDNAs recovered an additional 1.8 of ~10-kb specific sequence, and still others extended the 3' common sequence by 2.3 kb to a termination codon and putative 3'-untranslated region.

**Deduced Sequence of Syne-1 Proteins**—Together, the cDNAs we obtained encode at least 3 proteins (Fig. 2, *b* and *c*). The first is encoded by the ~4.7-kb RNA. It is 949 aa long, comprising 30 aa of ~4.7-kb specific sequence and 919 aa of common sequence. The open reading frame begins with a methionine that

is embedded in a consensus Kozak sequence and preceded by stop codons in all three frames. We refer to this form as Syne-1A. The second deduced protein is encoded by the ~10-kb RNA, and consists of >1989 aa, comprising >1070 aa of ~10-kb specific sequence plus the 919 aa of common sequence. We refer to this form as Syne-1B. We have not yet cloned the 5' end of the RNA that encodes Syne-1B. Finally, one cDNA that contained sequences common to Syne-1A and -1B lacked a 174-base pair stretch within the open reading frame. RNAs containing this sequence, which presumably arose by alternative splicing, would encode proteins lacking 58 aa in full-length forms. The position of this deletion is indicated in the Syne-1A sequence (Fig. 2*b*), but we do not know whether the resulting transcript is a variant of Syne-1A, Syne-1B or both.

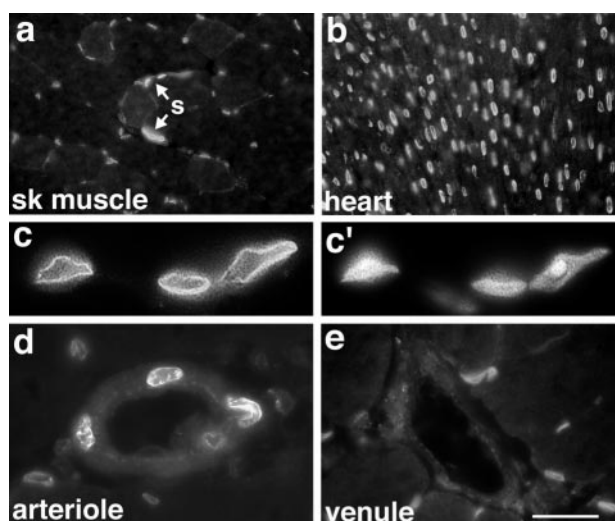
Analysis of the deduced Syne-1 sequence revealed two noteworthy features. The first, and more striking, was the presence of multiple “spectrin repeats” (22, 23). These ~100-aa long domains were first described in the cytoskeletal protein, spectrin; they have subsequently been found in numerous rod-shaped proteins that are components of or associated with the cytoskeleton. These include homologues of spectrin, such as fodrin; dystrophin and its homologue, utrophin (24); protein kinase A-associated protein;  $\alpha$ -actinin; the *Drosophila* protein Kakapo; and its mammalian orthologue, ACF7/MACF/macrophin/trabeculin- $\alpha$  (25–27). Syne-1B contains 15 spectrin repeats, of these, the final 6 are present in Syne-1A. Because our Syne-1B sequence is incomplete, and spectrin repeats extend to the 5' end of the known sequence, additional repeats may be present in the full-length protein. Seven of the repeats are most closely related to those in dystrophin or utrophin. The others are most closely related to those in protein kinase A-associated protein, spectrin or Kakapo/ACF7; in these cases, however, dystrophin or utrophin is the second closest relative. Thus, on the whole, the closest previously characterized relatives of Syne-1 are dystrophin and utrophin.

The carboxyl-terminal 60 aa of Syne-1 show a significant homology (41% identity, 58% similarity) to the carboxyl terminus of a *Drosophila* protein called Klarsicht (28, 29). This protein, which is otherwise devoid of recognizable motifs, is associated with nuclei in photoreceptor cells of flies. The similarity between Klarsicht and Syne-1 is modest in extent, but may be biologically significant in view of the nuclear localization of Syne-1 and the sequence of its homologues, described below.

**Association of Syne-1 with Nuclear Envelopes in Muscle Fibers**—We generated antisera to a bacterially produced fragment of Syne-1, then used the affinity purified antibodies to determine the subcellular localization of Syne-1. The fragment included 267 aa common to Syne-1A and -1B, and would therefore be expected to recognize both forms. When applied to cryostat sections of adult mouse skeletal muscle, anti-Syne-1 stained small elliptical structures that lay just beneath the plasma membrane of muscle fibers (Fig. 3*a*). The size, shape, and subsarcolemmal location of these structures suggested that they were myonuclei. This identity was confirmed by double staining with the DNA-binding dye, 4',6-diamidino-2-phenylindole. High magnification views suggested that Syne-1 is associated with the nuclear envelope (Fig. 3, *c* and *c'*).

Syne-1 was also associated with nuclei in cardiac myocytes (Fig. 3*b*) and in smooth muscle cells associated with intramuscular arterioles (Fig. 3*d*). On the other hand, nuclei were Syne-negative in a variety of non-muscle cells found within cardiac and skeletal muscles, including fibroblasts, glial (Schwann) cells of intramuscular nerves, or the cells of capillaries and venules (Fig. 3*e* and data not shown). Likewise, only faint cytoplasmic staining was observed in adult brain and kidney or in embryonic tissues other than muscles (data not shown). Thus Syne-1 is associated with nuclei in muscles of all three lineages (skeletal, smooth, and cardiac) but not in non-muscle cell types tested to date.

**Preferential Association of Syne-1 with Synaptic Nuclei**—As described under the Introduction, the few myonuclei apposed to the postsynaptic membrane at the NMJ are morphologically and functionally specialized. All myonuclei were faintly Syne-1-positive in adult skeletal muscles, but a small subset were stained far more intensely than the others (arrows in Fig. 3*a*). Because Syne-1 was isolated by virtue of its ability to interact with a synaptic protein, MuSK, we wondered whether the intensely stained nuclei were synaptic. To test this possibility, we double-stained sections with anti-Syne-1 plus rhodamine



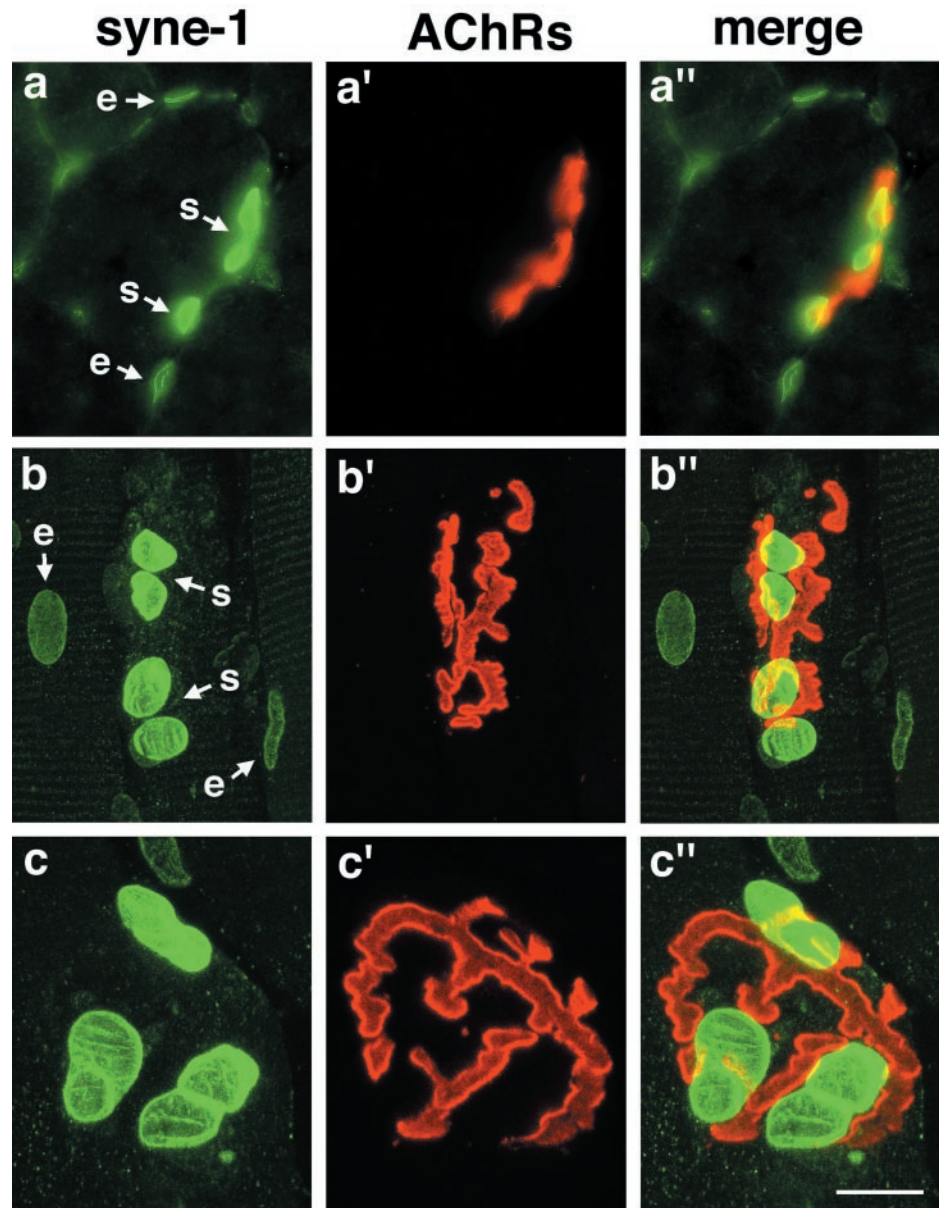
**FIG. 3. Syne-1 is associated with nuclear envelopes in muscle cells.** Cryostat sections of adult skeletal (*a* and *c-e*) and cardiac (*b*) muscle were stained with anti-Syne-1. The section in *c* was doubly stained with the DNA-binding dye, 4',6-diamidino-2-phenylindole (*c'*). Nuclei are labeled in skeletal and cardiac muscle fibers and in smooth muscle cells associated with intramuscular arterioles (*d*). In contrast, nuclei in venules are unstained (*e*). Arrow in *a* indicates position of a synaptic sites, as determined by counterstaining with rhodamine  $\alpha$ -bungarotoxin (see Fig. 4). Bar in *e* is 100  $\mu$ m for *a* and *b*; 25  $\mu$ m for *c*; and 45  $\mu$ m for *d* and *e*.

$\alpha$ -bungarotoxin, a toxin that binds tightly and specifically to AChRs in the postsynaptic membrane. The brightest Syne-1-positive nuclei in each section were invariably associated with synaptic sites (Fig. 4*a*). Confocal microscopy of double-labeled longitudinal sections confirmed that clusters of synaptic nuclei were intensely Syne-1-positive, whereas nearby nuclei in the same myofibers were only faintly Syne-1-positive (Fig. 4, *b* and *c*). Perisynaptic nuclei (those near to synapses) did not exhibit an intermediate level of staining, but rather resembled other extrasynaptic nuclei in their apparent level of Syne-1 immunoreactivity.

**Localization of Syne-1 in Developing and Denervated Muscles**—Synapse-associated myonuclei become transcriptionally specialized by E15, soon after synapses form (30, 31), and nuclear clusters form beneath the postsynaptic apparatus soon after birth (32). As a first step in determining whether Syne-1 might be involved in either of these developmental steps, we asked when Syne-1 became associated with synaptic and extrasynaptic nuclei. At E15, P0, and P7 all myonuclei were Syne-1 positive, but synaptic nuclei did not stain more intensely than extrasynaptic nuclei (Fig. 5*a* and data not shown). Some enrichment of Syne-1 in synaptic nuclei was evident by P14, and by the end of the first postnatal month, synaptic nuclei appeared richer in Syne-1 than any extrasynaptic nuclei in the same muscle fibers (Fig. 5*b* and data not shown). Thus high levels of Syne-1 are not required for transcriptional specialization or aggregation of synaptic nuclei, but might be involved in the maintenance of their specialized characteristics.

In embryos, Syne-1 appeared to be associated with nuclei of myotubes but not of myoblasts. However, distinction between these two cell types is difficult in cryostat sections. We therefore stained cultured myoblasts and myotubes of the myogenic cell line, Sol 8. Sol 8 myoblasts proliferate in rich medium, but fuse to form myotubes following withdrawal of serum (see “Experimental Procedures”). Nuclei in myotubes were clearly Syne-1 positive, whereas Syne-1 was barely detectable in nuclei of myoblasts (Fig. 5, *d* and *e*). Thus, Syne-1 may be up-regulated as or soon after myotubes form.

Although the entire postsynaptic apparatus is organized by



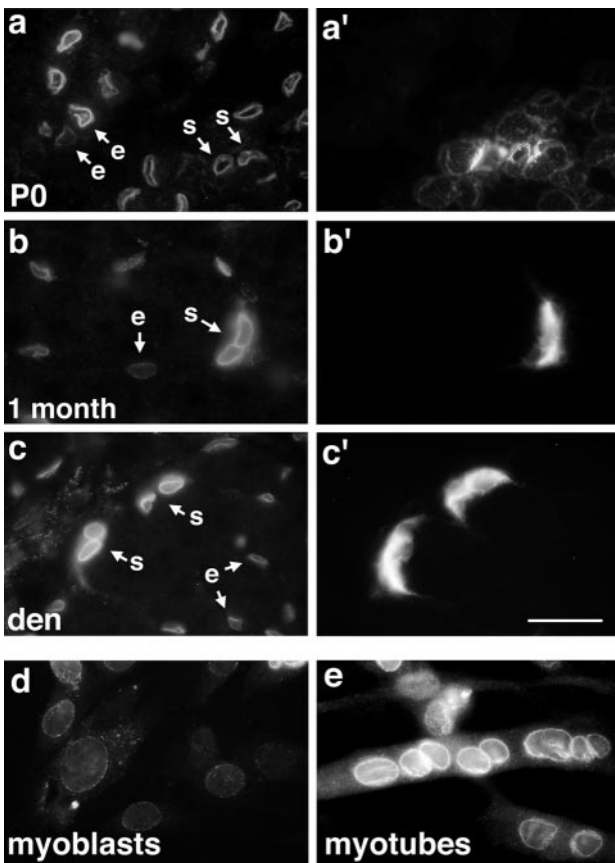
**FIG. 4. Syne-1 levels are higher in synaptic (s) than in extrasynaptic (e) nuclei of adult muscle fibers.** Cross-sections (*a*) or longitudinal sections (*b* and *c*) of adult muscle were double-stained with anti-Syne-1 (*a-c*) and rhodamine  $\alpha$ -bungarotoxin (*a'-c'*), then viewed in a compound (*a*) or confocal (*b* and *c*) microscope. Images are merged in *a''-c''*. Bar in *a* is 25  $\mu$ m for *a*; 30  $\mu$ m for *b*, and 20  $\mu$ m for *c*.

signals emanating from the motor nerve, some postsynaptic specializations are lost within days following denervation, whereas others are stable for weeks to months (reviewed in Ref. 7). To ask whether the selective association of Syne-1 with synaptic nuclei requires the continuous presence of the nerve, we stained muscles 3, 7, or 14 days following denervation. At all intervals, synaptic nuclei remained significantly richer in Syne-1 than extrasynaptic nuclei (Fig. 5c and data not shown).

**Localization of Syne-1 in Mutant Muscles**—Genetic and biochemical studies in mice have shown that the tyrosine kinase MuSK and the AChR-associated protein rapsyn are required for formation of the postsynaptic apparatus. No detectable synaptic differentiation occurs in the absence of MuSK (14). Because Syne-1 interacts with MuSK in non-muscle cells, it was possible that its accumulation or association with muscle nuclei was MuSK-dependent. To test this possibility, we stained muscles from neonatal  $\text{MuSK}^{-/-}$  mice with anti-Syne-1 antibody. Levels of Syne-1 did not differ detectably between  $\text{MuSK}^{-/-}$  mice and littermate controls, nor was association of Syne-1 with nuclei impeded in the absence of MuSK (Fig. 6a). Likewise, levels and nuclear association of Syne-1 were normal in neonatal mice lacking rapsyn (Fig. 6b), a component of the

postsynaptic cytoskeleton that associates with both MuSK and AChRs and is necessary for AChR clustering in the postsynaptic membrane (17, 33, 34). Unfortunately, both  $\text{MuSK}^{-/-}$  and  $\text{rapsyn}^{-/-}$  mice die at birth, so it was not possible to determine whether some nuclei became Syne-rich in the absence of other synaptic specializations.

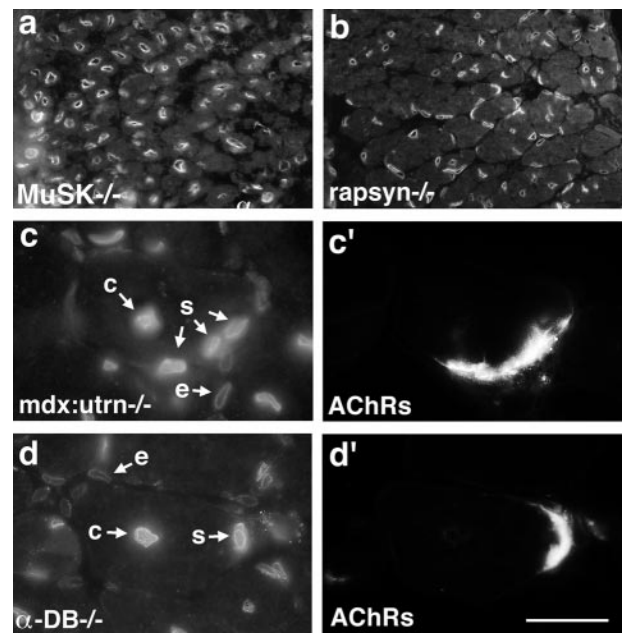
A second protein complex, the dystrophin-glycoprotein complex (DGC) is dispensable for formation of the NMJ, but required for its maturation and maintenance (35). Moreover, the DGC is critical for muscle stability, as shown by the dystrophic phenotype that results from mutation of each of several DGC components (36). Because Syne-1 becomes concentrated in synaptic nuclei as the NMJ matures, we wondered whether this concentration was DGC-dependent. We therefore examined the localization of Syne-1 in three strains of mutant mice lacking DGC components. The first were *mdx* mice, which lack dystrophin. These mutants have a moderate muscular dystrophy, but few synaptic defects. The second lacked the cytoplasmic DGC component,  $\alpha$ -dystrobrevin, which is critical for synaptic maturation. These mutants display a mild dystrophy as well as defects in maturation of the postsynaptic apparatus (35, 37). Finally, we examined double mutant mice lacking both dystro-



**FIG. 5. Syne-1 in developing and denervated muscle.** Synaptic (g) and extrasynaptic (e) nuclei show similar levels of Syne-1 at P0 (a), but synaptic nuclei were more intensely stained than extrasynaptic nuclei by 1 month of age. b, selective staining persisted following denervation for 7 (c) or 14 days (not shown). Sections in a-c were counterstained with rhodamine  $\alpha$ -bungarotoxin (a'-c'). d and e, levels of Syne-1 are far higher in myotubes (e) than in myoblasts (d) of the Sol 8 cell line. Bar in c is 50  $\mu$ m for all parts.

phin and its autosomal homologue, utrophin (38). The DGC is largely disassembled in these mice, which display synaptic defects similar to those in  $\alpha$ -dystrobrevin mutants but a much more severe muscular dystrophy. In all three mutant strains, synaptic nuclei were more intensely Syne-1 positive than extrasynaptic nuclei (Fig. 6, c and d, and data not shown). Thus, accumulation of Syne-1 at synapses is not DGC-dependent. Interestingly, however, the central nuclei present in regenerated fibers of all three genotypes were strongly Syne-1 positive (Fig. 6, c and d).

**Domains Required for Association of Syne-1 with Nuclei**—We used cultured cells to map the domain(s) that mediate the association of Syne-1 with nuclei. A FLAG epitope tag was added to the amino terminus of Syne-1A, and an expression vector encoding this fusion protein was introduced into Sol 8 myoblasts, Sol 8 myotubes, or QT-6 fibroblasts. Cells were then permeabilized and stained with a monoclonal antibody specific for the FLAG peptide. Recombinant Syne-1A was associated with nuclear envelopes in all three cell types (Fig. 7, b-d). Similar results were obtained with a FLAG-tagged construct that included all sequences common to Syne-1A and -1B, as well as some Syne-1B-specific sequences (Syne-1B $\Delta$ N in Fig. 7a; data not shown). In contrast, a Syne-1 construct lacking the carboxyl-terminal 710 amino acids was associated with nuclei in myotubes, but formed puncta throughout the cytoplasm of myoblasts and fibroblasts (Syne-1B $\Delta$ N $\Delta$ C;  $\Delta$ Fig. 7, e-g). The antibody to Syne-1 stained transfected cells in a pattern identical to that shown for anti-FLAG, but did not detectably stain



**FIG. 6. Syne-1 in mutant muscles.** a, P0 MuSK<sup>-/-</sup>. b, P0 rapsyn<sup>-/-</sup>. c, adult mdx:utrophin<sup>-/-</sup>. d, adult  $\alpha$ -dystrobrevin<sup>-/-</sup>. Syne-1 is present in myonuclei of muscles that lack MuSK or rapsyn. These mutants die at birth, before Syne-1 becomes synaptically concentrated in control muscle (see Fig. 5). Syne-1 is concentrated in synaptic nuclei(s) of muscles that lack dystrophin plus utrophin or  $\alpha$ -dystrobrevin. Concentrations of Syne-1 are also apparent in central nuclei (c) of regenerating myotubes in these mutants (arrows in c and d). Bar in d' is 60  $\mu$ m for all parts.

untransfected fibroblasts (data not shown). Together these results suggest that two distinct domains are required for nuclear localization of Syne-1. A carboxyl-terminal region, which includes the region of homology with the nucleus-associated protein Klarsicht (see above), is required for association of Syne-1 with nuclei in fibroblasts and myoblasts. However, additional sequences in the central region are sufficient to mediate association of Syne-1 with nuclei in myotubes.

Finally, we used transfected QT-6 fibroblasts to re-examine the association of Syne-1 with MuSK. In one set of experiments, QT-6 cells were transfected with expression vectors encoding MuSK, MuSK plus FLAG-tagged Syne-1A, or MuSK plus a FLAG-tagged control protein, NAB-1 (39). Cultures were lysed in nondenaturing detergent and subjected to immunoprecipitation with anti-FLAG; precipitated proteins were separated by gel electrophoresis and blots were probed with anti-MuSK. MuSK was clearly detectable in precipitates from cells that had been transfected with MuSK plus FLAG-Syne-1A, but not in precipitates from cells transfected with MuSK alone or MuSK plus FLAG-NAB-1 (Fig. 8). Thus, Syne-1 and MuSK can interact not only in yeast nuclei, but also in the cytoplasm of vertebrate cells. Second, we immunostained QT-6 fibroblasts that had been transfected with expression vectors encoding Syne-1 and/or MuSK, to ask whether expression of either protein affected the subcellular distribution of the other. In some experiments, vectors encoding rapsyn and/or AChRs were also co-transfected, based on the previous finding that rapsyn can co-cluster MuSK and AChRs (17, 34, 40). Neither MuSK nor Syne-1 detectably affected the distribution of its partner in this assay (data not shown).

**Orthologues and Homologues of syne-1**—A search of public data bases revealed several sequenced but uncharacterized cDNAs related to syne-1. A human cDNA called KIAA0796 was sequenced by Nagase *et al.* (41) as part of a project to identify novel genes expressed in human brain. The protein encoded by

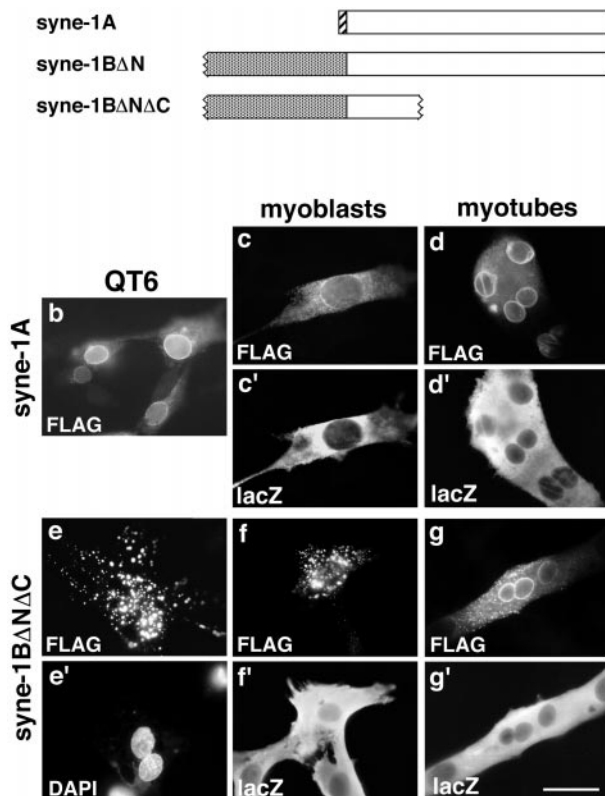


FIG. 7. Localization of recombinant Syne-1 in nuclei of Sol 8 myoblasts, Sol myotubes, and QT-6 fibroblasts. *a*, diagram of three epitope- (FLAG-) tagged constructs transfected into cultured cells. *b-g*, examples of cells co-transfected with vectors encoding  $\beta$ -galactosidase and either Syne-1A (*b-d*) or Syne-1B $\Delta$ N $\Delta$ C (*e-g*). Cells were permeabilized and stained with anti-FLAG. Cultures were counterstained with either anti-LacZ or DAPI as indicated (*b'-g'*). Localization of Syne-1B $\Delta$ N was similar to that of Syne-1A (not shown). Bar in *g'* is 30  $\mu$ m for *b-g*.

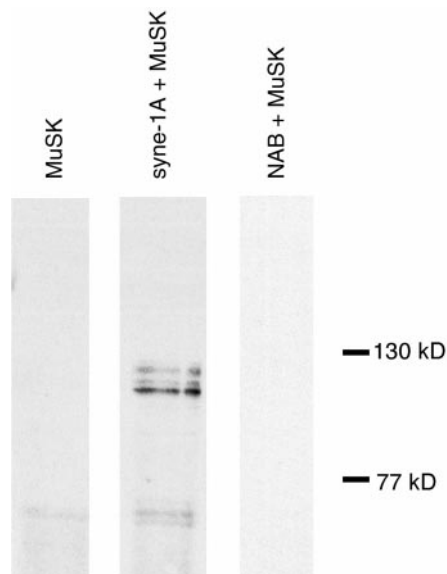


FIG. 8. Co-immunoprecipitation of MuSK with FLAG-tagged Syne-1 in co-transfected QT-6 cells (second lane). Lysates were incubated with anti-FLAG, and precipitates were probed with anti-MuSK. No MuSK was detected when cells were transfected with MuSK and FLAG-tagged-NAB-1 (third lane) or with MuSK alone (first lane).

KIAA0796 is 82% identical to aa 913–1989 of *syne-1B* (Fig. 9*a*). Based on this close relationship, we believe that KIAA0796 is the human orthologue of *syne-1B*, and refer to it as h-*syne-1B*.

Three additional cDNAs from human brain, identified in

large scale sequencing projects (KIAA1011, AL080133, and AL117404; Ref. 42), are closely related to human *syne-1*, but clearly derived from a distinct gene (Fig. 9*a*). Overlap among these cDNAs indicates that they are all derived from the same gene. Their sequence encodes a protein that is 49% identical (62% similar) to aa 1–1103 of h-*syne-1B*, and 46% identical (61% similar) to the corresponding stretch of mouse *syne-1B*. We refer to this putative protein as h-*syne-2*. Differences among *syne-2* cDNAs (*boldface* in Fig. 9*a*) indicate that this gene is subject to alternative splicing. Syne-2, like Syne-1 bears multiple spectrin repeats and a carboxyl-terminal segment that is homologous to the carboxyl terminus of Klarsicht. Importantly, Syne-2 is as similar to Klarsicht as is Syne-1 (Fig. 9*b*).

Northern analysis revealed the presence of an  $\sim$ 4.7-kb *syne-1* RNA in adult human skeletal muscle and heart, as well as a less abundant  $\sim$ 10-kb RNA in multiple tissues (Fig. 10*a* and data not shown). Thus, the distribution of Syne-1 is similar in mouse and human. However, *syne-2* RNA was undetectable in adult tissues by this method (data not shown). We therefore used a more sensitive method, RT-PCR, to assess the distribution of human *syne-1* and -2 RNAs in a panel of adult and fetal human tissues. As shown in Fig. 10*b*, for both *syne-1* and *syne-2*, two distinct PCR products were evident whose relative proportions varied among tissues. Sequencing of the purified products indicated that the variants differed by a single stretch of 69 nucleotides. This stretch occurred at the same position in both *syne-1* and *syne-2*, and corresponded to one of the alternatively spliced segments previously identified in *syne-2* by comparison of cDNAs (aa 652–675 in Fig. 9*a*). Thus, the similarity between Syne-1 and Syne-2 extends to a conserved pattern of alternative splicing.

RT-PCR revealed that *syne-1* and *syne-2* were expressed in overlapping but distinct patterns. For example, both genes were expressed in skeletal and cardiac muscle, but only *syne-1* was expressed in lung whereas only *syne-2* was expressed in placenta. Levels of *syne-2* RNA were lower than those of *syne-1* in most tissues, consistent with results from Northern analysis.

## DISCUSSION

In a screen for novel components of the postsynaptic apparatus at the NMJ, we identified a protein that we have named Syne-1. Syne-1 is associated with nuclear envelopes throughout muscle fibers, but is present at highest levels in the few specialized myonuclei that lie beneath the postsynaptic membrane. Syne-1 is the first protein shown to be concentrated in synaptic nuclei. Although its most prominent structural feature is a group of dystrophin-like spectrin repeats, a segment of Syne-1 is related to Klarsicht, a protein associated with nuclei and involved in nuclear migrations in *Drosophila*. Together, the structure and distribution of Syne-1 raise the possibility that it is involved in the migration and/or anchoring of myonuclei. The homologue of Syne-1, Syne-2, might also be involved in nuclear localization.

*Syne-1 and Myonuclei*—Syne-1 is associated with nuclear envelopes in skeletal, cardiac, and smooth muscle cells. Nuclear localization appears to involve at least two domains. Full-length Syne-1A associates with nuclei in multiple cell types whereas a truncated protein lacking a carboxyl-terminal segment localizes to nuclei in myotubes but not in myoblasts or fibroblasts. Interestingly, this segment includes a short stretch that is homologous to the predicted product of the *Drosophila klarsicht* (previously called *marbles*) gene (28, 29, 43). *Klarsicht* function is required for the basal to apical migration of nuclei in developing fly retinal photoreceptors, as well as for the central to apical transport of lipid droplets in gastrulating embryos. Moreover, Klarsicht protein is described as being





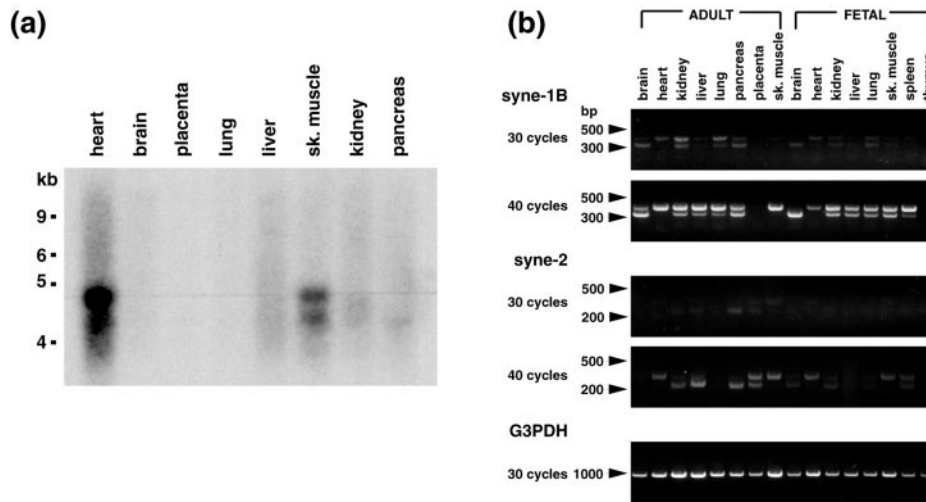


FIG. 10. **Expression of human syne-1 and -2.** *a*, Northern analysis of syne-1 expression in adult human tissues. As seen in mouse (Fig. 1), 4.7 kb RNAs were prominent in human skeletal muscle and heart. A less abundant ~10-kb RNA was detectable in multiple tissues in longer exposures (not shown). *b*, RT-PCR analysis of syne-1 and -2 expression in adult and fetal human tissues. In each case, products were analyzed after 30 and 40 cycles, to provide a measure of relative abundance. Paired bands reflect the existence of alternatively spliced forms that differ by 69 base pairs (see Fig. 9*b*). The fifth line shows expression of the ubiquitously expressed gene, glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) to show that similar amounts of template were amplified from each tissue.

and specialized in the postsynaptic apparatus (51, 52). In addition, syne-1 might be present in the postsynaptic cytoskeleton, bound to anchoring proteins that in turn bind nuclei. Indeed, Couteaux (53) has demonstrated the existence of filament bundles that stretch from the cytoplasmic surface of the postsynaptic membrane to the envelopes of synaptic nuclei. In either model, Syne-1 might be involved in nuclear migration in developing myotubes as well as in nuclear anchoring at synapses; similar molecular interactions could lead to migration or anchoring, depending on the nature and mobility of the proteins with which Syne-1 interacts in each location. The late postnatal increase in Syne-1 levels in synaptic nuclei, which occurs after nuclear clustering (32), might be involved in the stabilization of synaptic structure that is known to occur following the period of early postnatal growth and synaptic rearrangement (7).

**Syne-1 and MuSK**—We isolated Syne-1 by virtue of its association with the cytoplasmic domain of MuSK in yeast, and showed that a similar association occurs in transfected vertebrate cells. Moreover, both Syne-1 and MuSK are selectively associated with the postsynaptic apparatus of the NMJ. Yet at the synapse, MuSK is concentrated in the plasma membrane whereas Syne-1 is concentrated in nuclear envelopes. Do MuSK and Syne-1 interact *in vivo*? It is unlikely that membrane-associated MuSK binds directly to nuclear Syne-1 because MuSK is concentrated at the crests of the ~1  $\mu$ m-deep junction folds whereas nuclei lie beneath the folds. However, MuSK might be translocated to the nucleus under some circumstances or low levels of Syne-1 might be present at the postsynaptic membrane. There are now well documented cases in which barely detectable nuclear pools of a predominantly surface-associated protein (*e.g.* Notch), or barely detectable surface-associated pools of a predominantly intracellular protein (*e.g.* presenilin) are essential for biological function (54). Indeed, when we overexpress MuSK by transfection in muscle cells, we consistently observe immunoreactivity associated with nuclear envelopes, although attempts to increase this fraction by co-expression of Syne-1 have been unsuccessful.<sup>2</sup> Likewise, some Syne-1 is extranuclear in transfected cells (Fig. 8). At present, however, we cannot rule out the possibility that MuSK-Syne-1

interactions do not, in fact, occur *in vivo*.

Interestingly, a similar issue of subcellular localization has arisen with respect to the role of Klarsicht in the *Drosophila* eye (29). There, Klarsicht is associated with nuclei, yet the polarity of microtubules and distribution of dynein predicts that bioactive Klarsicht should be tethered to the apical plasma membrane, where it could act to “reel in” nuclei. The authors speculate that a small pool of Klarsicht is located apically, and that membrane-associated and nuclear Klarsicht both play roles in nuclear migration. A similar membrane/nuclear dual localization of Syne-1 might occur at the NMJ.

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**Syne-1, A Dystrophin- and Klarsicht-related Protein Associated with Synaptic Nuclei at the Neuromuscular Junction**

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