Regulated alternative splicing of avian cardiac troponin T (cTNT) pre-mRNA requires multiple intronic elements called muscle-specific splicing enhancers (MSEs) that flank the alternative exon 5 and promote muscle-specific exon inclusion. To understand the function of the MSEs in muscle-specific splicing, we sought to identify trans-acting factors that bind to these elements. MSE3, which is located 66–81 nucleotides downstream of exon 5, assembles a complex that is both sequence- and muscle-specific. Purification and characterization of the MSE3 complex identified one component as 5-aminooimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (PurH), an enzyme involved in de novo purine synthesis. Recombinant human PurH protein directly binds MSE3 RNA and PurH is the primary determinant of sequence-specific binding in the native complex. Furthermore, we show a direct correlation between the in vitro binding affinity of both the MSE3 complex and recombinant PurH with functional activation of exon inclusion in vivo. Together, these results strongly suggest that PurH performs a second function as a component of a complex that regulates MSE3-dependent exon inclusion.

Alternative splicing allows single genes to express multiple mRNAs. Splice site selection is often regulated in a cell-specific manner resulting in regulated expression of different protein isoforms (1–3). Genetic and biochemical studies in Drosophila have identified specific cis elements and trans-acting factors which mediate cell-specific splicing events (4). In vertebrates, cis elements that mediate cell-specific splicing events have been identified (5–12). Factors that bind to some of these elements have also been identified (13–18), but it remains unclear how these factors mediate cell-specific splicing events.

We are using the chicken cardiac troponin T (cTNT) gene to investigate the mechanisms of regulated splicing in striated muscle. cTNT expression is restricted to embryonic skeletal muscle and to embryonic and adult cardiac muscle. Exon 5 undergoes developmentally regulated splicing such that inclusion predominates in embryonic skeletal and cardiac muscle and skipping predominates in the adult (19). We have previously identified four cis-acting elements in the introns flanking exon 5 that function as muscle-specific splicing enhancers (MSEs) by transient transfection analysis of cTNT flanking exon 5 (5, 6). The MSEs are necessary for higher levels of exon inclusion in muscle cells than in fibroblast cells. Mutation of these elements causes exon skipping in muscle cells but has little effect on splicing in fibroblasts. These results have defined exon skipping as the default splicing pattern and indicate that exon inclusion requires positive-acting trans-factors present in muscle.

The four MSEs are designated 1 to 4. MSE1 is located in intron 4, immediately upstream of exon 5. MSE2, 3, and 4 are located in the first 130 nucleotides of intron 5 (5). MSE3 includes nucleotides 66–81 of intron 5 and was originally identified because of its conservation in sequence and position in four genes that undergo a similar pattern of developmentally regulated alternative splicing in muscle (Ref. 5 and data not shown). Six copies of MSE3 can functionally replace MSEs 2–4 for regulated splicing in transient transfection analysis of splicing minigenes indicating the importance of MSE3 as a target for muscle-specific regulatory factors (6).

Here we show that a sequence- and cell-specific complex is formed on MSE3 RNA in an electrophoretic mobility shift assay (gel-shift) in nuclear extracts from embryonic muscle tissue or tissue extracts from adult muscle tissue but not in nuclear extracts from fibroblast or HeLa cells. UV cross-linking analysis demonstrates that two proteins of approximately 40 and 70 kDa bind directly to MSE3 RNA. We have purified MSE3 binding activity from both embryonic and adult muscle and have identified the 70-kDa protein as 5-aminooimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (PurH), an enzyme involved in purine biosynthesis (20). Recombinant PurH binds MSE3 RNA with the same sequence specificity as the MSE3 complex purified from muscle. However, PurH alone forms a complex that is smaller than the MSE3 complex suggesting that the MSE3 complex contains additional proteins. The binding affinities of the MSE3 complex and PurH to five different sequences in vitro correlate with splicing enhancer activities of these sequences in vivo supporting a model in which binding of the PurH containing complex to MSE3 contributes to muscle-specific exon inclusion.

EXPERIMENTAL PROCEDURES

Cloning

Plasmids for in vitro transcription of MSE3 and MSE3m RNAs were constructed using the oligos CON (GTTGTTTCTGGTCGTTTCCCGTCTTATGATG) and MCON (GTTGACGACGATGACGCCTCCTCG-CTGATGATG), respectively. Oligos were kinased using polynucleotide kinase, and second strand synthesis was carried out by annealing the oligo CONR (ATCATCTAGACG) to both oligos and extending with

* This work was supported by National Institutes of Health Grant HL45565. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: cTNT, cardiac troponin T; MSE, muscle-specific splicing enhancer; RT-PCR, reverse transcriptase-polymerase chain reaction; DD, Dignam D buffer; MWCO, molecular weight cut-off; PAGE, polyacrylamide gel electrophoresis.
T4 DNA polymerase (21). The double stranded DNA was digested using XbaI. The resulting fragments were ligated into SacI (blunted using T4 DNA polymerase) and XbaI sites of Bluescript KS*-.

Plasmid templates for randomized MSE3-based RNAs were constructed by cloning PCR products which included the T7 RNA polymerase promoter and the MSE3 oligonucleotide (22). The plasmids were used to transcribe RNAs that are identical to MSE3 RNA except for 14 randomized nucleotides indicated as “N” in the sequence of CON/sel oligo, below and Fig. 9A. These positions were chosen since mutation of these positions inactivated MSE3 in vivo (5). Template plasmids were generated using CONssel oligo (5'-GGGGCAATTTGAGCTGNN(T)(C)(T)(G)CACTACACATAGGG-3') and 3SEL (5'-CTAGCGACCCG-3'). The PCR reaction was performed using 3.5 units Tag polymerase and buffer (Promega) with 2 mM MgCl₂ and 0.2 mM dNTPs. 30 cycles of the following PCR was performed: 1 min at 95 °C, 30 s at 54 °C, and 15 s at 72 °C. Following the last cycle an additional extension at 72 °C for 5 min was done. After PCR, the reactions were phenol/chloroform extracted and ethanol precipitated. The PCR product was treated with 26-gauge needle and rapidly pushed through cell volume of Dignam A (23) and allowed to swell on ice for 15 min. To lyse, the cells were drawn up in a syringe and rapidly pushed through a 26-gauge needle. This was repeated approximately 10 times. Nuclei were pelleted. The supernatant was removed and the nuclear pellet resuspended in 2/3 of the original packed cell volume of Dignam C. Extraction of nuclei was carried out on ice for 1.5 h with constant mixing. After extraction, nuclei were pelleted and the supernatant was diazylated against a modified Dignam D buffer (DG) that replaced the 100 mM KCl with 80 mM potassium glutamate (24).

Embryonic Muscle Nuclear Extract

Embryonic muscle nuclei were isolated from skeletal and heart muscle of 12 chicken embryos as described (25). Six to seven day embryos yielded 30-40 g of tissue. After obtaining the nuclear pellet, the proteins were extracted on ice with a roughly equal volume of Dignam C buffer containing 2 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 1.0 μg/ml aprotinin, and 0.7 μg/ml pepstatin for 1.5 h. Proteins were dialyzed against DG buffer containing protease inhibitors using a dialysis membrane with a molecular weight cut off (MWCO) of 3,500. A typical yield from 30 to 40 g of tissue was approximately 35 mg of total protein.

Adult Muscle Tissue Extract

Adult muscle extract was prepared from chickens based on the method of Skeek and Slater (26). Skeletal muscle (primarily breast with some leg) was isolated from freshly sacrificed adult hens. The tissue was placed into small pieces and homogenized in 30 mM KOH, 5 mM EDTA, and 1 mM dithiothreitol, pH ~4 (120 ml/100 g tissue) using a Waring blender. The homogenate was allowed to sit on ice for 15 min with occasional stirring. The homogenate was centrifuged for 20 min at 21,000 × g. The supernatant was poured off and saved, and the pelleted re-extracted in 2/3 of original volume for another 15 min. After centrifugation, both supernatants were pooled and passed through 4 layers of gauze. This crude extract was placed on ice and brought slowly to 40% (NH₄)₂SO₄ saturation with the addition of solid (NH₄)₂SO₄ (23 g solid/100 ml). After the addition was complete, stirring continued for an additional 30 min, then the precipitated proteins were removed by centrifugation at 15,000 × g for 30 min. The supernatant was brought to 65% (NH₄)₂SO₄ saturation (16.6 g solid/100 ml) as before and precipitated proteins recovered by centrifugation. The protein pellets were resuspended by rocking overnight in DG buffer without glycerol. The next day, the extract was dialyzed against DG buffer (which contains 20% glycerol) using a dialysis membrane with a MWCO of 3,500. For approximately 150 ml of extract, dialysis was carried out for a total of 10 h using 4 changes of 2 liters of buffer. Typically, 500 g of muscle tissue resulted in 140 ml of 40–65% extract with a protein concentration of 70–90 mg/ml.

Commercial Proteins

Rabbit muscle aldolase (A1893) and rabbit muscle pyruvate kinase (P7788) were purchased from Sigma. Before use, each was changed into the DG buffer using Ultrafree concentrating units with a MWCO of 10,000 (Millipore). The result of the buffer exchange (repeated dilution and concentration) was at least a 1:100,000-fold dilution of the original buffer.

Recombinant PurH

Recombinant human PurH and a pET-28a (Novagen) based expression plasmid (pET28a-hATIC) were kind gifts of S. Benkovic (Penn State University). For expression of recombinant protein, the h-ATIC plasmid was transformed into BL21 (DE3)/pLysS. Expression and purification of the His-tagged, recombinant protein was based on the manufacturer's instructions (Novagen pET system manual).

Gel-shift Analysis/Quantitation

Binding reactions were performed in 12.5-μl reactions using 45% extract and ~90,000 cpm of labeled RNA in the presence of 625 μM ATP, 25 mM MgCl₂, 25 mM creatine phosphate, 1 mM dithiothreitol, and 0.8% PEG. When competitor RNA was used, the competitor and labeled RNA were mixed prior to the addition of protein. Reactions were incubated at 30 °C for 15 min, placed on ice, and loaded directly onto a 6% acrylamide (55:1 acrylamide to bis) TGE (50 mM Tris, pH 7.5, 20 mM MgCl₂, 1.25 mM dithiothreitol, 4 μM each GTP, ATP, UTP, and CTP, 40 units of RNase, 6 μg of template DNA, and 800 units of T7 RNA polymerase. Reactions were incubated for 2 h at 37 °C then an additional 800 units of polymerase were added, and synthesis was continued for another 1–2 h. Reactions were phenol:chloroform extracted and ethanol precipitated. RNA was isolated from a denaturing polyacrylamide gel by UV shadowing. RNA was quantified by measuring absorbance at 260 nm.

Nuclear Extract from Fibroblasts

QT35 quail fibroblast cells were grown in F-10 media supplemented with 10% tryptose phosphate, 5% fetal calf serum, 1% chicken serum, 1% dimethyl sulfoxide, and 2 mM glutamine and then split 1:10. Cells were allowed to reach approximately 80% confluence in 100-mm plates prior to harvest. To harvest, the media was removed and plates were washed once and then scraped in ice-cold phosphate-buffered saline. During this time, plates were kept on ice. A cell pellet was obtained by centrifugation. Nuclear extract was prepared from the cells as described by Leon Col. (22). Briefly, the suspension was mixed with cell volume of Dignam A (23) and allowed to swell on ice for 15 min. To lyse, the cells were drawn up in a syringe and rapidly pushed through a 26-gauge needle. This was repeated approximately 10 times. Nuclei were pelleted. The supernatant was removed and the nuclear pellet resuspended in 2/3 of the original packed cell volume of Dignam C. Extraction of nuclei was carried out on ice for 1.5 h with constant mixing. After extraction, nuclei were pelleted and the supernatant was
Samples were UV irradiated 4 cm from a germicidal lamp (Phillips G15T8) for 6 min. Samples were digested with RNase Ti (0.5 μg) for 30 min at 37 °C. An equal volume of protein loading buffer was added to each sample, and samples were denatured at 100 °C. Proteins were resolved by 12.5% SDS-PAGE. Sizes were determined using precasted markers (Bio-Rad). For competition, the competitor RNA and the substrate were mixed prior to the addition of protein.

**Protein Purification**

**General**—All columns were run by gravity flow at 4 °C. Samples were loaded directly to the columns in DG buffer and eluted with NaCl in DG buffer without glycerol except where indicated. Samples were concentrated using Ultrafree units with a MWCO of 10,000. After each step in purification, samples were either extensively dialyzed against DG buffer using a dialysis membrane with 3,500 MWCO or by buffer exchange into DG buffer using the Ultrafree units. In the case of buffer exchange, there was at least a 1:20,000-fold dilution of the starting concentration.

**Embryonic**—Embryonic muscle nuclear extract was applied to a heparin-agarose column (Sigma or Bio-Rad) and washed with at least 5 ml volumes of DG buffer. The column was eluted with 100 μl NaCl and fractions containing protein pooled. The heparin-agarose fraction was loaded onto a Q-Sepharose (Amersham Pharmacia Biotech) column and binding activity was washed from the column without additional salt. The Q fraction was then applied to a poly(U)-agarose column (Sigma). The column was washed with at least 5 column volumes of DG buffer and eluted with 100 μl NaCl. This produced the final embryonic MSE3-binding fraction.

**Adult**—MSE3 binding activity was purified from a 40–65% ammonium sulfate fraction of adult muscle extract by modifying the scheme for purification from embryonic nuclear extract. Approximately 800 mg (10 ml) of the 40–65% fraction was loaded onto a 140-ml heparin-agarose column (Bio-Rad). The column was eluted with 100 mM NaCl in DG buffer containing 5% glycerol. Two-ml fractions were separated at 15 Watts constant power using a Rotorfor cell (Bio-Rad). Focusing was allowed to continue until the current remained constant for at least 20 min (approximately 4 h). Fractions were immediately tested for MSE3 binding activity and those with activity pooled.

Fractions from two isoelectric focusings were applied to a 140-ml Reactive Red 120 column (Sigma), and the column was washed with 250 mM NaCl until protein was no longer eluted as determined by measuring absorbance at 280 nm. The column was eluted with 750 mM NaCl, and fractions containing protein were pooled and saved.

The Reactive Red fraction was further separated on a 6-ml poly(U)-Sepharose column (Amersham Pharmacia Biotech). The column was eluted with a 70-ml 0–100 mM NaCl gradient in DG buffer containing 5% glycerol. Two-ml fractions were collected and assayed directly for MSE3 binding activity. Fractions containing the activity peaks were pooled, concentrated, and dialyzed.

The final step in purification of adult binding activity was to pool two poly(U) columns and apply them to a 6-ml heparin-agarose column (Bio-Rad). The column was eluted with a 70-ml linear gradient of 0–175 mM NaCl in DG buffer containing 5% glycerol. Two-ml fractions were collected, concentrated, and brought back to equal volumes during buffer exchange in DG buffer. Equal volumes were assayed by gel-shift and SDS-PAGE followed by Coomassie staining. Proteins were eluted from gels and protein sequencing was performed by the Protein Chemistry Facility at Baylor College of Medicine (Table 1).

**Transfections**—Calcium phosphate-mediated transfection into QT35 cells (fibroblasts) and primary embryonic skeletal muscle cultures, RNA extraction, and RTP-PCR have been previously described (6). Products of RT-PCR were quantitated directly from gels using a PhosphorImager.

Percent exon inclusion was calculated as [cpm exon inclusion / (cpm exon inclusion + cpm exon skipping)] × 100. Results presented are the average of at least three independent transfections.

**RESULTS**

**A Muscle- and Sequence-specific Complex Forms on a Muscle-specific Splicing Enhancer**—Trans-acting factors involved in regulating cTNT splicing are expected to bind the MSEs in a sequence-specific manner and to be present in nuclear extract from embryonic muscle cells, where exon inclusion is positively regulated, but absent in nuclear extract from QT35 quail fibroblast cultures or embryonic skeletal muscle tissue that were incubated with 10 fmol of labeled MSE3 RNA and 0, 2, 10, and 20 pmol of unlabeled wild type (MSE3) or mutant (MSE3m) competitor RNA. To reduce nonspecific binding to the RNA, heparin (0.48 mg/ml final concentration) was also added to the reactions. Complexes were separated on a native polyacrylamide gel. Sequences of MSE3 and MSE3m RNAs. Lowercase nucleotides are from the vector. Uppercase MSE3 or MSE3m are underlined.

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**FIG.1.** A sequence- and cell-specific complex is formed on MSE3 RNA. A, equal amounts of nuclear extract (22.4 μg of protein) from QT35 fibroblast cultures or embryonic skeletal muscle tissue were incubated with 10 fmol of labeled MSE3 RNA and 0, 2, 10, and 20 pmol of unlabeled wild type (MSE3) or mutant (MSE3m) competitor RNA. To reduce nonspecific binding to the RNA, heparin (0.48 mg/ml final concentration) was also added to the reactions. Complexes were separated on a native polyacrylamide gel. Sequences of MSE3 and MSE3m RNAs. Lowercase nucleotides are from the vector. Uppercase MSE3 or MSE3m are underlined.
to compete MSE3 binding (data not shown) further demonstrating sequence specificity. These results demonstrate that MSE3 forms a complex that is both sequence- and muscle-specific; therefore, the factors forming the MSE3 complex are strong candidates for regulating muscle-specific exon inclusion.

Two Proteins Bind Directly to MSE3 RNA—Using day 12 embryonic chicken muscle nuclear extract, an initial purification scheme for MSE3 binding activity was devised (Fig. 2A, "Experimental Procedures"). After each step in purification, fractions were assayed for MSE3 binding activity after each chromatography column. Sequence specificity was assayed using 10 fmol of labeled MSE3 RNA and competitions with 5 pmol of MSE3 or MSE3m RNA. Sequence specificity was assayed using 10 fmol of labeled MSE3 RNA and competions with 5 pmol of MSE3 or MSE3m RNA. MSE3 forms a complex that is both sequence- and muscle-specific manner. Proteins of approximately 70 and 40 kDa were competed more efficiently by wild type MSE3 RNA than mutant MSE3m RNA (Fig. 3). While cross-linking of proteins to MSE3 was very inefficient and could not be improved by using all four labeled nucleotides or 5-bromouridine in substrate RNA, the 70- and 40-kDa bands were consistently seen (data not shown). Furthermore, the levels of unlabeled RNA required to compete binding in the cross-linking assay are the same as the levels required for competition of the MSE3 complex in a parallel gel-shift assay (data not shown) which strongly suggests that the cross-linked proteins are components of the MSE3 complex.

MSE3 Binding Activity Is Also Present in Adult Muscle Tissue—We found MSE3 binding activity in a 40–65% ammonium sulfate fraction from adult muscle tissue whose MSE3 complex precisely comigrated with the MSE3 complex from embryonic extract. To determine if it was the same complex, the 40–65% ammonium sulfate fraction was subjected to the same series of chromatography columns as the embryonic nuclear extract and the resulting fractions were assayed with MSE3 RNA in a gel-shift assay (Fig. 4A). Sequence specificity of the adult MSE3 complex was tested using competitions with wild type and mutant RNA as above (Fig. 4B). The MSE3 complex present in extract from adult muscle tissue shares the same mobility, chromatographic properties, and sequence specificity as the complex originally isolated from embryonic muscle nuclear extract. Therefore, we conclude that the same MSE3 binding activity is present in both embryonic and adult muscle suggesting that quantitative differences and/or contributions of additional factors are responsible for developmental switch of exon 5 use (see "Discussion").

\[\text{FIG. 2. Purification of MSE3 binding activity from embryonic muscle.} A, \text{purification scheme of MSE3 binding activity from chicken embryonic striated muscle nuclear extract.} B, \text{fractions were assayed for MSE3 binding activity after each chromatography column. Sequence specificity was assayed using 10 fmol of labeled MSE3 RNA and competitions with 5 pmol of MSE3 or MSE3m RNA.}\]

\[\text{FIG. 3. UV cross-linking of the MSE3 complex.} \text{The poly(U) fraction from embryonic muscle nuclear extract was cross-linked to labeled MSE3 RNA with 0, 5, 10, and 25 pmol of wild type (MSE3) or mutant (MSE3m) competitor RNA in the binding reactions. Proteins were separated by SDS-PAGE on a 12.5% gel. Numbers are apparent molecular weights in kDa.} \]
Peptide sequencing was used to identify the three proteins copurifying with MSE3 binding activity through the final heparin-agarose column. All peptides had 100% identity to previously characterized proteins (Table I). The 40- and 61-kDa proteins are aldolase and pyruvate kinase, respectively. The 69-kDa protein is PurH, an enzyme which catalyzes the last two steps of de novo purine biosynthesis. PurH is the only protein of the three that was also identified by peptide sequencing in partially purified MSE3 binding activity from embryonic nuclear extract (data not shown).

**PurH Is Sufficient for Sequence-specific Binding of MSE3**—To determine the role of each protein in MSE3 complex formation, the proteins were individually tested for MSE3 binding activity and complex formation. Purified aldolase and pyruvate kinase, both from rabbit muscle, were purchased from Sigma. Recombinant human PurH (r-hPurH) was expressed in bacteria and purified using an N-terminal His tag (protein and expression plasmid from S. Benkovic, Penn State University). Increasing amounts of each protein were assayed for MSE3 binding activity in a gel-shift assay (Fig. 7). Of the three proteins identified in the fractions containing MSE3 binding activity, only r-hPurH was able to bind directly to MSE3. Although the complex formed by r-PurH was smaller than the MSE3 complex, it comigrated with a minor complex that became more prominent during purification suggesting that PurH is only one component of the MSE3 complex (Fig. 7, lanes 17 and 18). Addition of aldolase and pyruvate kinase to r-hPurH had no effect on the mobility of the complex formed by r-hPurH alone (Fig. 7, lanes 13–16). Like the MSE3 complex isolated from both embryonic and adult muscle, r-hPurH bound preferentially to the wild type MSE3 sequence (Fig. 8, compare lanes 1–5 and lanes 21–25).
The Binding Affinities of the MSE3 Complex and PurH Correlate with Enhancer Activity in Vivo—

MSE3 was originally defined as one of four MSEs required for enhanced exon inclusion in muscle using transfected splicing minigenes. Binding of the MSE3 complex to the wild type MSE3 sequence versus the MSE3m mutant correlates with the ability of MSE3 but not MSE3m to regulate exon inclusion in transfected minigenes (5). This result suggested that the MSE3 complex functions to increase exon inclusion during splicing of pre-mRNA. To provide additional evidence for this model, we sought to further correlate the binding affinity of the MSE3 complex with MSE3 splicing enhancer activity in vivo by using more variants of the MSE3 sequence.

To isolate sequences with a range of binding affinities for the MSE3 complex, the central 14 nucleotides of the MSE3 sequence were replaced with a random cassette (see "Experimental Procedures," and Fig. 9A). The affinity of individual sequences for the MSE3 complex was measured by their ability to compete MSE3 complex assembly in a Reactive Red fraction from adult muscle. The effect of each competitor on the assembly of the MSE3 complex was quantitated using a PhosphorImager and results are expressed as percent of total counts in the MSE3 complex normalized to no competitor in the reaction Fig. 9. Actual values varied somewhat between individual experiments, however, the order and fold difference between the different binding curves remained constant. Using the number of picomoles of competitor required to reduce the MSE3 complex by 50% as a measure of binding affinity, MSE3 has an approximately 10-fold greater affinity for the MSE3 complex than does the original MSE3m mutant. The mutant r3-51 has a 2-fold higher affinity for the complex than the natural MSE3 (0.2 versus 0.4 pmol) while r3-49 has a slightly lower affinity than MSE3 (0.7 versus 0.4 pmol). The r0-2 mutation has no apparent affinity for the MSE3 complex.

Six copies of MSE3 can replace MSEs 2–4 in transfected minigenes to regulate enhanced exon inclusion in muscle as long as MSE1 remains in the upstream intron; whereas, six copies of MSE3m are not able to regulate exon inclusion in vivo (6). The six copies of the natural MSE3 were replaced with six copies of the new mutant MSE3 sequences r0-2, r3-49, and r3-51. These new minigenes were tested by transient transfection in fibroblast and embryonic muscle cells and assayed for exon inclusion using RT-PCR (Fig. 10). In fibroblasts, both r3-51x6, containing concatamers of the highest affinity sequence, and r3-49x6, a sequence with an intermediate binding affinity, show increased exon inclusion compared with MSE3mx6 minigene. The minigene r0-2x6, which does not bind the MSE3 complex, does not show a significant difference in levels of exon inclusion compared with the MSE3mx6 minigene. These results demonstrate a correlation between binding affinity of the MSE3 complex in vitro with increased exon inclusion in vivo. However, unlike the natural MSE3, increased exon inclusion is not restricted to muscle cells (see "Discussion").

Having established a strong correlation between MSE3 complex binding affinity and in vivo exon inclusion activity, we investigated the binding affinity of r-hPurH using the same set of mutant competitors. As with the MSE3 complex, r-hPurH binds MSE3 with an approximately 10-fold higher affinity than...
MSE3m, r3-51 slightly better than the natural MSE3 sequence and r3-49 with an affinity between that of the wild type and MSE3m mutant. Also in agreement with the binding studies of the MSE3 complex, r-hPurH does not bind to the r0-2 sequence (Fig. 8). Thus PurH binding affinity precisely correlates with the MSE3 complex, r-hPurH does not bind to the r0-2 sequence (Fig. 8). Thus PurH binding affinity precisely correlates with that of the purified MSE3 complex affinity. This result strongly suggests that PurH is responsible for the binding specificity in the MSE3 complex, and its binding is functionally involved in cTNT exon 5 inclusion.

**DISCUSSION**

Increased inclusion of cTNT exon 5 requires at least three of four intronic MSEs which act in a positive manner in muscle cells while having no effect in fibroblasts, consistent with a model in which positive-acting factors in muscle bind to the MSEs to increase exon inclusion (5, 6).

Cell-specific regulation of alternative splicing by multiple *cis* elements is seen with several other pre-mRNAs (7, 8, 10–12, 14, 16, 29, 30). Regulation of neuron-specific inclusion of the N-1 exon of *c-src* also requires sequences both upstream and downstream of the alternative exon. The downstream control region is required for inclusion of the N-1 exon in neuronal cells and forms a specific complex with neuronal but not HeLa nuclear extract in vitro (15). Many of the factors in this complex have been identified (13, 15, 18); however, the factor(s) responsible for the neuron-specific assembly of the complex has not yet been identified. The best characterized example of regulated alternative splicing is the inclusion of the female-specific exon 4 of the *doublesex (dsx)* gene in *Drosophila*. Inclusion of this exon requires the activation of a weak 3′ splice site (31, 32) and is controlled by three distinct *cis* regulatory sequences: the $\text{dsx}$ repeat elements (31, 33–35), a purine-rich exon splicing enhancer (36), and a sequence just upstream of the 3′ splice site (37). Of the three types of elements, only the repeat elements are recognized by a female-specific factor. The female-specific *Transformer (Tra)* protein binds to the $\text{dsx}$ repeats in a complex with the ubiquitously expressed Tra-2 and members of the SR protein family (34–36, 38, 39).

We hypothesize that the regulation of exon inclusion by the MSEs is similar to the regulation seen in *c-src* and *dsx*. It is not necessary for all of the MSEs to bind a muscle-specific factor to be involved in muscle-specific activation of exon inclusion; however, it seems likely that at least one MSE is recognized by factors present in muscle but not fibroblast cells. Since we are most interested in finding muscle-specific splicing regulators, we sought to identify factors that bind to the MSEs that are both sequence- and cell-specific. Both criteria are fulfilled since MSE3 forms a sequence-specific complex in embryonic muscle nuclear extract but not in nuclear extract from fibroblasts or HeLa cells.

Alternative splicing of exon 5 results in high levels of exon inclusion in embryonic striated muscle that decreases to undetectable levels of inclusion in the adult (19). During our characterization of embryonic MSE3 binding activity, we identified the same MSE3 binding activity in adult muscle extract. It is important to note that the MSEs were defined as being required for exon inclusion in embryonic muscle. The basis for the developmental transition to exon skipping is unknown. The simplest model of developmental regulation is the loss of a positive-acting factor in adult muscle. While the MSE complex was detected in extracts from embryonic and adult muscle, it is possible that the MSE3 complex is more abundant in the embryo. However, the differences in starting materials, nuclear extract for embryonic and whole tissue for adult, precludes any conclusions on the relative abundance of MSE3 binding activity.
at these two developmental stages. An alternative model is that the developmental switch involves more than loss of a positive acting factor. One possibility is the acquisition of negative regulatory factors to block or inactivate the function of the MSE3-specific complex. In support of this model, there are mutations within introns 4 and 5 that lead to increased exon inclusion in fibroblasts (6). In addition, we have found that polypyrimidine tract-binding protein plays a role in repressing cTNT exon inclusion.\(^2\) Thus, the presence of the MSE3 complex in adult muscle does not rule out a role in regulating embryo-specific splicing.

Final purification of MSE3 binding activity from adult muscle resulted in the identification of three proteins copurifying with binding activity: aldolase, pyruvate kinase, and PurH. PurH, with an apparent molecular mass of 69 kDa, corresponds to the size of the largest cross-linked protein and is the only one of the three proteins that directly binds MSE3 RNA. PurH was also identified in the final MSE3-binding fraction from embryonic nuclear extract (data not shown). The 40-kDa cross-linked protein corresponds in size to aldolase; however, aldolase does not interact with MSE3 by itself or in combination with the other proteins (Fig. 7). Thus, we have not yet identified the 40-kDa protein that cross-links to MSE3 which is probably obscured by the high levels of aldolase still present after the final heparin-agarose column. As PurH is not restricted to muscle, the unidentified 40-kDa cross-linked protein is a candidate for a factor that allows the MSE3 complex to form in muscle extract but not in extract from fibroblast or HeLa cells.

PurH copurifies with the MSE3 complex from both embryonic and adult muscle, corresponds in size to the larger cross-linked protein in the native MSE3 complex (Fig. 3), and is sufficient for sequence-specific binding of RNA (Fig. 8). Together, these results provide strong evidence that PurH is one component of the MSE3 complex found in muscle. PurH and the native MSE3 complex also have identical binding preferences for five different RNA sequences (compare Figs. 8 and 9). Furthermore, differences in the binding affinity of the recombinant protein and of the complex with these five sequences are similar. These results confirm that PurH is a component of the native MSE3 complex and indicate that PurH is the primary determinant of binding specificity in the MSE3 complex. Coexpression of PurH with cTNT minigenes in fibroblasts and muscle cultures resulted in only modest increases in exon inclusion (data not shown) indicating that PurH is not the limiting component of the complex which mediates exon inclusion. Consistent with this conclusion, Western blot analysis of PurH expression during skeletal muscle development reveals no changes in protein abundance (data not shown).

The binding affinity of both the MSE3 complex and PurH for a number of MSE3-based sequences was demonstrated to generally correlate with the ability of these sequences to enhance exon inclusion (Figs. 9 and 10). The MSE3 complex has an affinity for r3-49 that is between that of the wild type MSE3 and the original MSE3m mutation and a slightly higher affinity for the r3-51 sequence than for the natural MSE3 sequence. When these sequences are used to replace concatamers of MSE3 in splicing minigenes, there is an overall increase in the level of exon inclusion compared with the MSE3mx6 minigene. Equally important, the r0-2x6 minigene, which has no affinity for the MSE3 complex, does not show an increase in exon inclusion over the MSE3mx6 minigene. This correlation between binding affinity \textit{in vitro} and ability to function as a splicing enhancer \textit{in vivo} with five different sequences strongly suggests that PurH and the MSE3 complex contributes to enhanced exon inclusion \textit{in vivo}.

The MSE3 complex is muscle-specific, and MSE3 concatamers enhance muscle-specific exon inclusion (6). In contrast, r3-49 and r3-51 increase exon inclusion in both cell types (Fig. 10). There are several possible reasons why there is not a strict correlation between binding affinity and a cell-specific response. First, MSE3 may also serve as a target for negative as well as positive regulatory factors to prevent exon inclusion in non-muscle cells, and binding sites for these factors could be absent in the r3-49 and r3-51 sequences. Several cell-specific cassette-type alternative exons require positive regulatory elements for exon inclusion and are actively repressed in cells undergoing exon skipping (11, 12, 14, 29, 40). In support of this model, we have found that polypyrimidine tract-binding protein represses exon inclusion in these fibroblasts.\(^2\) It is possible that the lack of polypyrimidine tract-binding protein-binding sites in R3-49 and R3-51 allows PurH in fibroblasts to increase exon inclusion. A strong increase in exon inclusion in fibroblasts would, however, be prevented by the absence of the muscle-specific factor. In addition, the absence of enhanced inclusion in muscle may be due to subtle changes in spacing and orientation on the different sequences leading to disruption of necessary interactions between the MSE3 complex and other splicing factors.

PurH, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (AICARFT/IMPChase), catalyzes steps 9 and 10 of \textit{de novo} purine synthesis. The product of step 9 serves as the substrate for step 10 and studies of human PurH indicate that the two enzymatic activities are in non-overlapping domains (41). Sequence comparisons of the human and chicken PurH genes shows 81% amino acid identity. The conservation between eukaryotes and prokaryotes is also striking; human PurH shares 38% amino acid identity with PurH from \textit{Escherichia coli}.

It is not unprecedented for an enzyme to have multiple distinct and unrelated functions. In addition to its function in glycolysis, glyceraldehyde-3-phosphate dehydrogenase can bind tRNA (42) and regulate viral translation initiation (43), mRNA stability (44), and ribozyme catalysis (45). It also has uracil glycosolase activity in the nucleus (46). The protein pterin-4a-carbinolamine dehydratase (PCD)/dimerization cofactor of HNF1 (DCoH) also functions as an enzyme and as a regulator of gene expression. As an enzyme, PCD/DCoH is required in the cytoplasm for regeneration of tetrahydrobipterin (47). In the nucleus, PCD/DCoH is found in a 2:2 heterotetrameric complex with HNF1 (hepatocyte nuclear factor 1) where it is required to stabilize HNF1 binding to its target sequences for activated transcription (48–50). Thymidylate synthetase forms a complex with p53 mRNA \textit{in vivo} that is thought to regulate p53 translation (51). The best studied example of an enzyme having a role in RNA metabolism is the iron-binding protein 1. Iron-binding protein 1 binds to iron response elements in 5′- and 3′-untranslated regions of iron responsive mRNAs to regulate translation initiation and mRNA stability. After purification, iron-binding protein 1 was identified as cytosolic aconitase (52).

The identification of PurH as part of a muscle-specific complex binding to MSE3 and the correlation of its binding affinity to levels of exon inclusion suggest that PurH has two very diverse functions. During evolution, PurH enzymatic function in nucleotide biosynthesis would have been required to precede a function in RNA processing. It is interesting to note that eukaryotic PurH proteins have two regions that are not present in PurH proteins from prokaryotes, where splicing does not occur. UV cross-linking and gel shifts also indicate that a second protein of approximately 40 kDa is part of the muscle-specific MSE3 complex. Together with PurH, this MSE3 com-

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\(^2\) N. Charlet-B and T. A. Cooper, manuscript in preparation.
plex is likely to have a role in muscle-specific enhanced exon inclusion and regulated alternative splicing.

Acknowledgments—We thank Dr. Steve Benkovic (Penn State University) for the PurH cDNA expression clones and Bill Mattox, Miles Wilkinson, and members of the Cooper lab for critical reviews of the manuscript. We also thank Claire Lo for excellent technical assistance.

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